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DIPLOMARBEIT

Titel der Diplomarbeit

„M-Zell-targeting mit Neuraminidase-funktionalisierten
Mikropartikeln zur oralen Behandlung von
Nahrungsmittelallergien“

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1 Einleitung

Essen ist eines der wichtigsten Dinge in unserem Leben, genauso wie Atmen oder Schlafen. Wir essen, um zu überleben und unseren Körper mit Vitaminen, Mineralstoffen und Energie zu versorgen. Ebenso ist Essen auch ein Genuss und bereitet uns Vergnügen.

Dieses Vergnügen kann aber von sehr unangenehmen Erfahrungen getrübt werden, wenn man an einer Nahrungsmittelallergie leidet. Die Lebensqualität wird dadurch stark eingeschränkt und es kann im schlimmsten Fall sogar ein lebensbedrohlicher anaphylaktischer Schock auftreten (1).

Eine Allergie ist eine immunologische Reaktion des Körpers auf verschiedenste Proteine aus Pflanzen oder Tieren. Diese harmlosen Eiweißstoffe werden dabei vom Immunsystem als bedrohlich erkannt. Dadurch wird in der Sensibilisierungsphase die Bildung von IgE- Antikörpern induziert (2), welche an Rezeptoren der Mastzellen binden und Entzündungsmediatoren wie Histamin freisetzen.

Die Allergie ist in Europa eines der häufigsten chronischen Leiden. In Österreich sind circa zwei Millionen Menschen an einer Allergie erkrankt, was 25% der Bevölkerung repräsentiert. 2004 starben acht Menschen an Status asthmaticus und weitere zwei Menschen erlitten einen schweren anaphylaktischen Schock. Wird dieser Trend nicht gestoppt, wird laut Prognosen im Jahr 2015 jeder zweite Mensch an einer Allergie leiden (3). Statistiker reihen die Allergie an den dritten Platz der chronischen Gesundheitsprobleme. Außerdem wird bestätigt, dass diese Menschen um 30 % weniger reaktiv und denkfähig sind und die Erkrankung zu erheblichen Kosten für das Gesundheitswesen beiträgt (4). Das Phänomen der zunehmenden allergischen Erkrankungen kann man vor allem in westlichen Ländern beobachten und wird neben vielen anderen Faktoren mit Hygiene und Exposition gegenüber Pathogenen in Verbindung gebracht (1).

Was ist also eine Allergie? Der Erste, der das Wort Allergie verwendete, war der Wiener Pädiater Clemens von Pirquet im Jahr 1906 (5). Er leitete diesen Begriff aus den griechischen Wörtern „allos“, das „verschieden oder anders“ bedeutet, und

„ergon“, das mit „Aktivität, Tätigkeit, Verrichtung oder Handlung“ übersetzt wird, ab. Für ihn bedeutete Allergie, im Gegensatz zur heutigen Definition, dass der Körper in seiner Reaktionsfähigkeit bzw. seinen aktiven Vorgängen beschleunigt oder eingeschränkt ist. Und zwar war das für ihn sowohl in zeitlicher, als auch in qualitativer und quantitativer Hinsicht zu verstehen. Neben vielen anderen Forschungsansätzen arbeitete er mit kutanen Tuberkulinen, die er mittels Hauttests frühzeitig nachweisen konnte. 1911 übernahm er von seinem früheren Lehrmeister Escherich eine Kinderklinik in Wien, wo er bis zu seinem frei gewählten Tod weiter an der Allergie forschte (6).

Nahrungsmittelunverträglichkeiten werden wie folgt unterteilt:

