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Research Paper

Physicochemical and Immunological Characterization of N,N,N-Trimethyl Chitosan-Coated Whole Inactivated Influenza Virus Vaccine for Intranasal Administration

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Purpose. The purpose of this study was the development and physicochemical and immunological characterization of intranasal (i.n.) vaccine formulations of whole inactivated influenza virus (WIV) coated with N,N,N-trimethyl chitosan (TMC).
Methods. Synthesized TMCs with a degree of quarternization of 15% (TMC15) or 37% (TMC37) were tested *in vitro* for their ability to decrease the transepithelial resistance (TEER) of an epithelial cell monolayer. TMC15- and TMC37-coated WIV (TMC15-WIV and TMC37-WIV) were characterized by zeta potential measurements, dynamic light scattering, electron microscopy and gel permeation chromatography. Mice were vaccinated i.n. with selected vaccine formulations and immunogenicity was determined by measuring serum hemagglutination inhibition (HI) and serum IgG, IgG1 and IgG2a/c titers. Also a pulse-chase study with TMCs in solution administered i.n. 2 h prior to WIV was performed. Protective efficacy of vaccination was determined by an aerosol virus challenge.
Results. TMC37 induced a reversible decrease in TEER, suggesting the opening of tight junctions, whereas TMC15 did not affect TEER. Simple mixing of (negatively charged) WIV with TMC15 or TMC37 resulted in positively charged particles with TMCs being partially bound. Intranasal immunization with TMC37-WIV or TMC15-WIV induced stronger HI, IgG, IgG1 and IgG2a/c titers than WIV alone. TMC37-WIV induced the highest immune responses. Both TMC15-WIV and TMC37-WIV provided protection against challenge, whereas WIV alone was not protective. Intranasal administration of TMC prior to WIV did not result in significant immune responses, indicating that the immunostimulatory effect of TMC is primarily based on improved i.n. delivery of WIV.
Conclusions. Coating of WIV with TMC is a simple procedure to improve the delivery and immunogenicity of i.n. administered WIV and may enable effective i.n. vaccination against influenza.

KEY WORDS: immunogenicity; influenza; nasal vaccination; physicochemical characterization; trimethyl chitosan (TMC).

INTRODUCTION

Currently most inactivated influenza vaccines on the market are administered via the intramuscular (i.m.) or subcutaneous (s.c.) route. Although these routes of administration can induce strong systemic humoral immune responses, the intranasal (i.n.) route has several advantages when compared to i.m. vaccination (1). It allows simple, non-invasive, needle-free administration which decreases infection risks due to the reuse of needles, enables large scale vaccinations without trained personnel and increases compliance. Furthermore, the i.n. route theoretically induces less local adverse effects than i.m. needle injections and is easily accessible, making administration by a trained professional unnecessary. Furthermore, i.n. immunization has the potential of inducing mucosal immunity, and it has been suggested that nasal vaccination may induce broader protection against heterologous influenza viruses, a feature that is very important in providing protection against seasonal as well as pandemic influenza (2,3).

Live attenuated influenza virus (LAIV) has been researched extensively and is currently on the market as i.n. seasonal influenza vaccine in the US and Russia. The efficacy of i.n. LAIV is comparable to or even better than that of i.m. split vaccines (4). Nonadjuvanted inactivated i.n. influenza vaccines on the other hand, are generally not as immunogenic

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as i.m. vaccines or as promising as LAIV for i.n. vaccination. On the other hand, inactivated vaccines are generally regarded as safer than LAIV, especially for infants and immunocompromised individuals.

From the inactivated influenza vaccines, whole inactivated virus (WIV) formulations seem to be the most promising (5–8). In comparison to subunit and split influenza vaccines, WIV induces much stronger humoral and cellular immune responses via various immunization routes (8–11). Furthermore, WIV can induce cross-protection, especially when administered via the mucosal route, which may be especially important in the development of a pandemic vaccine (2,5,12,13). The superior, Th1-biased immune responses can be explained by the presence of viral RNA inside the particulate viral structure of WIV, a TLR 7 ligand, which acts as an adjuvant (10).

Although these features of WIV are promising, the immunogenicity of plain WIV after i.n. administration, especially the systemic humoral immune responses, can still be improved (8). The use of adjuvants is a common approach to improve vaccination and different types of adjuvants have been tested for i.n. vaccination, like *Escherichia coli* heat labile enterotoxin (LT), and cholera toxin (CT) derivatives, various toll-like receptor ligands like CpG motifs, LPS and poly I:C (as reviewed in (1)). In the case of i.n. vaccination, however, the induction of relatively low systemic immune responses cannot be fully explained by a lack of intrinsic immunogenicity of the vaccine, illustrated by the strong immune responses induced by these vaccines after i.m. administration, especially in the case of WIV (10,14–16). It is therefore likely that inefficient delivery of antigens to antigen presenting cells, caused by mucosal barriers and rapid clearance from the nasal cavity, is a limiting factor in the induction of stronger immune responses. Use of mucoadhesive polymers may overcome these problems by their interaction with the mucosal surfaces in the nasal cavity, leading to an increased residence time and enhanced uptake of the antigen (17).

Chitosan is a mucoadhesive polymer that has been studied for many applications (18,19), including mucosal vaccine delivery (20–22). The unfavorable pH-dependent water solubility of chitosan has prompted the development of chitosan derivatives (23), like *N,N,N*-trimethyl chitosan (TMC) (24,25). TMC is a cationic and mucoadhesive polymer that has been tested as additive in mucosal vaccines, both as solution in oral, pulmonary and nasal vaccination (26) and as nanoparticles with associated antigen (27–31). TMC can be characterized by the degree of quaternization (DQ) of amines, which determines the charge density of TMC. The DQ of TMC can be tailored during synthesis and influences chemical and biological properties of TMC. Amidi *et al.* demonstrated the superior immunogenicity of i.n. administered influenza antigens that are encapsulated in TMC nanoparticles when compared to antigens admixed with TMC solutions (27).

Because WIV vaccines were found to be the most immunogenic of the nonadjuvanted influenza vaccines (8,15,16,32), it is a logical step to use WIV vaccines for optimization with TMC for i.n. vaccination. In this study, we propose TMC-coated WIV (TMC-WIV) as i.n. influenza vaccine by combining the mucoadhesive properties of TMC with the particulate structure of WIV. This approach exploits the ability of TMC to interact not only with negatively charged mucus and mucosal surfaces, but also with negatively charged virus particles. We used *O*-methylated TMCs with a DQ of 15% (TMC15) and 37% (TMC37) to coat WIV. The preparation and characterization of TMC15-coated WIV (TMC15-WIV) and TMC37-coated WIV (TMC37-WIV) vaccines are presented and it is analyzed whether these novel vaccine formulations significantly improve the immunogenicity and protective efficacy of i.n. administered whole inactivated influenza virus. Additionally, a pulse-chase study is performed with TMCs in solution and WIV to determine if TMC requires coadministration with WIV to exert its adjuvant effect.

