



Characterization of *Vibrio cholerae* neuraminidase as an immunomodulator for novel formulation of oral allergy immunotherapy

Susanne C. Diesner^{a,b}, Cornelia Bergmayr^a, Xue-Yan Wang^c, Denise Heiden^a, Sarah Exenberger^a, Franziska Roth-Walter^{a,d}, Philipp Starkl^a, Davide Ret^{a,e}, Isabella Pali-Schöll^{a,d}, Franz Gabor^c, Eva Untersmayr^{a,*}

^a Institute of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria

^b Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria

^c Department of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

^d The Interuniversity Messerli Research Institute of the University of Veterinary Medicine Vienna, Medical University Vienna and University Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria

^e Institute of Applied Synthetic Chemistry, Vienna University of Technology, Getreidemarkt 9, 1060 Vienna, Austria

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ABSTRACT

To improve current mucosal allergen immunotherapy *Vibrio cholerae* neuraminidase (NA) was evaluated as a novel epithelial targeting molecule for functionalization of allergen-loaded, poly(D,L-lactide-co-glycolide) (PLGA) microparticles (MPs) and compared to the previously described epithelial targeting lectins wheat germ agglutinin (WGA) and Aleuria aurantia lectin (AAL).

All targeters revealed binding to Caco-2 cells, but only NA had high binding specificity to α -L fucose and monosialoganglioside-1. An increased transepithelial uptake was found for NA-MPs in a M-cell co-culture model. NA and NA-MPs induced high levels of IFN- γ and IL10 in naive mouse splenocytes and CCL20 expression in Caco-2. Repeated oral gavage of NA-MPs resulted in a modulated, allergen-specific immune response. In conclusion, NA has enhanced M-cell specificity compared to the other targeters. NA functionalized MPs induce a Th1 and T-regulatory driven immune response and avoid allergy effector cell activation. Therefore, it is a promising novel, orally applied formula for allergy therapy.

1. Introduction

Oral immunotherapy is of special interest as an alternative therapeutic approach for allergy treatment as it can elicit systemic as well as mucosal immune responses. Its main advantage over subcutaneous injections is the painless application resulting in a higher patient compliance [1]. For oral application, drugs have to fulfill several criteria, such as digestion stability and sufficient bioavailability by overcoming the first pass metabolism [2].

One promising strategy is the use of poly(D,L-lactide-co-glycolide) acid (PLGA) particles as a carrier system. The particles protect encapsulated proteins against gastrointestinal degradation [3] and are fully biocompatible being eliminated via the Krebs cycle with no

polymer-specific immune response [2,4,5]. However, the immune system is shifted towards a Th1 milieu depending on particle size [4–6]. The efficacy of PLGA carriers can be enhanced targeting intestinal microfold cells (M-cells) by particle surface modification, as demonstrated for AAL [7].

M-cells located within the follicle associated epithelium are responsible for uptake and transport of intact particulate structures to the mucosa-associated lymphatic tissue [8,9]. Since they have a plasma membrane rich in specific carbohydrate residues, such as α -L fucose and monoganglioside (GM1) [10–12], lectins binding to the M-cell glycocalyx may serve as specific targeters on carrier systems for antigen or drug delivery to the immune system [9,13–15]. Therefore, they are perfect targets for allergen entry in oral immunotherapy.

Abbreviations: AAL, Aleuria aurantia lectin; CT, Cholera toxin; DAPI, 4',6-diamidino-2-phenylindole; FS, Fluospheres; GM1, Monosialoganglioside 1; LPS, Lipopolysaccharide; M-cell, Membranous or Microfold cells; MP, Microparticles; NA, Neuraminidase; OVA, Ovalbumin; PEMA, Poly-ethylene-alt-maleic anhydride; PLGA, Poly(D,L-lactide-co-glycolide) acid; RANTES, Regulated on activation, normal T cell expressed and secreted; RT, Room temperature; SGF, Simulated gastric fluid; SIF, Simulated intestinal fluid; TCT, N,N',N"-triaceetylchitotriose; TEER, Transepithelial electrical resistance; TMB, Tetramethylbenzidine; TSLP, Thymic stromal lymphopoietin; WGA, Wheat germ agglutinin; ZO, Zonula occludens

* Corresponding author.

E-mail address: eva.untersmayr@meduniwien.ac.at (E. Untersmayr).

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In previous experiments, functionalization of PLGA particles with AAL increased the transepithelial transport mainly via M-cells and induced a Th1-dominated immune response in peripheral blood mononuclear cells of allergic patients [7,16]. Moreover, oral application of allergen-loaded, AAL-coated PLGA-MPs to birch pollen-sensitized mice beneficially modulated an established Th2 biased immune response [13].

In this study, we aimed to exploit neuraminidase (NA) of *Vibrio cholerae* as a targeting agent due to its structural similarities with AAL [16]. In this context the structure of *Vibrio cholerae* NA is of special interest, as the catalytic center is flanked by two identical lectin-like domains [17]. Moreover, it has a high cleaving specificity removing sialic acid residues to unmask its main interaction partner GM1 [18]. As it should, thus, be able to especially bind to M-cells, we aimed to identify whether NA is superior as targeting molecule with special focus on its immunomodulatory capacity, compared to wheat germ agglutinin (WGA), as a binding molecule targeting epithelial cells, and to AAL, which is known for an enhanced M-cell specific binding [7]. Safety and suitability of oral application of NA as targeting molecule have been assessed in vitro and in vivo in the present work.

2. Materials and methods

2.1. Characterization of the targeter NA

The targeting molecule NA from *Vibrio cholerae* (Type III, sterile filtered, 1–5 units/mg protein, N7885, Sigma-Aldrich, St. Louis, MO) was tested for its gastric stability in simulated gastric fluid (SGF) as described previously and compared to the lectins AAL (L-1390) and WGA (L-1020, both from Vector Laboratories, Burlingame, CA, USA) [19]. Digestion was stopped with 1 M NaOH after 60, 120 and 180 min. The molecules were subsequently digested in simulated intestinal fluid (SIF) using 3.2 mg/ml pancreatin in a ratio of 1:5 w/w (Kreon 10.000 IE, Abbott Products GmbH, North Chicago, IL). After 5, 10, 15, 30 or 45 min digestion was stopped by non-reducing SDS-PAGE buffer and boiling, according to a modified protocol [20]. Protein integrity was evaluated by SDS-PAGE using Coomassie brilliant blue staining and silverstaining.

Binding of NA to intestinal epithelial cells in comparison to WGA and AAL was investigated in vitro by flow cytometry. Colon carcinoma cells Caco-2/Tc7 (3×10^5 cells, a kind gift of Monique Rousset, INSERM, Paris, France), with a small intestinal phenotype [21] were cultured in Dulbecco modified minimal essential cell culture medium (Gibco®, ThermoFisher Scientific, Vienna, Austria) supplemented with 10% fetal calf serum, 1% non-essential amino acids, 10 mM HEPES, 10 mM L-glutamine, 1 U/ml penicillin and 1 µg/ml streptomycin in a humidified 5% CO₂/95% air atmosphere at 37 °C. Single Caco-2 cells were washed with PBS and incubated with 16, 32, 64 or 128 µg FITC-NA or Biotin-AAL and Biotin-WGA (each 16 µg) for 30 min in suspension. For determination of background staining, cells were incubated with or without biotin-labeled IgG being followed by FITC-Avidin (Sigma-Aldrich). Acquisition was performed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) and calculated with FlowJo 9.3.3 software (Tree Star, Franklin Lakes, NJ).