MATERIALS & METHODS

Materials

Chitosan was purchased from Primex (Siglufjordur, Iceland). N-Methyl-2-pyrrolidone (NMP), thiazolyl blue tetrazolium bromide (MTT), sodium acetate, acetic acid (anhydrous), sodium hydroxide and hydrochloric acid were obtained from Sigma-Aldrich Chemical Co (Zwijndrecht, the Netherlands). Dulbecco's modified Eagle's medium (DMEM) and Hank's balanced salt solution (HBSS) were purchased from Invitrogen (Breda, the Netherlands). Sodium dodecyl sulfate (SDS) was ordered from Merck (Darmstadt, Germany). Linear polyethylene imine (PEI) was kindly provided by S. van der Wal (Utrecht University, the Netherlands). Iodomethane 99% stabilized with copper was obtained from Acros Organics (Geel, Belgium). Live, egg-grown, mouse adapted influenza A/PR/8/34 virus (A/PR/8/34) and purified, cell culture-grown (Madin–Darby Canine Kidney (MDCK) cells), β-propiolacton (BPL)-inactivated influenza A/PR/8/34 virus, as well as polyclonal rabbit anti-A/PR/8/34 serum were kindly provided by Nobilon International BV (Boxmeer, The Netherlands). PO-labeled goat anti mouse -IgG (H+L), -IgG1, -IgG2a/c and -IgA(Fc) were purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands). All other chemicals were analytical grade.

Synthesis of TMC15 and TMC37

TMC15 was synthesized by methylation of chitosan as described previously (25,33). Briefly, 1.0 g of chitosan (number average molecular weight (Mn)=25 kDa, weight average molecular weight (Mw)=42 kDa, determined by gel permeation chromatography (GPC), as described in the next section) and 2.4 g of sodium iodide were added to 60 ml NMP and 6.0 ml 15% weight/weight (*w*/*w*) NaOH solution and stirred for 20 min. Then, 6.0 ml of methyl iodide was added and the mixture was refluxed for 60 min. The obtained TMC with a DQ of 15 was precipitated in diethyl ether and washed thoroughly with diethyl ether. Finally, the precipitated TMC was dissolved in 10% (*w*/*v*) NaCl solution and stirred for at least 18 h for ion-exchange. This solution was dialyzed afterwards at room temperature against deionized water for 3 days by changing buffer twice daily, filtered through a

0.8 μm filter and freeze dried. TMC37 was synthesized from chitosan (Mn=46 kDa, Mw=75 kDa, determined by GPC) in a similar way, but in an extra step, 3.0 ml 15% (*w*/*w*) NaOH solution and 3.0 ml iodomethane were added and stirred for another 60 min before the TMC was precipitated and washed with diethyl ether. In addition to trimethylation of the amines, these reaction conditions lead to substantial methylation of the C3 and C6 hydroxyl groups (33). Polymers were characterized by GPC and NMR as described previously (25,33). Polymer characteristics are summarized in Table I.

GPC Analysis of Chitosans and TMCs

The Mn and Mw of chitosans and the synthesized TMC15 and TMC37 were determined by GPC on a Viscotec triple detection system using a Shodex OHPak SB806 column (30 cm) and 0.3 M sodium acetate buffer pH=4.4 as running buffer as described earlier (33). Briefly, polymer samples were dried overnight in a vacuum oven at 40°C. Then, samples were dissolved overnight in running buffer at a concentration of 5 mg/ml, filtered through a 0.2 μm filter and injected (50 μl) onto the GPC column. Flow was 0.7 ml/min. Mw and Mn were determined using the Viscotec analysis software that integrates refractive index, viscosity and right (90°) and low angle (7°) scattering data.

MTT Cell Toxicity Assay

MTT cell toxicity assay was performed according to Mosmann (34). Caco-2 cells were seeded in a 96-well plate at a density of 4×10^4 cells per well and incubated for 2 days at 37°C and 5% CO2 in culture medium (DMEM, high glucose, 10% FCS, L-glutamine, pyruvate, non-essential amino acids). The medium was removed and the cells were incubated for 2.5 h with 100 μl TMC solutions in HBSS (TMC concentrations were 0.1, 1 and 10 mg/ml, pH set at 7 with 0.1 M NaOH). SDS (10 mg/ml) was used as positive control and HBSS as reference for 100% cell viability. Then, the HBSS was removed and the cells were washed with phosphate buffered saline. The cells were incubated with 100 μl freshly prepared solution of 0.5 mg/ml MTT in DMEM, without any additions for 3 h at 37°C and 5% CO2. Subsequently, the wells were emptied, the formed formazan crystals were dissolved with 100 μl of DMSO and the absorbance was read at 595 nm.

Transepithelial Electrical Resistance (TEER) Measurements

TEER measurements were performed as described earlier (33,35). Shortly, Caco-2 cells were cultured on 12-transwell plates with a microporous membrane in Dulbecco's modified Eagle's medium (DMEM) until a confluent cell layer was formed. The medium was replaced by Hank's balanced salt solution (HBSS) at the basolateral side 10 min before the start of the experiments. Then, 0.5 ml solution of TMC15 and TMC37 (2 mg/ml in HBSS, pH adjusted to 7 with 0.1 M NaOH) was applied at the apical side of the cell monolayers. SDS (10 mg/ml) was used as positive control and HBSS as reference. The TEER of the Caco-2 cells was measured with a Millicell-ERS (Millipore, Billerica, USA) measuring device at certain time points (0, 15, 30, 45, 60 and 90 min) after addition of the stimuli. After 90 min, the cells were washed with HBSS and incubation of the cells was continued in DMEM for 24 h at 37°C and 5% CO2 to determine the recovery of the TEER.