To identify and characterize binding partners of NA and the lectins AAL and WGA on Caco-2 cells, inhibition experiments were performed. Caco-2 cells (1×10^5 /well) were grown for 14 days until monolayer formation in a 96-well tissue culture microplate, according to previous protocols [16]. Cells were washed and blocked with 2% bovine serum albumin in PBS for 30 min. Subsequently, biotinylated NA, AAL or WGA (all 3.47 µM) pre-incubated 1:1 (85 + 85 µl) with increasing concentrations of α-L fucose, N,N',N"-triacylchitotriose (TCT; both 79, 158, 318 µM), glycosylated or deglycosylated GM1 from bovine brain (GM1; all from Sigma-Aldrich; 0.5, 5, 10, 25 and 50 µg/ml) were added to Caco-2 cells for 60 min. FITC-Avidin (200 pmol/well) was added for 30 min and fluorescence intensity was measured at 485 nm excitation

and 530 nm emission. Values of remaining binding in the presence of inhibition molecules were calculated as percentage of the binding of non-inhibited targeting substances. Three independent sets of experiments were performed.

2.2. Preparation of functionalized particles

OVA-loaded PLGA microparticles (PLGA-MPs) were prepared by the water-in-oil-in-water solvent-evaporation technique (Supplementary Fig. 1A).

Aqueous solution of OVA (400 µl, 500 mg/ml, Grade V, lyophilized powder > 98%, Sigma-Aldrich, Lot SLBD2312V and 076 K7045) was emulsified with 400 mg PLGA in ethyl acetate by sonication for 2 min. After adding 8 ml aqueous solution of Poly-ethylene-alt-maleic anhydride (PEMA) (0.5% in w/v), the emulsion was sonicated to yield the w/o/w emulsion. After pouring the mixture into 100 ml of a 0.25% aqueous solution of PEMA, the particle suspension was stirred at 600 rpm for 1 h at room temperature (RT) in order to remove the residual ethyl acetate. The MPs were re-suspended in 20 mM HEPES/NaOH-buffer pH 7.0 and washed several times with the same buffer to remove non-encapsulated OVA. Total PLGA content was determined gravimetrically after lyophilization. Particle size distribution was determined using a Malvern Mastersizer 2000 laser particle size analyzer (Malvern Instruments). NA, AAL or WGA were covalently coupled to the surface of PLGA-MPs using a modified carbodiimide method (Supplementary Fig. 1B) [22]. As the H-type of PLGA (Boehringer Ingelheim, Ingelheim, Germany) with free carboxyl end groups was used as a matrix, amine-containing ligands were bound to the superficial protruding polymer chains via amide bonds. The carboxylates were activated with carbodiimide to yield an active ester intermediate that readily reacts with primary amines resulting in covalently linked ligand. PLGA-MP suspension (5 ml) in 20 mM HEPES/NaOH-buffer pH 7.0 was activated for 2 h at RT by adding 5 ml of a solution of 1400 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 59 mg N-hydroxysuccinimide in the same buffer. In order to remove excess reagents, the MPs were washed three times with 20 mM HEPES/NaOH-buffer pH 7.4. MPs were resuspended in 5 ml of the same buffer, 29.4 µM NA-, AAL- or WGA solution was added and incubated under rotation at RT. Non-reacted binding sites were saturated by incubation with 3 ml glycine solution (200 mg/ml) for 5 h at RT. MPs were washed three times by centrifugation to remove excess reagents, resuspended in 5 ml isotonic 20 mM HEPES/NaOH pH 7.4, and stored at –80 °C. The concentration of NA-, AAL-, and WGA on grafted PLGA-MPs was gravimetrically determined after lyophilisation. As a reference, uncoated PLGA MPs (Plain-MPs) were treated as above but solely buffer added. Concentration of encapsulated OVA was measured after dissolving the MPs in 5% SDS/0.1 M NaOH for 3 h at RT by MicroBCA protein assay kit following manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). Covalent coupling to carboxylate-modified fluorospheres (FS) (Invitrogen, Carlsbad, CA, USA) was done according to manufacturer's instructions.

2.3. Characterization of uncoated or NA-coated MPs as allergen carrier systems

SGF, SIF and SDS-PAGE experiments were done with MP preparations (NA-, AAL, WGA-grafted MPs filled with OVA) as described above (2.1). OVA was released from MPs by incubation in 5% SDS/0.1 M NaOH for 3 h at RT and protein integrity was evaluated by SDS-PAGE followed by Coomassie brilliant blue staining.

The presence of NA, WGA and AAL on the surface of MPs was confirmed by ELISA and Western blot. For both techniques, MPs were dissolved in 5% SDS/0.1 M NaOH for 3 h at RT. The dissolved MPs were coated on a plate overnight or were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking, particle-surface bound functionalization substances were detected by using NA,

WGA- or AAL-specific polyclonal mouse antibodies, followed by anti-mouse IgG horseradish-peroxidase-labeled antibodies (Abcam, Cambridge, UK). TMB (BD Biosciences) was used as substrate for ELISA, Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used for Western blot.

To rule out a strong endotoxin contamination, lipopolysaccharide (LPS) levels of the targeters and the functionalized microparticles were determined using Endosafe-PTS cartridges (Charles River Laboratories, Wilmington, MA, USA) according to manufacturer's instructions.

2.4. Binding of NA-coated particles on epithelial Caco-2 cells and M-cells

To test whether NA-coated FS bind to intestinal epithelial cells, Caco-2 cells were grown on uncoated glass chamber slides (SPL Lifesciences, Pocheon, Korea) overnight. Cells were washed with ice-cold PBS and coated FS were added for 60 min at 4 °C. Cells were fixed, permeabilized and unspecific binding was blocked with 1% bovine serum albumin in PBS for 30 min at RT. Cells were stained with an α -tubulin antibody followed by an anti-mouse IgG Alexa Fluor 568 antibody (Life Technologies) for detection. The nucleus was visualized with DAPI (4',6-diamidino-2-phenylindole, 1:5000, Life Technologies) staining. Acquisitions were done using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany).

For co-culture experiments, Caco-2 cells (5×10^5 cells/filter) were seeded on transwell filters (Corning Incorporated Nr 3402, Costar Transwell 12 wells, 3 μ m pore size, diameter 12 mm), cultured for 21 days until transepithelial resistance (TEER) reached 300–400 Ω /cm² and then co-cultured with Raji-B cells (1×10^6 Raji cells/filter; ATCC Raji (ATCC® CCL-86)) for the last 4 days of incubation period on the basolateral side of the epithelial layer as described [23]. Tight junction integrity was confirmed by TEER measurements and M-cell formation was verified by measurement of alkaline phosphatase activity (Phosphatase substrate, 5 mg tablets, Sigma Aldrich) [23,24]. After 1 h incubation with phenolred-free RPMI, NA-, AAL- and WGA-coated FS (150 μ g/ml, 500 μ l per well) were added to the apical side of the epithelium for 120 min. FS transported through the epithelium against gravitation were collected from the basolateral side, centrifuged and dissolved in 0.1 M NaOH. The fluorescence intensity of transported FS was quantified at 480/530 nm. As control, uptake studies were performed with Caco-2 cells cultured without Raji-B cells. Statistics were calculated from 6 wells per condition.

2.5. Real-time PCR of stimulated Caco-2 cells

To investigate the effects of particle stimulation on gene expression of intestinal epithelial cells, Caco-2 cells (2.28×10^5 cell/well) were seeded in 12-well tissue culture plates and cultivated for 21 days. Cells were stimulated in four independent experiments either with the targeters NA, AAL or WGA (10 μ g/ml) or with NA-, AAL-, WGA-coated or uncoated OVA-loaded MPs (10 μ g/ml) for 1, 2, 3 and 6 h or were left unstimulated as negative control. Cells were harvested with 700 μ l TRIzol reagent and RNA was isolated according to RNeasy Mini Kit protocol (Qiagen) without DNase treatment. Final RNA was eluted in 30 μ l RNase free water and RNA concentrations as well as purity were measured with nanodrop ND-1000 Spectrophotometer (peqLab Biotechnologie GmbH). Reverse transcriptase PCR was performed using the High Capacity cDNA Reverse Transcription Kit without RNase Inhibitor (Applied Biosystems) and final cDNA was diluted 1:5 in nuclease free water. Quantitative realtime PCR according to SYBR Green Mix protocol in 384 well plates (384 well plate micro AMP Optical 384 well reaction plate with barcode; Applied Biosystems; 4,309,849) using the following primers: Claudin-1 (5'-CAGTCAATGCCAGGTACGAA TTT-3'; 3'-AAGTAGGGCACCTCCCAGAA-5'), claudin-4 (5'-TGTACCA ACTGCCTGGAGGAT-3'; 3'-GACACCGGCACTATCACCATAA-5'), ZO-1 (5'-ACAGTGCCTAAAGCTATTCCTGTGA-3'; 3'-TCGGGAATGGCTCCTT GAG-5'), ZO-2 (5'-CGGTAAATACCGCTGAGGCAAA-3'; 3'-GGGAACCA

CTGGGTGTAATTCA-5') (all from Sigma Aldrich) was performed with 7900HT Fast Real Time PCR machine (Applied Biosystems) to investigate the changes of tight junction expression after cell stimulation. To determine the changes of cytokine expression in Caco-2 cells after stimulation quantitative realtime PCR according to TaqMan Universal Master Mix Protocol (Applied Biosystems) using the following TaqMan assays: CCL20 (TaqMan Hs01011368_m1), RANTES (TaqMan Hs00982282_m1), TSLP (TaqMan Hs00263639_m1), β -actin (TaqMan Hs99999903_m1) (all FAM labeled assays from Applied Biosystems) was performed using β -actin as endogenous control. PCR reactions were performed in technical triplicates for each of the four independent experiments. Significant outliers were calculated and excluded from statistical analysis.

2.6. Animals

Healthy female BALB/cAnNCrl mice (6–8 weeks, 15–20 g, provided with health report certificate) were purchased from Charles River Laboratory or from the Institute of Laboratory Animal Science and Genetics (Medical University of Vienna, Himberg, Austria) and housed under conventional conditions (at 22 °C at a 12 h light/dark cycle) in groups of 5–8 in polycarbonate Makrolon type II cages with filter tops and espen wood bedding (both Ehret GmbH, Emmendingen, Germany) enriched with nesting material. Mice were fed with a special egg-free diet (Ssniff, Soest, Germany) with free access to food and water. Experimental procedures were started after a 2-weeks acclimation period in a separate animal experimentation room by treating animals in random order within each group. Sample size calculation was based on previous own data [19] with a power calculation based on two-sided, two-sample *t*-test. The concept of replacement, refinement and reduction had a fundamental impact on study design of the approved ethical protocol. Primary outcome of the animal studies was to investigate immunomodulatory capacity and safety of oral application of OVA-loaded NA-MPs in comparison to WGA- and AAL-MPs.

Animals were treated according to European Union rules of animal care and with approval by the local ethics committee and the Austrian Federal Ministry of Science and Research (permission numbers GZ BMWF-66.009/0051-II/10b/2008 and BMWF-66.009/0070-II/3b/2012).

2.7. In vitro spleen cell stimulation experiments

Naïve mouse spleen cells, isolated as described previously [19], were stimulated in a 96-well round-bottom tissue culture-plate (4×10^5 cells/well) either with NA, WGA, AAL or the functionalized, OVA loaded microparticles, OVA alone or the positive control concanavalin A (ConA, Sigma-Aldrich) (all 5 μ g/ml) or medium as negative control (ctrl) for 72 h. Supernatants were analyzed for their cytokine levels using IL4, IFN- γ and IL10 Ready-set-go ELISA kits (eBioscience, San Diego, CA) following manufacturer's instructions. Cell supernatants were diluted 1:2. Cytokine concentrations were calculated according to a standard curve.

2.8. Mouse mast cell protease-1 measurement after single MPs gavage

For analysis of immediate mast cell activation, mice were gavaged once with the respective MPs preparations and sera taken 1 h after gavage were screened for mouse mast cell protease-1 (mMCP-1) using the mouse MCPT-1 Ready-set-go ELISA Kit (eBioscience, San Diego, CA) according to manufacturer's instructions. Rectal body temperatures were measured 30 min, 1 h, 3 h, 6 h, 8 h, 24 h, 48 h and 72 h after single gavage using a Thermalert TH-5 thermometer (Physitemp Instruments Inc., Clifton, NJ).

2.9. Safety evaluation of repeated gavages of MPs in naïve mice

To evaluate the safety of repeatedly orally applied MPs, naïve mice ($n = 8$) were fed 6 times every other week with Plain- or NA-, AAL- or WGA-MPs containing 200 µg OVA on 3 consecutive days. Serum was collected before and 14 days after the last MP application (Supplementary Fig. 2). Sixteen days after the last oral application cycle, mice were orally challenged (OC) with OVA (50 mg per mouse). Rectal temperature as indicator of anaphylaxis was measured before and 15 and 30 min after OC.

2.10. Safety evaluation of repeated MPs gavages in OVA-allergic mice

To further evaluate the safety of repeatedly orally applied OVA-loaded MPs, mice ($n = 8$) were immunized intraperitoneally (i.p.) with 2 µg OVA adsorbed to 2% aluminum hydroxide solution (1.3 µg Al (OH)₃) in 2 weeks intervals. Thereafter, mice were orally challenged (OC) with PBS or 50 mg OVA to induce local inflammation and to evaluate the allergic immune response. Thereafter, mice were fed 6 times every other week with Plain- or NA-, AAL- or WGA-MPs containing 200 µg OVA on 3 consecutive days. Serum was collected before and during sensitization as well as before, during and 14 days after the last MP application (Supplementary Fig. 3). After the last oral application cycle, mice were orally re-challenged (OC) with PBS or OVA (50 mg per mouse). Rectal temperature as indicator of anaphylaxis was measured before and 15 and 30 min after OC.

2.11. mMCP-1 and OVA-specific IgG1, IgG2a, IgA and IgE ELISA

Mouse mast cell protease-1 (mMCP-1) levels in serum taken 1 h after OC were detected as described above. Sera taken after the last oral application from immunization described in paragraph 2.9. or after sensitization (MIS 6) and after MP gavages (MIS 12) from immunization described in paragraph 2.10. (Supplementary Figs. 2 and 3) were diluted 1:100 and concentrations were calculated according to a standard curve. Sera were screened for OVA-specific IgE and IgA by ELISA, as described previously [19]. Sera were diluted 1:200 for IgA and 1:20 for IgE. Rat anti-mouse IgA and IgE (all from BD Biosciences) were diluted 1:500 and horseradish peroxidase-labeled goat anti-rat IgG (GE Healthcare, Buckinghamshire, UK) 1:1000, respectively. Sera taken before immunizations were defined as background levels and were subtracted from sera post immunizations. Concentrations were calculated according to a standard curve.