Preparation of Vaccine Formulations

Purified, cell culture-derived, BPL-inactivated A/PR/8/34 suspended in a 10 mM phosphate buffered saline solution (150 mM NaCl, pH=7) (PBS) was concentrated by centrifugation at 22,000×*g* for 30 min at 4°C and resuspended in 5 mM HEPES buffer (pH 7.4). The WIV concentration is expressed as mg total protein/ml as determined by DC protein assay (Bio-Rad, Hercules, CA, USA). The amount of hemagglutinin (HA) was approximately 35% of the total protein content, as determined previously (8). The TMC15-WIV and TMC37-WIV vaccines were prepared by simply adding equal volumes of TMC15 or TMC37 solution (in 5 mM HEPES, pH 7.4) to a WIV dispersion using a Gilson pipette while gently mixing for 5 s. The (*w*/*w*) ratio of TMC/WIV was varied between 0 and 12 by adding the TMCs at different concentrations while keeping the volume the same. The formulations were prepared at a final WIV concentration of 0.156 mg/ml for the initial characterization of TMC-coated WIV formulations over a broad range of TMC/WIV (*w*/*w*) ratios. For i.n. vaccination, the vaccine formulations were prepared at a concentration of 1.25 mg/ml in 5 mM HEPES, pH 7.4 and were also characterized at this concentration. The TMC solutions for the pulse-chase study were prepared at a concentration of 1.25 mg/ml in 5 mM HEPES, pH 7.4.

Characterization of Vaccine Formulations

Particle size was measured by dynamic light scattering (DLS) using a Malvern ALV CGS-3 (Malvern Instruments, Malvern, UK). DLS results are given as a *z*-average particle size diameter and a polydispersity index (PDI). The PDI

Table I. Polymer Characteristics of Synthesized TMC15 and TMC37

Polymer	DQ[a] (%)	DOM[a] C3 (%)/C6 (%)	DAc[a] (%)	Mn[b] (kDa)	Mw[b] (kDa)
TMC37	37	9/14	4	44	94
TMC15	15	4/6	16	30	63

DQ degree of quarternization, *DOM* degree of *O*-methylation, *DAc* degree of acetylation, *Mn* number average molecular weight, *Mw* weight average molecular weight
[a] As determined by NMR
[b] As determined by GPC

can range from 0 (indicating monodisperse particles) to 1 (a completely heterodisperse system).

Zeta potentials of WIV and TMC-WIV vaccines were measured using a Zetasizer Nano (Malvern Instruments, Malvern, UK). The integrity of the viral structure was investigated using negative stain transmission electron microscopy (TEM). Therefore WIV formulations (1.25 mg/ml) with and without TMC coating (TMC/WIV ratio of 1) were stained using 2% uranyl acetate as described previously (36).

The WIV-associated fraction of TMC15 and TMC37 was determined by quantification of the free TMC, present in the supernatant of centrifuged TMC-WIV vaccine, using the GPC method described above. Briefly, 75 μl TMC solution (in 5 mM HEPES buffer, pH 7.4) was added to 75 μl WIV (2.5 mg protein/ml in 5 mM HEPES, pH 7.4) and mixed by pipeting and vortexing gently for 3 s. TMC-WIV formulations were centrifuged for 40 min at 22,000×*g* at 4°C and the supernatant was collected. Prior to injection, 20 μl GPC running buffer was added to 100 μl supernatant to adjust the pH of the sample. The sample concentration was determined using the refractive index.

The WIV-associated TMC content as percentage of the total TMC in the formulation (TMC_{bound} (%)) was calculated as:

$$TMC_{bound}(\%) = 100 - \frac{\left(TMC_{free}(g) \times 100\right)}{total\ TMC\ (g)}$$

The WIV-associated TMC as TMC/WIV (*w*/*w*) ratio (TMC_{bound}/WIV (*w*/*w*)) was calculated as:

$$TMC_{bound} \Big/ WIV(w/w) = \frac{\left(total\ TMC(g) - TMC_{free}(g)\right)}{WIV(g)}$$

Immunization Protocol

Animal experiments were conducted according to the guidelines provided by the Dutch Animal Protection Act and were approved by a Committee for Animal Experimentation. For all experiments 6–8 weeks old female C57-BL/6 mice (Charles River) were used. Mice were housed in groups of 8–11 mice and food and water were provided *ad libitum*. Prime and boost immunizations at day 0 and 21, respectively, were performed without anesthesia. Groups of 11 mice were vaccinated i.n. with WIV, TMC15-WIV or TMC37-WIV at a dose of 12.5 μg WIV (corresponding to approximately 4.3 μg HA). As a negative control, a group of 11 mice was vaccinated i.n. with PBS. TMC15-WIV and TMC37-WIV vaccines were freshly prepared from WIV dispersion and TMC15 or TMC37 solution. For i.n. immunization, mice were held in supine position without anesthesia and the vaccine was administered to the left and right nostril in a total volume of 10 μl. As a reference, one group of mice was vaccinated i.m. with WIV at a dose of 12.5 μg protein in a volume of 100 μl in the left and right quadriceps for prime and boost vaccination, respectively.

Additionally, a pulse-chase study was performed in which two groups of mice were first administered 10 μl of a 1.25 mg/ml TMC37 or TMC15 solution i.n., followed 2 h later by 10 μl of 1.25 mg/ml WIV i.n.

Blood Sampling and Nasal Washes

Blood samples were collected by orbital puncture in MINICOLLECT® serum separator tubes coated with SiO_2 (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) 20 days after prime vaccination and boost vaccination. Coagulated blood samples were centrifuged at 6,500×*g* for 8 min at room temperature to obtain serum samples. Individual serum samples were stored at −20°C until further analysis. 20 days after boost vaccination, three mice from each group were anesthetized with diethyl ether and bled by orbital puncture. The trachea was cannulated towards the nasopharyngeal duct with a PVC tube (inner/outer diameter 0.5/1.0 mm). 500 μl PBS containing complete Mini, EDTA free protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA) at a concentration of one tablet/7 ml PBS was flushed through the nasal cavity and collected from the nostrils.

Challenge

22 days after the boost vaccination, mice challenged with 50 ml ($8.8\times10^8\times$the 50% egg infectious dose (EID50)/ml) aerosolized, egg-grown influenza A/PR/8/34 using a DeVilbiss Ultra-Neb 2000 ultrasonic nebulizer (Direct Medical Ltd, Lecarrow, Ireland) for 25 min. After challenge, mice were put back in their cages and any observed signs of illness like lethargy, standing fur and curved back were recorded. Additionally, their body weight was monitored daily for 15 days. For comparison of loss in body weight, the average area under the curve (AUC) was calculated for each group from body weight curves of individual mice. All i.n. groups were compared to the negative control group (PBS i.n.) and the positive control group (WIV i.m.) by the average (AUC) of individual mice using a one-way ANOVA and Bonferroni's correction for multiple comparisons.

Hemagglutination Inhibition Test

First, 25 μl serum was incubated for 18 h at 37°C with 75 μl Receptor Destroying Enzyme (RDE) solution (Denka Seiken UK Ltd, Coventry, UK) to suppress nonspecific hemagglutination inhibition. RDE was then inactivated by incubating the mixture for 30 min at 56°C. Next, 150 μl PBS was added to obtain a final ten-fold serum dilution. 50 μl diluted serum was transferred in duplicate to V-bottom 96-wells plates (Greiner, Alphen a/d Rijn, The Netherlands) and serially diluted twofold in PBS. Next, four hemagglutination units (HAU) Influenza A/PR/8/34 (in 25 μl PBS) were added to all wells and the mixtures were incubated for 40 min at room temperature. Finally, 50 μl 0.5% (*v*/*v*) chicken erythrocytes in PBS were added to all wells and plates were incubated for 1 h at room temperature. The HI titer was expressed as the reciprocal value of the highest serum dilution capable of completely inhibiting the virus-induced agglutination of chicken erythrocytes. If no complete inhibition could be detected in the first lane, serum was arbitrarily scored 5. Comparison between different experimental groups from the same dose was made by a one-way ANOVA test and the Bonferroni correction for multiple comparisons on the log transformed HI titers.

Antibody Assays

Antigen specific serum antibody responses were determined by a sandwich ELISA. Maxisorp ELISA plates (Nunc, Roskilde, Denmark) were coated overnight with polyclonal rabbit anti-A/PR/8/34 serum (dilution 1:1,620). Plates were washed in between all prescribed steps with wash buffer (0.64 M NaCl, 3 mM KCl, 0.15% polysorbate 20 in 10 mM phosphate buffer pH=7.2) using a Skanwasher 300 (Molecular Devices, Sunnyvale, CA, USA). Next, plates were incubated for 1 h at 37°C with blocking buffer (0.2% (*w*/*w*) casein, sucrose 4% (*w*/*w*), Triton X-100 0.05% (*w*/*w*) and sodium azide 0.01% (*w*/*w*) in 30 mM TRIS pH=7.4), followed by incubation with egg-grown, BPL-inactivated A/PR/8/34 (8 HAU/ml) for 1 h at 37°C. Plates were then incubated with twofold serially diluted sera (100 µl/well) for 1 h at 37°C. Next, plates were incubated with 100 µl of a 1:2,500 dilution of horseradish peroxidase linked goat anti mouse -IgG (H+L), -IgG1, -IgG2a/c or -IgA(Fc) (Nordic Laboratories, Tilburg, the Netherlands) for 30 min, and washed twice. Finally, 100 µl 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added and plates were incubated for 15 min at room temperature before enzymatic conversion was stopped by adding 50 µl 2 M sulfuric acid. Optical density (OD) was then measured at 450 nm using a Tecan Sunrise plate reader (Tecan Trading AG, Zurich Switzerland). Titers are given as the reciprocal sample dilution corresponding to 20% of the maximal ELISA signal above background. Comparison between different experimental groups was made by a one-way ANOVA test and the Bonferroni correction for multiple comparisons.

RESULTS

Effect of TMCs on Epithelial Cell Toxicity and TEER *In Vitro*

The *in vitro* cell toxicity of the TMCs was determined using the MTT cell toxicity assay. The results (Fig. 1) show that TMC37 was slightly more toxic than TMC15, but far less than PEI. Even at the highest concentration (10 mg/ml) tested, the TMCs were less toxic than PEI at 0.2 mg/ml.

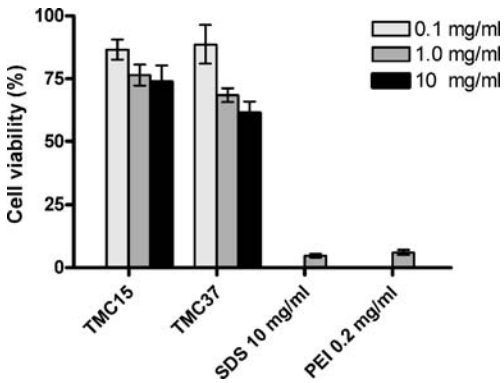

Fig 1. Effect of TMC15 and TMC37 on the viability of Caco-2 cells (MTT assay) at different concentrations. Error *bars* represent 95% confidence intervals (n=6). For comparison, polyethylene imine (*PEI*) (0.2 mg/ml) and SDS (10 mg/ml) were used as controls known to be toxic.

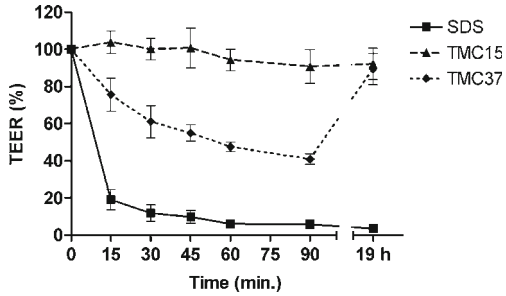

Fig 2. Effect of TMC15 and TMC37 on the TEER of Caco-2 cells at a concentration of 2 mg/ml. 10 mg/ml sodium dodecyl sulfate (*SDS*) was used as a control known to be toxic. After 90 min, the cells were washed with HBSS and incubation of the cells was continued in DMEM to determine the recovery of the TEER. The TEER of cells treated with HBSS was set at 100%. Error *bars* represent 95% confidence intervals (n=6).

TEER measurements were performed to investigate the ability to open tight junctions. As shown in Fig. 2, TMC37 induced a reversible decrease in TEER, suggesting that TMC37 has the ability to open tight junctions. TMC15 on the other hand, did not decrease the TEER at any measured time point, indicating that TMC15 cannot open tight junctions.

Zeta Potential and Particle Size of TMC-WIV

TMC15 and TMC37 were mixed with WIV at different (*w*/*w*) ratios and the zeta potential and particle size were determined. Uncoated WIV, with an average diameter of 160 nm (PDI 0.11), had a negative zeta potential of approximately −20 mV, as shown in Fig. 3. By increasing the concentration of TMC, the zeta potential of the TMC15-WIV and TMC37-WIV increased, initially resulting in a neutral zeta potential and instant aggregation. This indicates that the colloidal WIV formulation is stabilized by repulsive electrostatic forces between the particles. At higher TMC/WIV (*w*/*w*) ratios, above 0.9 and 0.2 for TMC15 and TMC37, respectively, positively charged particles were formed with an average diameter between 260 and 350 nm for TMC15-WIV and 400–500 nm for TMC37-WIV (PDI ≤ 0.23, indicating fairly homogeneous particle sizes). A maximum zeta potential was reached above +30 mV at a TMC/WIV (*w*/*w*) ratio of 12 for TMC15-WIV and TMC37-

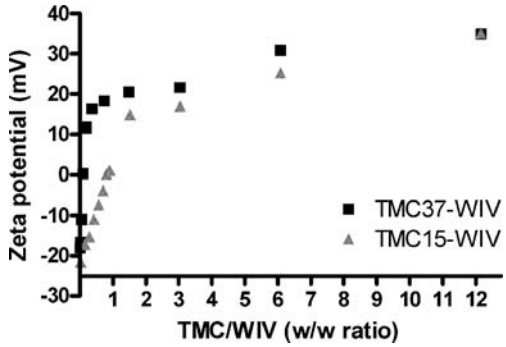

Fig 3. Zeta potential of WIV at a concentration of 0.156 mg/ml, formulated with TMC15 or TMC37 at different TMC/WIV (*w*/*w*) ratios in 5 mM HEPES pH 7.4.