2.12. Total and OVA-specific IgA and IgE in intestinal lavages

After sacrifice, intestines were removed and flushed with 2 ml ice-cold PBS containing protease inhibitors (Roche, Rotkreuz, Switzerland). Intestinal lavage fluid was screened for mucosal total IgA. Microtiter plates were coated with rat anti-mouse IgA (0.1 µg/well, BD Biosciences). After blocking, standard dilution series or mucosal lavage fluid (dilution 1:1000), were added. For detection, a biotin-labeled anti-mouse IgA antibody (0.1 µg/well, BD Biosciences) was used, followed by Streptavidin-HRP (eBioscience). As substrate TMB (BD Biosciences) was used and stopped with hydrochloric acid before measuring optical density at 450–630 nm. OVA-specific IgA was measured in intestinal lavage fluid (undiluted samples) as described above for serum OVA-specific IgA.

2.13. Cytokine determinations

After sacrifice, mouse splenocytes were isolated and stimulated as described above either with OVA or the positive control concanavalin A (ConA, Sigma-Aldrich) (all 5 µg/ml) or medium as negative control for 72 h. Cytokine levels of supernatants were analyzed for IL4, IFN-γ and IL10 using the Ready-set-go ELISA kits (eBioscience, San Diego, CA) as

described above. Cytokine concentrations were calculated according to a standard curve.

2.14. Statistical analysis

Data of inhibition experiments were statistically compared with the GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) software using the two-way ANOVA and Bonferroni post-test. Fluorescent intensity results of different FS and real-time PCR experiments as well as antibody concentration, comparison of body temperature and cytokine levels of in vivo studies were analyzed by one-way ANOVA and Tukey's multiple comparison test if normally distributed. For non-parametric testing Dunn's multiple comparison test was used. Co-culture results were statistically compared using two-way ANOVA after logarithmic transformation. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of NA

3.1.1. In vitro characterization of NA as novel targeting substance

To evaluate its suitability for oral application and, thus, its digestion stability, NA was subjected to simulated digestion experiments (SGF and SIF) and compared to the lectins AAL and WGA, which have been used in previous experiments for microparticle functionalization [13,16]. In SGF experiments (Fig. 1A) all three coupling molecules remained stable for up to 180 min. Protein bands of NA ranged from 20 to 90 kD under denaturing conditions. AAL revealed its expected monomeric protein band at 36 kD and WGA, which appears as a dimer at 36 kD under neutral pH conditions, formed a monomeric protein band at 18 kD in SDS-PAGE [25,26]. In subsequent SIF experiments, the targeters remained stable for up to 45 min (data not shown).

The binding of the targeting molecules to epithelial cells was investigated using Caco-2 cells, which exhibit a small intestinal epithelial phenotype and which have been widely used for investigating the potential of lectins as targeting molecules [27–29]. FITC-labeled NA bound to Caco-2 cells in a dose dependent manner (Fig. 1B) as analyzed by flow cytometry. In these experiments, biotinylated WGA and AAL revealed an even higher fluorescence intensity compared with NA, which might be explained by signal enhancement via the FITC-streptavidin-biotin complex in comparison to NA being FITC labeled alone.

The specific binding partners of the targeting molecules were investigated in cellular inhibition experiments. Binding of NA to Caco-2 cells was inhibited in a concentration dependent manner (Fig. 1C) by the addition of α-L fucose (reduced binding up to 40%; mean inhibition with 318 µM α-L fucose) and by GM1 (inhibition up to 25.4%; mean inhibition with 50 µg/ml), surrogating also an affinity to glycosylated GM1, but not by TCT. The binding of AAL to α-L fucose was lower (reduced binding up to 30%; mean inhibition with 318 µM α-L fucose). TCT expectedly reduced the binding of WGA substantially (up to 50%; mean inhibition with 318 µM TCT). No binding inhibition of AAL or WGA was found with GM1 (data not shown).

3.1.2. Immunomodulatory effect of NA on Caco-2 cells and splenocytes in vitro

The immunomodulatory capacity of NA in comparison to WGA and AAL on lymphocytes and epithelial cells was investigated. Spleen cells of naïve BALB/c mice were stimulated with the targeters and cytokines were measured. NA and AAL induced a significant up-regulation of the Th1 cytokine IFN-γ compared to WGA and the negative medium control (Fig. 2A). The Th2 cytokine IL4 was not induced by any of the targeting molecules (Fig. 2B). Only NA increased the T-regulatory cytokine IL10 in comparison to WGA, AAL and the control (Fig. 2C). ConA was used as positive control and induced significantly higher cytokine levels compared to all other stimulation conditions (data not shown).

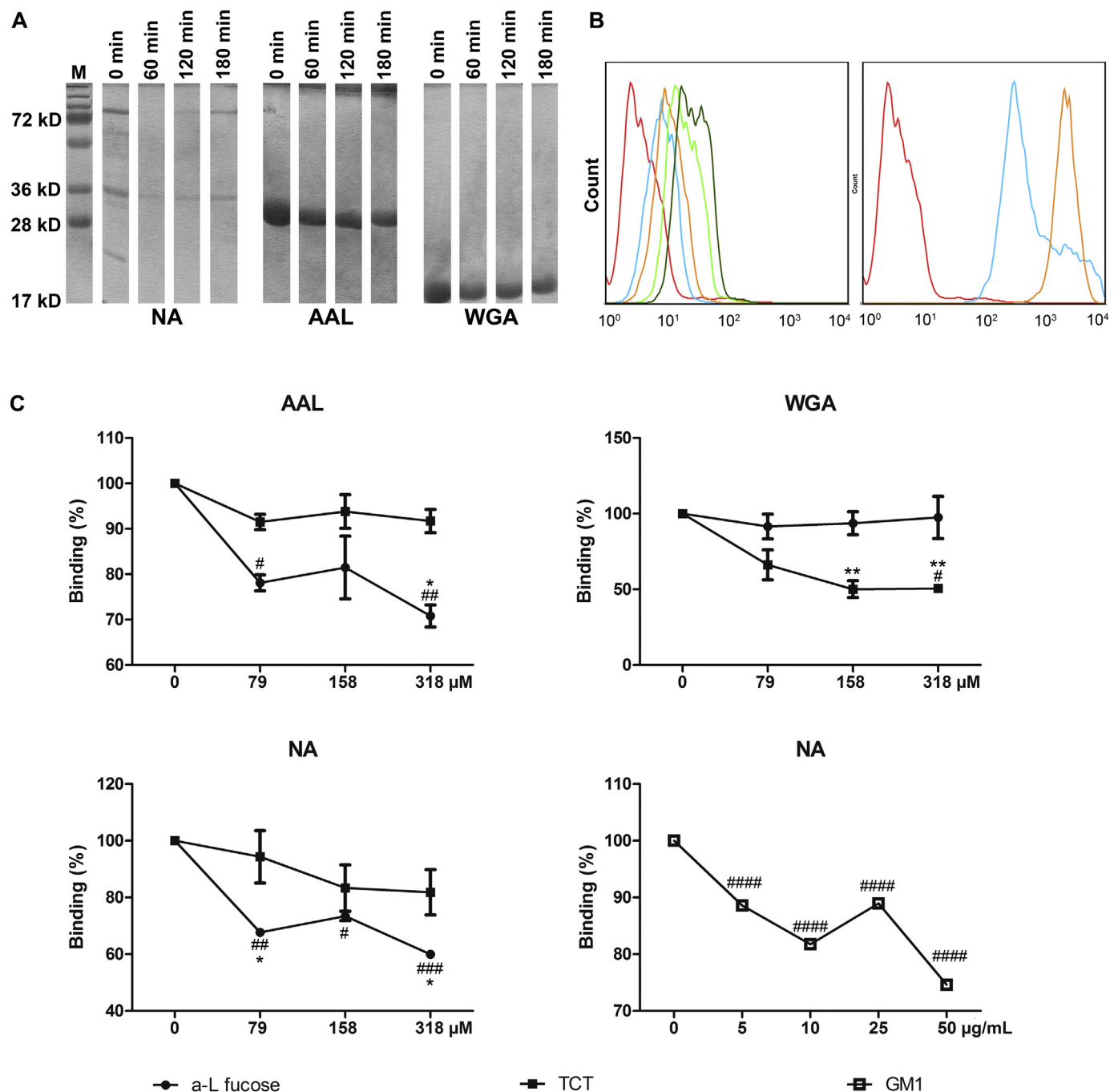


Fig. 1. Characterization of NA as functionalization substance.

NA, AAL and WGA remained stable up to 180 min in SGF experiments (A) and bound to Caco-2 cells in a dose-dependent manner (B; FITC-NA: 16 µg: blue line, 32 µg: orange line, 64 µg: light green line, 128 µg: dark green line versus MFI control (red line) left panel, WGA: light blue line, AAL: orange line versus MFI control (red line). AAL was inhibited by α-L fucose and WGA by TCT. GM1 and α-L fucose inhibited NA binding to Caco-2 cells in a dose-dependent manner (C, n = 3). Data are presented as mean ± standard error of mean (SEM). [#]P, statistical significant differences to the uninhibited control (0 µM); *P, significant differences between α-L fucose and TCT inhibitions at same concentrations. *P/[#]P < 0.05, **P/[#]P < 0.01, ***P < 0.001, ****P < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The immunomodulatory capacity of NA compared to AAL and WGA on epithelial cells was assessed in vitro using Caco-2 cells. Significant upregulation was observed only for CCL20 after NA stimulation, with a maximum after 3 h, compared to AAL and WGA (Fig. 2D). No significant differences between the targeter groups were found for TSLP, RANTES and the tight junction molecules zonula occludens-1 (ZO-1) and ZO-2, as well as for claudin-1 and claudin-4 (data not presented). IL8 was substantially induced after NA stimulation but levels were not significantly different from AAL and WGA stimulation (data not

shown).

3.2. Characterization of NA-functionalized particles

3.2.1. Preparation of NA- and lectin-functionalized microparticles

NA or the lectins WGA and AAL were coupled to PLGA microparticles, which serve as antigen carrier being loaded with OVA as representative allergen (Supplementary Fig. 1). The mean diameter of OVA-loaded MPs was 3.9 µm and the allergen-content, measured before

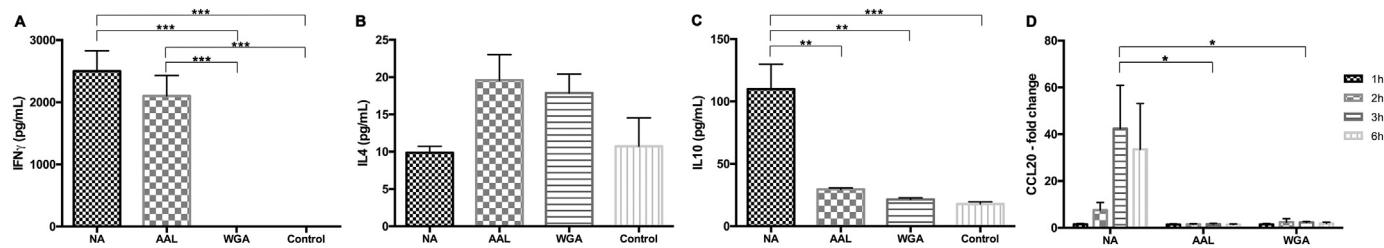


Fig. 2. Safety evaluation and immunomodulatory properties of the targeting molecules NA, AAL and WGA.

NA induced significantly higher IFN- γ (A) and IL10 (C) production in naive spleen cells, while IL4 was not increased (B). Caco-2 cell stimulated with the targeting molecule NA revealed significantly higher induction of CCL20 compared to AAL and WGA with a maximum peak after 3 h (D). (n = 4), data are mean + SEM, *P < 0.05, **P < 0.01, ***P < 0.001.

functionalization, was $164.2 \pm 9.3 \mu\text{g}$ OVA/mg particles.

Efficient coupling of NA, WGA and AAL to MPs was confirmed using a biconchonic acid assay as well as by ELISA and Western Blot. No changes in the diameter after NA-coupling were observed.

3.2.2. Epithelial binding of NA-functionalized particles

After coupling, we aimed to test the binding of the functionalized particles to epithelial cells in vitro. The functionalization of particles increased the binding to Caco-2 cells compared to plain particles (representative images are shown in Fig. 3A). Especially NA- and AAL-coated particles showed an enhanced binding to Caco-2 cells.

However, NA does not only bind to epithelial cells, but shows a significantly enhanced transepithelial uptake via M-cells in a human M-cell like in vitro model [23]. An intact epithelial monolayer was confirmed by TEER measurements ($300\text{--}400 \Omega/\text{cm}^2$ before uptake studies). When M-cells are present, the transepithelial uptake of NA-particles and AAL-particles was significantly enhanced compared to monoculture of Caco-2 cells alone, where M-cells are missing (P = 0.003) (Fig. 3B). The M-cell binding of NA-particles was even significantly higher compared to particles coated with the well characterized M-cell binding lectin AAL, indicating superiority of NA over AAL coupling with regard to M-cell targeting.

3.2.3. Digestion stability of encapsulated OVA

As encapsulation should protect the antigen from digestion, OVA-loaded Plain-, NA-, AAL- or WGA-MPs were tested for their stability in SGF experiments. SDS-PAGE analysis of samples exposed to SGF revealed that encapsulated OVA (represented as a band of 45kD) remained stable for up to 120 min of SGF digestion representing the average gastric transit time (Fig. 4) and up to 45 min in simulated intestinal digestion (data not shown). In contrast “unprotected” OVA proteins were degraded within 30 min. Coupling of NA to particles reduced endotoxin-levels 19-fold compared to free, unbound NA, emphasizing that observed immunological changes were not due to high LPS content of MPs. Endotoxin levels of WGA- and AAL-MPs was below the detection limit (data not shown).

3.3. In vitro and in vivo safety assessment of OVA-loaded NA-MPs

3.3.1. Immunomodulatory capacity of OVA-loaded NA-functionalized MPs

Naive spleen cells were stimulated with the respective MP formulations as described above for the targeting molecules alone. Only NA-MPs induced a significantly higher OVA-specific production of the Th1 cytokine IFN- γ (Fig. 5A) and the T-regulatory cytokine IL10 (Fig. 5C) compared to all other MP formulations and to medium alone (control). The Th2 cytokine IL4 was not upregulated in the tested groups (Fig. 5B).