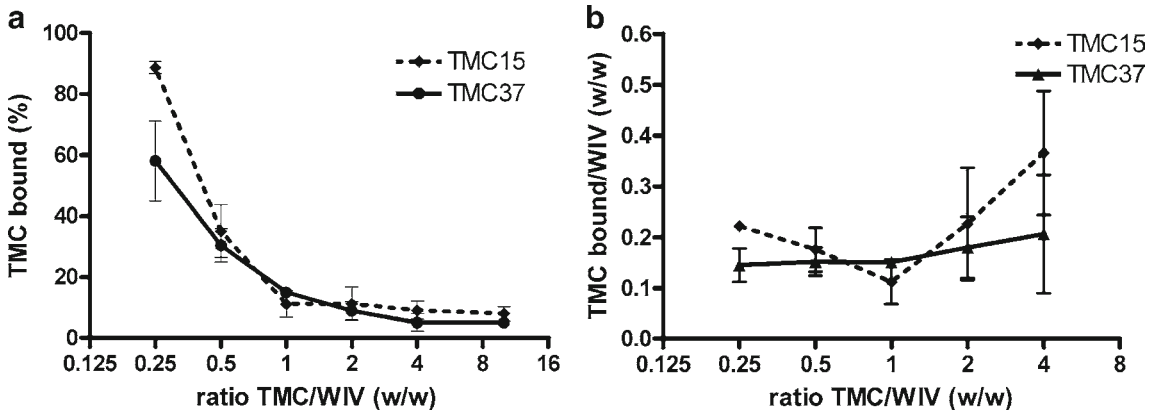

Fig 4. WIV-associated TMC expressed **a** as percentage of total TMC content in the formulation and **b** as TMC/WIV (*w*/*w*) ratio. TMC-WIV formulations were prepared at a WIV concentration of 1.25 mg/ml. Error *bars* indicate the 95% confidence intervals of three independent measurements.

WIV. Fig. 3 also shows that less TMC37 was needed to obtain positively charged particles as compared to TMC15.

Binding of TMC15 and TMC37 to WIV

The binding of TMCs to WIV was quantified using GPC. The percentage of TMC that is associated to WIV depended strongly on the amount of TMC added (Fig. 4a). On the other hand, the amount of TMC15 and TMC37 that is associated to WIV was rather constant between 0.15 and 0.25 g/g WIV, at TMC/WIV ratios ranging from 0.25 to 2 (Fig. 4b). At higher (*w*/*w*) ratios the WIV-associated fraction of TMC15 and TMC37 was very small. Therefore the data points were less reliable, because the standard deviations, derived from measurements of the supernatant, were relatively large (see calculation in MATERIALS & METHODS), leading to unreliable results. When adding more TMC, at TMC/WIV ratios above 1.0 (*w*/*w*), the excess TMC appeared to remain in solution. The constant amount of WIV-associated TMC suggests that the amount of TMC bound to WIV had reached saturation levels.

The zeta potential did not decrease when TMC15-WIV or TMC37-WIV (1:1 (*w*/*w*)) formulated at a WIV concentration of 1.25 mg/ml was diluted up to 32-fold with 5 mM HEPES buffer (Fig. 5). This indicates that the WIV-associated TMC does not readily dissociate and that the association of TMC with WIV is not strongly dependent on the free TMC concentration in solution. The slight increase in zeta potential upon dilution, especially in the TMC37 sample, may be caused by the dilution of residual ions from residual PBS after pelleting and resuspending the WIV.

Based upon these results, TMC15-WIV and TMC37-WIV formulations with a TMC/WIV (*w*/*w*) ratio of 1.0 were selected for an immunization study. At this (*w*/*w*) ratio, the particles were positively charged and the WIV seemed maximally coated with the cationic polymers. Moreover, the physicochemical characteristics of these formulations were comparable, as shown in Table II.

TMC induced no observable changes to the viral ultra structure visualized by TEM (Fig. 6). In our setting the TMC coating could not be observed, likely due to the nature of the negative stain or the limitations of the microscope. TMC coated grids stained with uranyl acetate did not show any recognizable structure (data not shown).

Immunization Study

The novel TMC-WIV vaccines were compared to plain WIV in a vaccination study. Mice were vaccinated twice with a 3 week interval and sera were sampled 20 days after prime and boost vaccination. Although i.m. vaccination with plain WIV induced higher HI titers than any of the i.n. vaccinations, coating of WIV with TMC37 significantly enhanced the induction of HI titers after i.n. administration (Fig. 7). Coating with TMC15, on the other hand, hardly improved the induction of HI titers of plain WIV.

In contrast to WIV, strong antigen-specific IgG responses were developed after i.n. prime vaccination with TMC37-WIV in all mice, which were further increased after boost vaccination (Fig. 8a). TMC15-WIV also induced substantial antigen-specific IgG responses, especially after boost vaccination, though not as pronounced as TMC37.

To determine the influence of TMC15 and TMC37 on the type of immune responses against WIV, IgG1 and IgG2a/c titers were determined by ELISA (Fig. 8b, c, respectively). For the formulations that showed detectable HI and IgG responses (i.m. WIV, i.n. TMC37-WIV and i.n. TMC15-WIV),

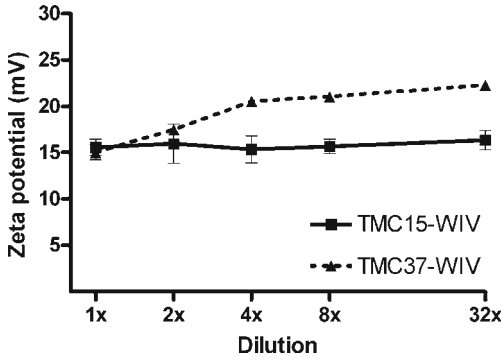

Fig 5. Zeta potential of TMC15-WIV and TMC37-WIV formulated at 1.25 mg/ml WIV at a TMC/WIV (*w*/*w*) ratio of 1.0, after different dilution steps with 5 mM HEPES pH 7.4. Error *bars* indicate the standard deviations of three measurements.