The immunomodulatory capacity of the respective MPs formulations on Caco-2 was evaluated in real-time PCR experiments. CCL20 was highly upregulated during NA-MPs stimulation (mean CCL20 fold increase 28 after 3 h stimulation, Fig. 5D), which was not observed for

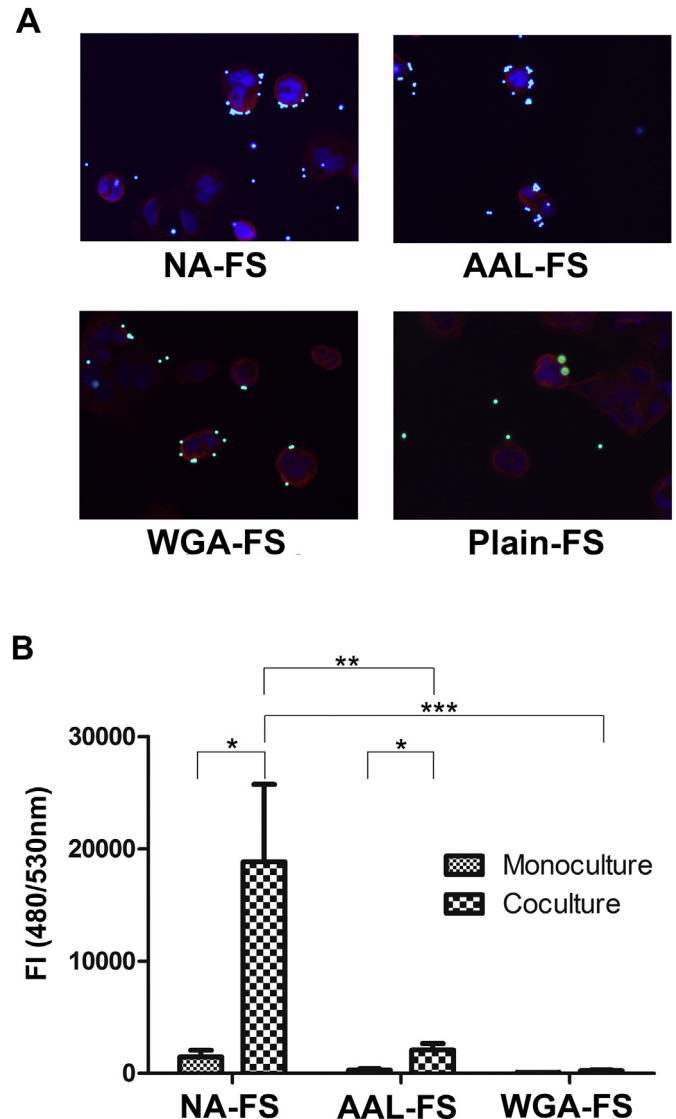


Fig. 3. Efficient binding of NA-FS to Caco-2 cells and M-cells.

Binding of FS to Caco-2 cells was higher when the particle surface was functionalized with NA, AAL or WGA compared to the negative control (Plain-FS) (A). The transepithelial uptake of both, NA- and AAL-FS, was significantly higher when M-cells are present (co-culture vs. monoculture) (B). In the presence of M-cells (co-culture) NA-FS were more efficiently transported through the epithelium compared to AAL-FS and WGA-FS. (n = 3), data are mean + SEM, *P < 0.05, **P < 0.01, ***P < 0.001.

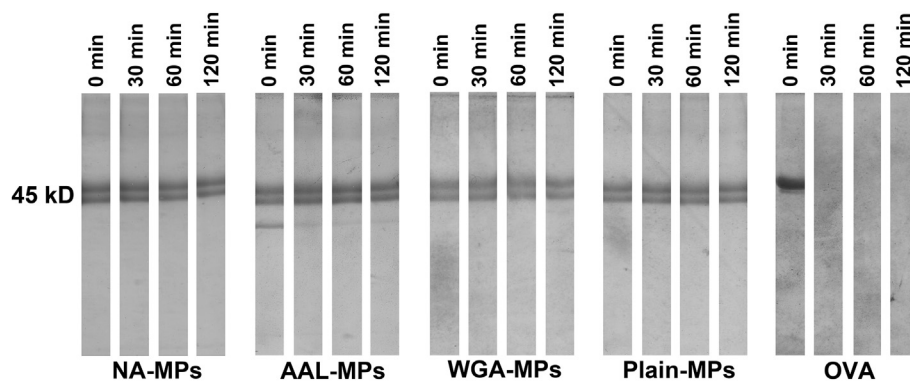


Fig. 4. Digestion evaluation of functionalized MPs.

Encapsulation of OVA in uncoated MPs (Plain-MPs) or functionalized MPs (NA-MPs, AAL-MPs, WGA-MPs) ensured protein integrity up to 120 min in SGF experiments in contrast to unprotected OVA.

the other formulations. AAL-MPs induced a mean CCL20 fold increase of 16 after 2 h of stimulation. No differences between the groups were found for the tight junction molecules ZO-1, ZO-2, claudin-1 and claudin-4 or for TSLP and RANTES (data not shown).

3.3.2. In vivo safety evaluation

For safety evaluation in vivo, naïve BALB/c mice received a single oral gavage of the respective OVA-loaded Plain-, NA-, AAL- or WGA-MPs preparations.

Single oral application of OVA-loaded NA-MPs did not induce mast cell activation, indicated by negative mMCP-1 levels in sera (data not shown). This was accompanied by a stable rectal temperature up to 72 h, as a marker of anaphylaxis, after oral MP gavage (data not shown).

For further safety evaluation, naïve BALB/c mice were repeatedly fed with OVA-loaded functionalized MPs (Supplementary Fig. 2).

Sera after the last MPs gavage were screened for OVA-specific antibody levels. No OVA-specific IgE was induced as measured titers were significantly lower in the groups fed NA-MPs, WGA-MPs and Plain-MPs than in naïve animals, the latter representing background IgE levels (Fig. 6A).

Significantly higher levels of OVA-specific IgA were observed in the groups fed NA-MPs and AAL-MPs compared to WGA-MPs, Plain-MPs and naïve animals (Fig. 6B), while OVA-specific IgG1 and IgG2a levels were not significantly different between the groups (data not shown). Mice were further orally challenged with OVA after the repeated MP gavages to evaluate the potential of anaphylaxis in these animals. No decline of body temperature as marker of anaphylaxis was observed in any of the groups. Additionally, no activation of mucosal mast cells, indicated by mMCP-1 levels, was found after oral OVA challenge (data not shown).

Screening intestinal lavage after oral provocation for antibody levels revealed significantly lower OVA-specific IgA titers in the group fed AAL-MPs compared to naïve animals, while all other groups were only

substantially, but not significantly lower (Fig. 6C). Total IgA in intestinal lavage was comparable between all groups (Fig. 6D). No OVA-specific induction of cytokines (IFN γ , IL4 or IL10) was observed (data not shown).

In a third in vivo approach, systemically sensitized, OVA-allergic mice were fed 6 times with OVA-loaded functionalized MPs (Supplementary Fig. 3). After MP gavages, there was a slight decrease of IgE in NA-MP fed mice (Fig. 7A) and a marginal increase of IgA antibody levels in all allergic mice (Fig. 7B) comparing titers from before to after MP gavages, without reaching statistical significance. Comparable results were observed for IgG1 and IgG2a with no significant changes from before to after MP gavages (data not shown). To evaluate the potential of anaphylaxis induction by repeated oral gavages of OVA-loaded MPs in highly allergic animals, core body temperature was measured. The different MPs had no effect on core body temperature from before to after oral MP feedings (data not shown). Additionally, we did not observe elevated mediator release (mMCP-1) upon oral OVA challenge following 6 cycles of MP gavages (data not shown). While no differences were found for IL4 titers between the different groups (data not shown), significantly elevated OVA-specific IFN γ and IL10 levels were measured in splenocyte supernatants in the group receiving 6 cycles of NA-MPs.

4. Discussion

In this study we investigated the suitability, safety and functionality of NA as a novel PLGA microparticle functionalization substance for epithelial targeting with future implication as oral immunotherapy of allergy.

NA is encoded within a pathogenicity island of the *Vibrio cholerae* genome [30]. It is able to hydrolyze complex gangliosides on intestinal epithelial cells to yield GM1 resulting in high local concentrations of GM1 [31,32]. Thereby, NA enhances its own binding partner on enterocytes and M-cells and might increase the intestinal residence time.

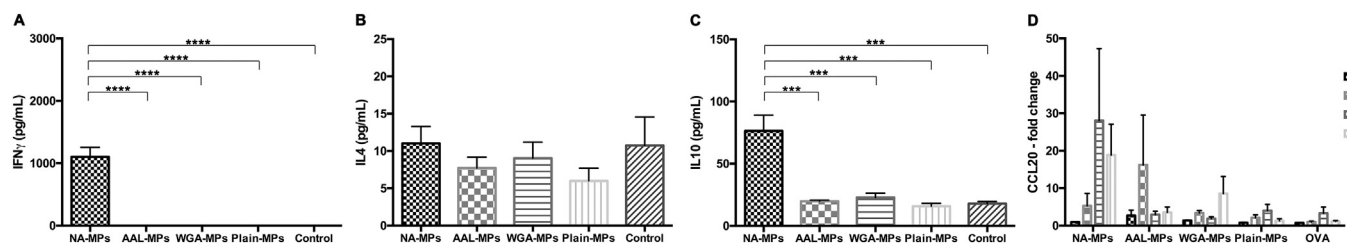


Fig. 5. Safety and immunomodulatory properties of NA-MPs.

Spleen cells of naïve mice were stimulated with the respective functionalized MPs preparation. NA-MPs induced significantly higher levels of IFN- γ and IL10 measured in spleen cell supernatants (A and C), but did not affect IL4 levels (B). Caco-2 cell experiments revealed an induction of CCL20 after 3 h of NA-MPs stimulation compared to the other MP preparations (D). (n = 4), data are mean + SEM, ***P < 0.001. ****P < 0.0001.

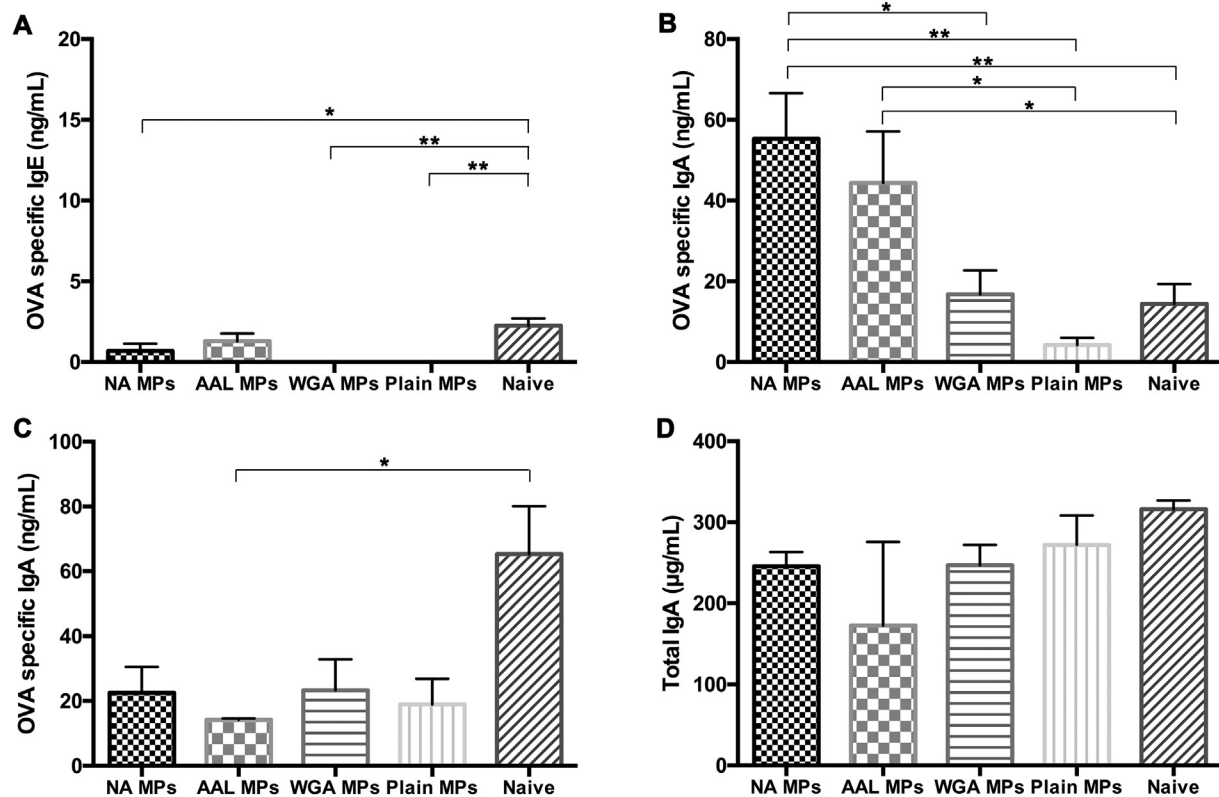


Fig. 6. Repeated gavages of NA-MPs do not induce IgE responses.

After 6 cycles of oral application of MPs preparations (see Supplementary Fig. 2), no induction of OVA-specific IgE in serum was found (A). OVA-specific IgA was significantly higher in the NA-MPs and AAL-MPs fed mice (B), with the latter MPs inducing also significantly reduced OVA-specific IgA in intestinal lavages compared to naive animals (C). No significant differences were observed for total IgA in intestinal lavage (D). (n = 8), data are mean + SEM, *P < 0.05, **P < 0.01.