Table II. Properties of i.n. Administered Vaccine Formulations

Formulation	Concentration WIV (mg protein/ml)	TMC:WIV ratio (*w*/*w*)	TMC bound[a] *w*/*w* (± sd)	Zeta potential[a] (mV)	Radius (PDI)
WIV	1.25	0	–	−13.3 (±0.9)	93.2 (0.18)
TMC15-WIV	1.25	1	0.112 (±0.017)	15.6 (±0.4)	196.0 (0.24)
TMC37-WIV	1.25	1	0.151 (±0.002)	15.1 (±0.9)	269.1 (0.32)

[a] Average of three measurements

IgG2a titers after prime vaccination were higher and detectable in a larger number of mice than IgG1 titers. After boost vaccination with these three vaccines, more mice developed IgG1 as well as IgG2a titers. These findings indicate that coating of WIV with TMC15 or TMC37 does not influence the quality of humoral systemic immune responses, but rather improves the delivery of WIV after i.n. administration.

Additionally, a pulse-chase study was performed to study whether the adjuvant activity was only observed when TMCs were coadministered with WIV. In this study the time between the administration of TMC and WIV should be as short as possible, but long enough to avoid interaction between TMC and WIV after administration. Therefore, as opposed to others who used a time period of 24 h (22), we chose 2 h. This should be sufficiently long to prevent WIV and TMC to interact after administration, anticipating that the cationic TMC will be rapidly neutralized by the abundantly present negatively charged mucins and/or cleared by mucociliary activity.

In the pulse-chase study with TMC15 and TMC37 (indicated in Figs. 7, 8 and 9 with "TMC37sol-2 h WIV" and "TMC15-2 h WIV", respectively), none of the mice developed detectable HI titers (Fig. 7) and only a few mice that received TMC37 solution i.n. 2 h before the WIV developed weak IgG titers after boost vaccination (Fig. 8a–c). Mice that received TMC15 solution prior to WIV i.n. vaccination were unresponsive. These results clearly indicate that TMC37 and TMC15 are not effective when administered 2 h prior to the i.n. administration of WIV, confirming our conclusion that the adjuvant effect is mainly based on improved delivery and less on immune stimulation by TMC.

Challenge with Live, Aerosolized Virus

To determine the protective effect of vaccination, eight mice in each group were challenged with homologous, egg grown influenza virus. Mice were monitored for signs of illness like standing fur, grouping together and lethargic behavior. Additionally, body weight was recorded daily for 15 days after challenge as a measurable sign of illness. As shown in Fig. 9, all mice in the positive control group, vaccinated i.m. with WIV, were protected. Mice did not show any signs of illness and maintained body weight over the period of 2 weeks after challenge. On the other hand, mice in negative control group that received PBS i.n., lost weight from day 2 to day 10, became lethargic and had a curved back and standing fur. Two mice in this group were sacrificed at day 9 and the other mice regained weight from this day, indicating recovery from viral infection. Mice vaccinated i.n. with WIV alone (Fig. 9a) were also not protected. They lost body weight in a similar pattern as the control group but started to regain body weight 2 days earlier than the control group. Furthermore, all mice vaccinated with WIV i.n. showed signs of illness from day 6 and 8, respectively, until day 13.

In contrast, i.n. vaccination with TMC37-WIV protected all mice against signs of illness and loss of body weight (Fig. 9b). Furthermore, all challenged mice in the TMC15-WIV i.n. group that were seropositive (six out of seven) were also protected against symptoms of illness (Fig. 9c). Like the i.m. WIV group, both groups had a significantly higher average relative body weight than the PBS i.n. group.

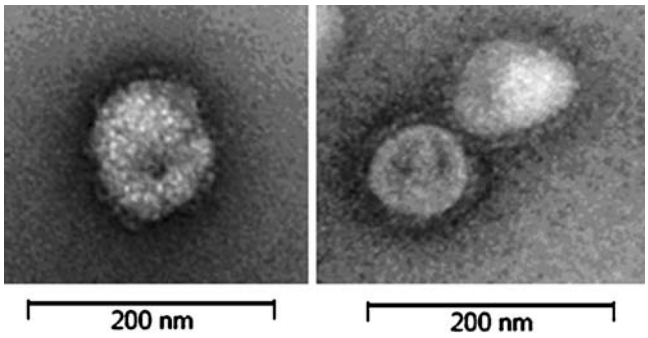

Fig 6. Negative stain transmission electron microscopy picture of WIV (*left*) and TMC37-WIV (*right*).

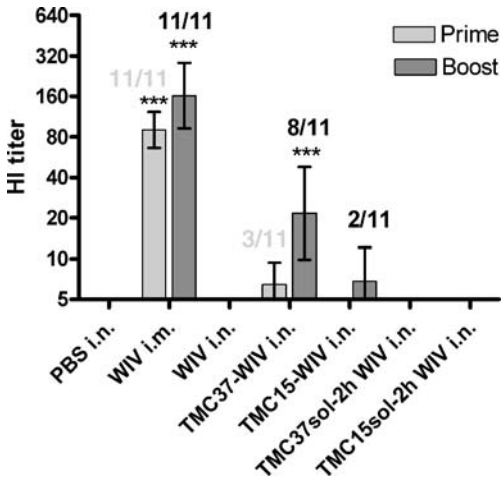

Fig 7. Geometric mean HI titers 20 days after prime and boost vaccinations. Negative serum samples were arbitrarily assigned a titer of 5 for calculation purposes. Error *bars* indicate 95% confidence intervals. Indicated above the *bars* are the number of mice that developed detectable HI titers (e.g. 3/11 indicates three out of 11 mice). $n=11$ for all groups. All HI titers after prime and boost vaccination were compared using a one-way ANOVA test and Bonferroni's correction for multiple comparisons. ***$P<0.001$ indicate titers that are significantly higher than those of the WIV i.n. group.