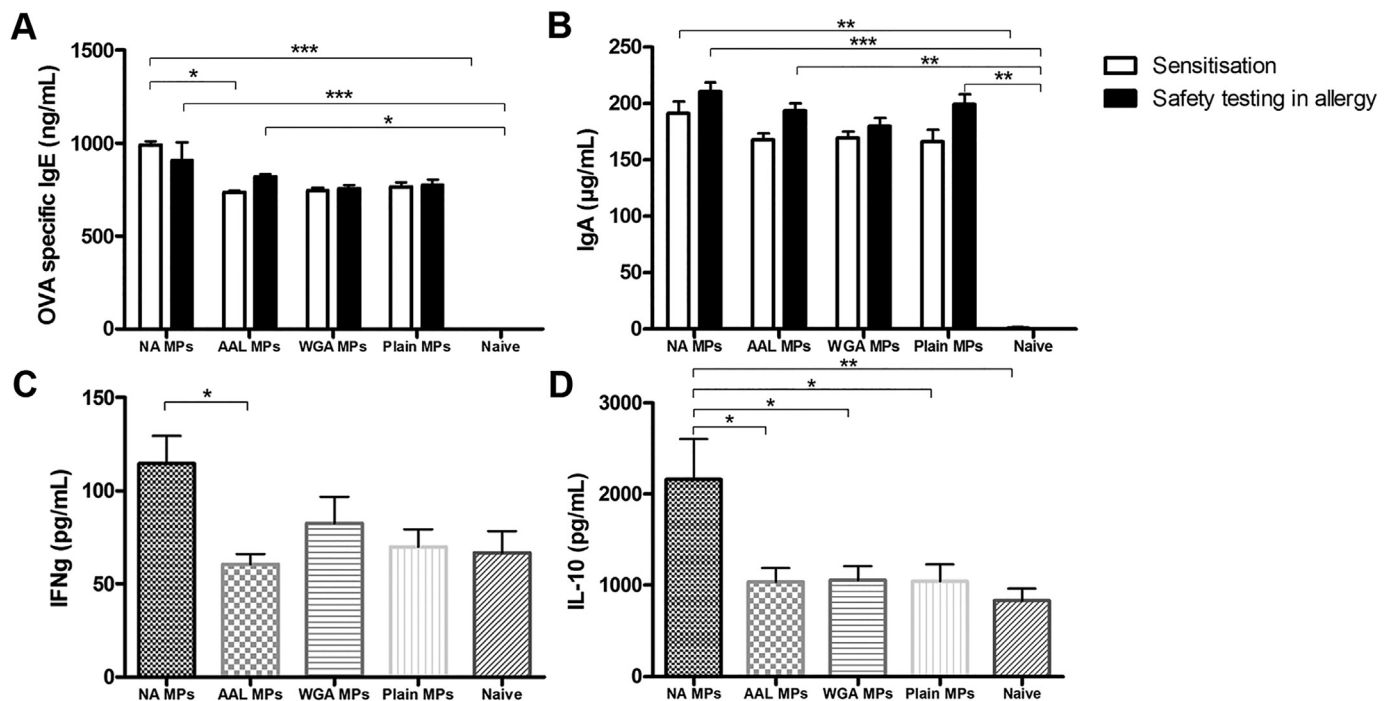


Fig. 7. Systemic immunomodulation is observed after repeated gavages of NA-MPs.

After 6 cycles of oral application of MPs preparations to allergic mice (see Supplementary Fig. 3), no elevation of OVA-specific IgE (A) and IgA (B) serum levels was measured. Six cycles of oral feeding of OVA-loaded NA-MPs was associated with elevated levels of IFN-γ (C) and IL10 (D) production by mouse splenocytes upon OVA stimulation. (n = 8), data are mean + SEM, *P < 0.05, **P < 0.01, ***P < 0.001.

NA is part of the mucinase complex, and as such, is an important virulence factor, which enhances the pathogenicity of *Vibrio cholerae*. By degrading the mucin layer of the gastrointestinal tract, it facilitates bacterial penetration. Thus, NA-coated particulate carriers might have improved access to intestinal cells, and in particular to M-cells, which are protected by only a thin mucus layer [17]. NA was even reported to promote cholera toxin (CT) binding to GM1 [32], which might contribute to the pro-allergic effect of CT induced by cAMP overproduction [33]. However, due to its functionality NA is not able to stimulate cAMP overproduction like CT, which was confirmed by the absence of pro-allergic effects in our results. Our in vitro data support the assumption that, similarly to the lectin AAL, NA binds through α -L fucose and additionally through GM1. As α -L fucose and GM1 are both highly expressed on M-cells [34,35], NA might represent a promising tool not only to enhance the residence time on intestinal epithelial cells but also to target M-cells directly leading to an enhanced transepithelial uptake of allergen loaded microparticles. Indeed, binding and enhanced transepithelial transport of NA-MPs compared to the epithelial binding of the lectins WGA but also AAL, which has been shown to have M-cell specificity in previous studies [7], was confirmed using the human Caco-2 cell line co-cultured with Raji B-cells for an M-cell-like model [23,24]. This emphasizes the superiority of NA-functionalization for PLGA particles over the previously described lectins WGA and AAL.

PLGA microparticles protected OVA, as our model food allergen from gastric and intestinal degradation due to encapsulation, ensuring that intact allergens reach the intestinal immune system. In addition, NA was revealed to be extremely stable under gastric and intestinal proteolytic conditions and, thus, represents an ideal targeting molecule for the oral route [19,36]. The particulate nature of MPs ensures uptake via the Peyer's patches and enables the release and presentation of intact allergen to immune cells residing underneath the M-cells.

However, as NA is part of the pathogenicity island of *Vibrio cholerae*, a thorough safety evaluation of this novel therapeutic formulation was pivotal. NA per se was able to induce secretion of Th1 as well as immunosuppressive cytokines like IFN- γ and IL10 in vitro, which are beneficial in allergen immunotherapy to counterbalance the Th2-biased immune response present in allergy. This cytokine induction was even more profound with NA compared to the control lectin AAL. NA and NA-MPs were capable of inducing CCL20 expression in Caco-2 cells in our study. In literature, CCL20 and IL8 were associated with a pro-inflammatory response upon incubation of Caco-2 cells with entero-invasive bacteria, such as *Salmonella* and *Shigella* [37]. CCL20 triggers the chemotaxis of dendritic cells, which in combination with an induction of Th1 and T-regulatory immunity might be beneficial in cases of an ongoing allergic response. However, we do not anticipate that NA is a strong pro-inflammatory trigger on intestinal epithelial level, as we observed no significant effects on tight junction integrity and molecules such as ZO-1, ZO-2 and claudin-1 and claudin-4. Additionally, CCL20 is regarded as a crucial chemokine for M-cell differentiation and regulates lymphocyte recruitment in a CCL20-CCR6 dependent manner [38]. As this chemokine is, thus, involved in MALT formation, it is tempting to speculate that NA-coated MPs induce formation of intestinal structures, which are the main, immunologically active target of this novel treatment strategy.

Based on the here presented results, NA was proven to be suitable for oral application due to its digestion stability and additionally might have adjuvant, immunomodulatory properties on epithelial and lymphoid cells.

Single and long-term, repeated oral application of NA-functionalized MPs were safe as no adverse effects were observed in naïve and allergic mice with regards to the formation of allergen-specific IgE or the activation of allergy effector cells, such as mast cells. Additionally, an allergen-specific immunomodulatory potential in an ongoing systemic Th2 immune response was observed.

In conclusion, we propose NA of *Vibrio cholerae* as a suitable, highly efficient and safe bioadhesive targeting molecule at mucosal sites.

Coating of MPs with NA increases intestinal uptake, protects the antigens against degradation and ensures presentation of intact antigens to immune cells at mucosal induction sites. NA and NA-functionalized MPs are able to induce a profound Th1 and T-regulatory environment, which, in the case of allergy, might counteract an ongoing Th2 response. Additionally, only NA induces CCL20, which recruits DCs and lymphocytes to the subepithelial layer and initiates MALT formation. Thus, stimulation of the Th1 and T-regulatory cells might counterbalance an ongoing Th2 response.

All these data support the advantage of NA as functionalization substance for PLGA-MPs with implication as allergy treatment. However, the clinical efficacy of oral immunotherapy with NA-functionalized MPs in situations where a local Th2 driven immune response is present, has to be the focus of future research.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clim.2018.03.017>.

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Conflict of interest

The authors declare that they have no conflict of interest. FRW and FG are inventors at the patent family EP1638531A1, held by Biomedical International R + D, Vienna, Austria.

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