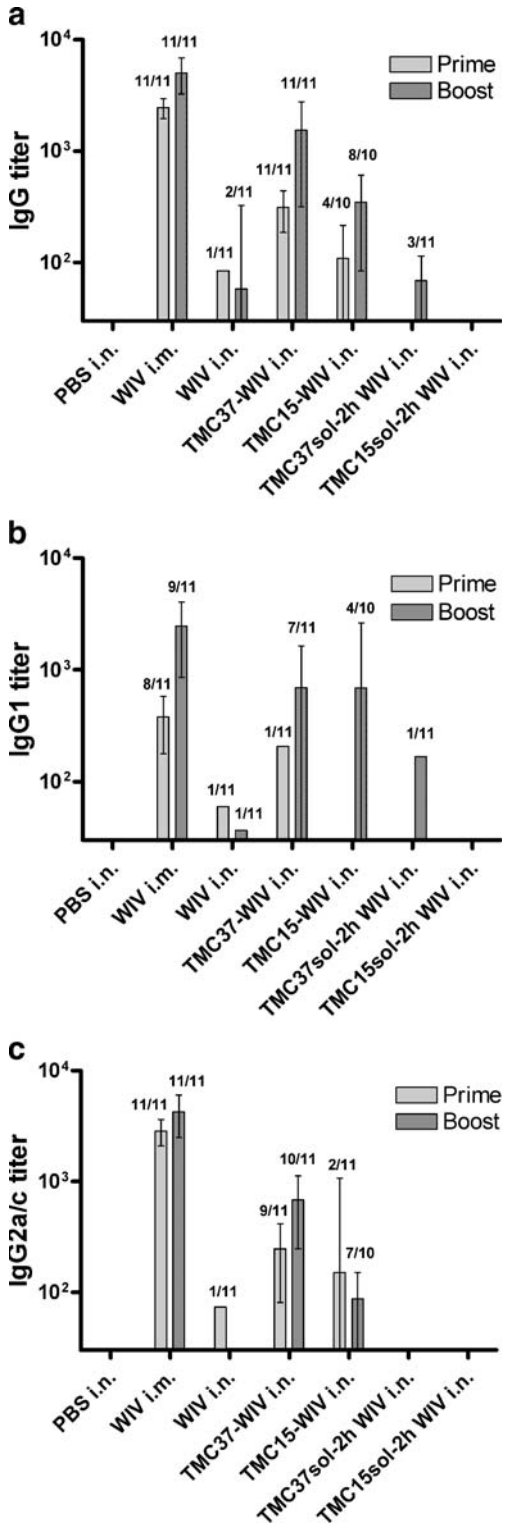

Fig 8. Geometric mean antigen specific serum IgG titers (**a**), IgG1 (**b**) and IgG2a/c (**c**) of seropositive mice 3 weeks after prime and boost vaccination. Error *bars* indicate 95% confidence intervals. Indicated above the *bars* is the number of mice that developed detectable IgG titers after boost vaccination (e.g. 3/11 indicates three out of 11 mice). All formulations were compared using a one-way ANOVA test and Bonferroni's correction for multiple comparisons.

In the pulse-chase study with TMC37, all mice lost a considerable amount of body weight. Body weight loss was more severe in the pulse-chase study with TMC15 (Fig. 9e) than with TMC37 (Fig. 9d). Moreover, most mice in the pulse-chase study with TMC15 and one mouse in the pulse-chase study with TMC37 developed clear signs of illness. These results may suggest that the TMC37 solution reduced the severity of infection slightly, although the AUC data of the TMC15 and the TMC37 group were not significantly different from that of the negative control group that received PBS i.n. So, TMC should be administered i.n. together with WIV to provide complete protection against weight loss and clinical signs of illness.

DISCUSSION

In a previous study we have shown that the most promising inactivated nonadjuvanted i.n. influenza vaccines are based on WIV (8). To further optimize WIV for i.n. vaccination, the use of the cationic mucoadhesive polymer TMC was explored in this study. We demonstrated that coating of WIV with TMC substantially improves the immunogenicity of i.n. WIV vaccine. Although some reports have been published on TMC as a nasal adjuvant (27,30), this is the first time that TMC in solution was formulated with WIV as an antigen, resulting in TMC-coated WIV. Because of the particulate character of WIV, soluble TMC can simply be added to obtain a positively charged, nanoparticulate antigen delivery system. The way to produce TMC-coated WIV formulations is extremely simple and fast, making this procedure very suitable for upscaling to large scale production. For other nanoparticulate vaccines, the large scale production can be too difficult or costly to make it to the market (37). A simple formulation procedure is especially important in the case of seasonal and pandemic flu, since the timelines for development and production of flu vaccines are extremely short (38). Future studies should be performed to determine the stability upon storage.

The presented TMC-WIV vaccines contain both WIV-associated TMC and TMC in solution. It should be further investigated what the role of free TMC is in the induction of immune responses as well as stabilization of the formulation. Both the soluble fraction and the coated TMC may contribute to the adjuvant effect, since both TMC solution and TMC nanoparticles have been reported to increase immune responses (27,30,35).

TMC15-WIV and TMC37-WIV i.n. induced strong immune responses and complete protection, whereas WIV i.n. hardly induced detectable HI and antibody titers and was not protective. Although expected from other reports (26,27,29,31) and from earlier observations in our own lab, no sIgA could be detected in the nasal washes. We can, however, not exclude the sampling and/or detection methods were suboptimal.

Our results show that TMC37-WIV induced superior immune responses to TMC15-WIV, indicating that the polymer characteristics determine their adjuvanticity. The TMC15 and TMC37 used had different molecular weights in addition to different DQs (see Table I). The differences in Mn and Mw are caused by differences in chain length of the chitosans used, chain scission during trimethylation and DQs

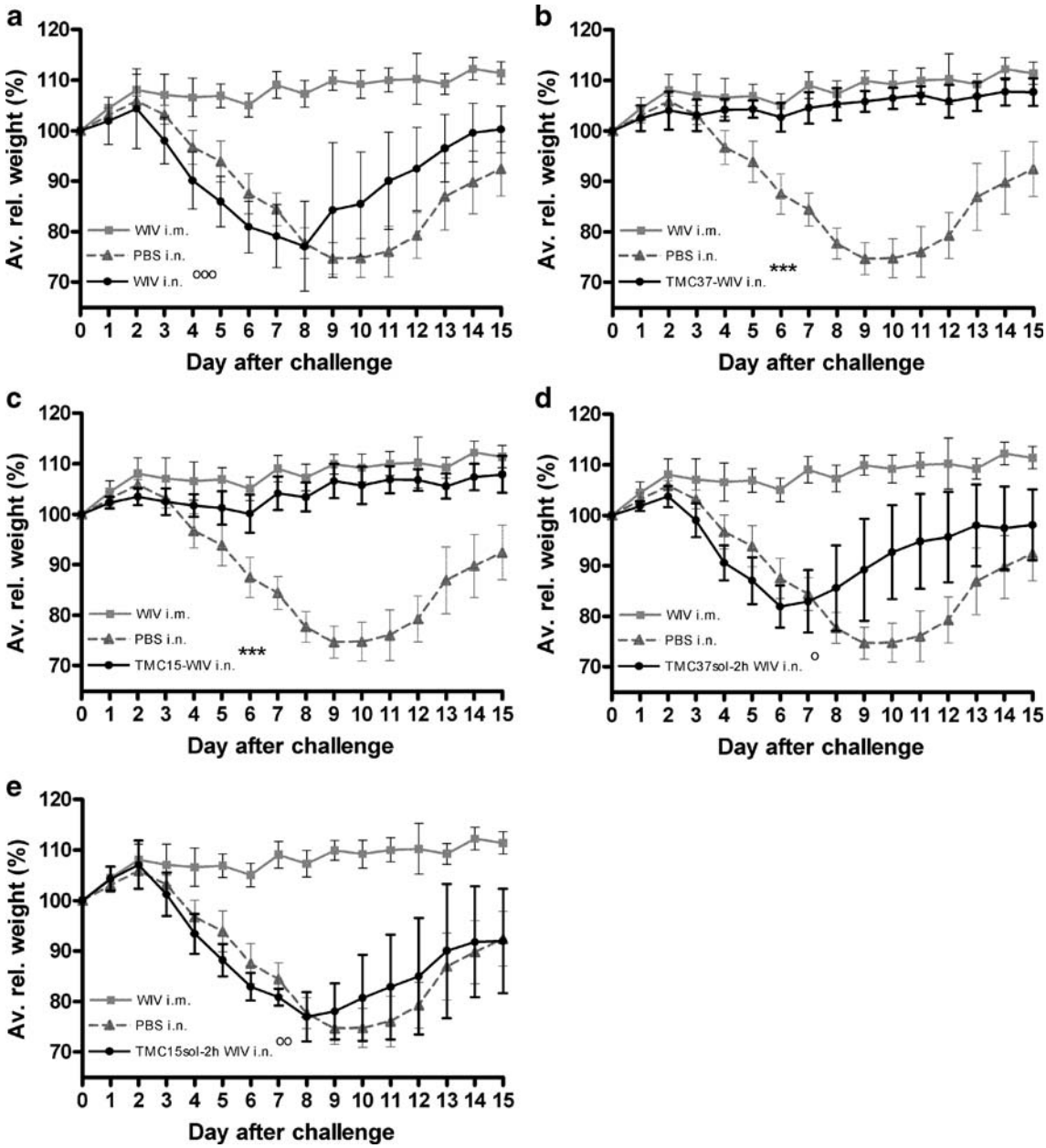

Fig 9. Average relative body weight curves after challenge of mice vaccinated with: **a** WIV i.n.; **b** TMC37-WIV; **c** TMC15-WIV; **d** TMC37sol-2 h WIV i.n. and **e** TMC15sol-2 h WIV i.n.. For comparison, each *panel* contains the curves of WIV i.m. and PBS i.n.. During the study some mice were sacrificed (§) or died due to handling (†): **a** WIV i.n., one mouse (day 9, §); **b** TMC37-WIV i.n., one mouse (day 0, †); **c** TMC15-WIV i.n., one mouse (day 14, §); PBS i.n., two mice (day 10, §). $N=8$ for all groups except for TMC15sol-2 h WIV ($n=7$). One nonresponding mouse in the TMC15-WIV group that lost weight and showed signs of illness, was sacrificed at day 15 and not included in the average body weight curve. Error *bars* indicate the 95% confidence intervals. Asterisks indicate that average AUCs significantly (***$P<0.001$) higher than the PBS i.n. group. *Circles* indicate that average AUC is significantly ($^{o}P<0.05$; $^{oo}P<0.01$ and $^{ooo}P<0.001$) lower than the WIV i.m. group.

(39). Systematic studies about the influence of molecular weight on the adjuvant properties of TMC are lacking. However, the effect of the Mw of TMC and chitosan on cell toxicity, ability to open tight junctions and drug uptake has been described (40–44). These studies indicate that small differences in Mw have a negligible influence on these parameters. Therefore, it can be anticipated that the observed differences between TMC15 and TMC37 regarding their effect on cell viability, TEER and adjuvant effect can be attributed to the different DQs.

The relation between the DQ of TMC and opening of tight junctions as well as toxicity has been studied thoroughly (28,33,45–47). TMC with a DQ of ca. 40%, has been proposed to have the strongest effect on opening of tight junctions (45,46), the strongest adjuvant properties (26) and an acceptable toxicity profile. Our results, showing increased immunogenicity and stronger decrease in TEER for TMC37 when compared to TMC15, are in line with these findings.

The quality of serum humoral immune responses was hardly affected by TMC15 and TMC37. Both TMC15-WIV

and TMC37-WIV induced a mix of IgG2a/c and IgG1 immune response, just like i.m. WIV. This is indicative of mixed Th1/Th2 immune responses, which are more favorable than biased Th2 immune responses typically elicited by nonadjuvanted subunit and split vaccines (8,48). Furthermore, these results are in line with the notion that TMC acts as an adjuvant by improving the delivery of antigens to the immune system rather than providing an immunostimulatory signal, which is the mechanism action of other adjuvants like TLR ligands and toxins. The results from the pulse-chase study also support this idea. Mice that received either TMC15 or TMC37 i.n., 2 h before WIV was administered i.n., hardly developed any immune responses. Likely, the TMC solution is already cleared from the nasal cavity or neutralized by the abundantly present, negatively charged mucus or mucosal surfaces, prior to the WIV administration. These findings are in line with the results of an earlier i.n. pulse-chase study where chitosan solution was administered 24 h prior to the antigen (22). So, TMC exerts its adjuvant effect mainly by improving the antigen delivery and must therefore be formulated and/or administered together with WIV to enhance the induction of immune responses.

Recently, two papers were published on dry powder formulations of WIV with several mucoadhesive compounds including chitosan (49,50). In these studies, a spray freeze dried WIV-chitosan formulation induced significantly lower serum IgG titers after i.n. boost vaccination than plain WIV without any mucoadhesives. This might be due to the poor solubility of chitosan at physiological pH, the very reason why we used TMC instead. Also, TMC was recently shown to enhance immune responses elicited by a powder vaccine formulation after pulmonary delivery (29).

In conclusion, TMC-coated WIV elicited strong humoral immune responses in mice upon i.n. vaccination and provided protection against signs of illness after a homologous aerosol challenge. This, together with the ease of production and the clear advantages of the intranasal route of administration, holds great promise for the further development of effective inactivated i.n. influenza vaccines. Since WIV i.m. was more potent than any of the i.n. administered vaccines, these promising results call for more research. Studies should be performed to determine the influence of other polymer characteristics on the adjuvant properties of TMC. Recent developments in TMC synthesis facilitate this research (33). Moreover, the design of TMC-WIV particles combined with other adjuvants like TLR-ligands may further improve the potency of i.n. WIV vaccines.

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