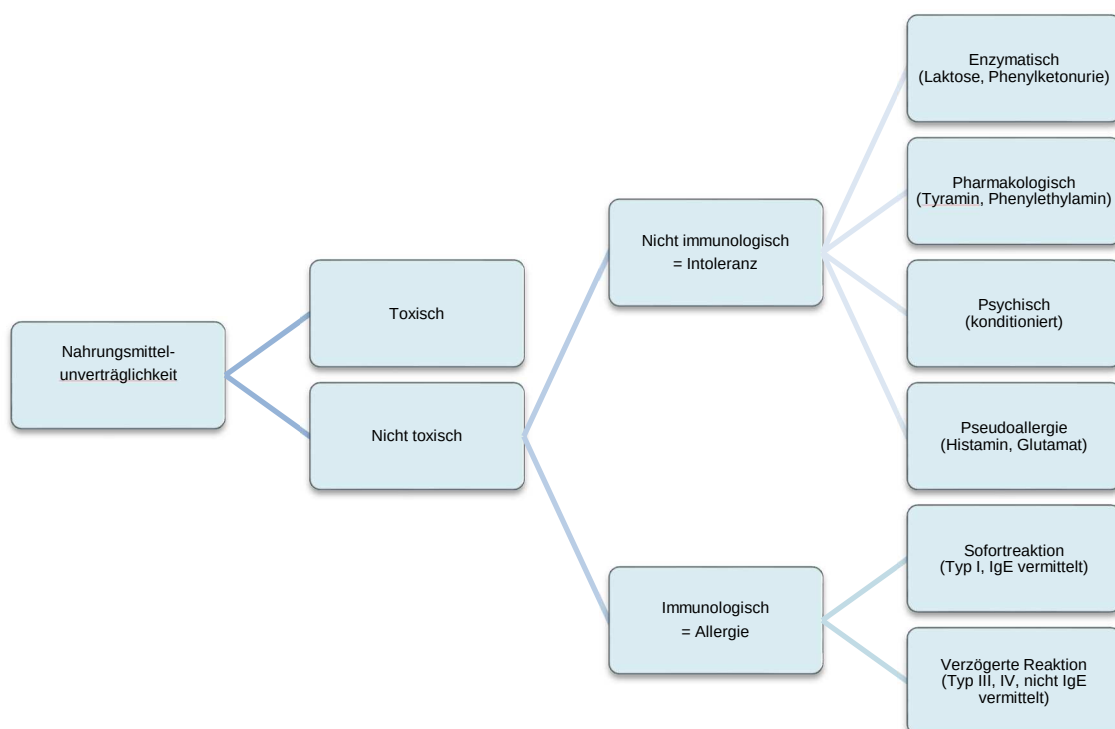


Abbildung 1: Klassifizierung der Nahrungsmittelunverträglichkeiten.

Dabei sind Typ I: Sofortreaktionen,
 Typ II: zytotoxische Reaktionen,
 Typ III: Immunkomplexreaktionen und
 Typ IV: verzögerte Reaktionen.

Im Rahmen meiner Diplomarbeit haben wir uns mit Typ I Reaktionen gegen Nahrungsmittel, im speziellen mit der Allergie gegen das wichtige Eiallergen Ovalbumin (OVA) beschäftigt. Diese allergische Reaktion wird durch spezifische IgE Antikörper vermittelt, tritt nach einer ersten symptomlosen Sensibilisierung, wenige Minuten nach Kontakt mit dem Allergen auf. Dabei kann es sich einerseits um lokale Reaktionen im Gastrointestinal Trakt, wie Schmerz, Erbrechen und Durchfälle handeln. Andererseits kann es zu systemischen Reaktionen wie Nesselausschlag, Ekzeme, Schwellungen, Asthmaanfällen oder sogar schweren anaphylaktischen Schocks kommen (7).

Für den Fall, dass solche schweren Reaktionen auftreten, sollte jeder betroffene Allergiker mit lebensrettenden Sofortmaßnahmen, wie einer waagrechten Lagerung und der Anwendung eines EpiPen[®], eines Adrenalininjektionssystems zur subkutanen Verabreichung, vertraut sein (8,9).

Ovalbumin ist das Hauptallergen im Eiklar und ein ernährungsphysiologisch sehr wertvolles Glykoprotein mit einer Molmasse von 45kDa. Aber nicht nur OVA kann eine Allergie auslösen, auch Ovomucoid, Lysozym und Ovomucin sind wichtige Allergene im Ei. Neben Milch und Nüssen zählen Eier gerade bei Kindern zu den häufigsten Allergie-auslösenden Nahrungsmitteln (10).

Da es derzeit kaum Möglichkeiten einer kausalen Therapie von Nahrungsmittelallergien gibt, war es das Ziel der vorliegenden Studie eine neue Therapieoption, nämlich mit Neuraminidase (NA) beschichtete Mikropartikel, die mit dem Modellallergen OVA beladen waren, auf ihre therapeutische Effizienz zu überprüfen (11,12). Zu diesem Zweck wurden Mausversuche durchgeführt, wo die Auswirkung der Verabreichung von Allergen-beladenen PLGA (poly D, L- Milchsäure-co- Glycolsäure) Mikropartikeln bei Nahrungsmittel-allergischen Tieren untersucht wurden (13).

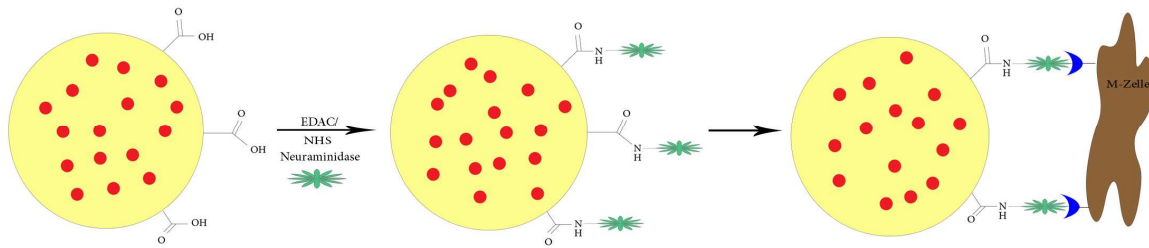


Abbildung 2: Schematische Darstellung der Herstellung von NA-funktionalisierten, Allergen-beladenen Mikropartikeln (EDAC - 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid; NHS - N-Hydroxy-succinimid).

Im Rahmen dieser Studie wurden Mäuse durch Suppression der Magensäure mit einem Protonenpumpenhemmer gegen OVA allergisiert (14,15). Protonenpumpeninhibitoren sind Prodrugs, die im sauren Milieu der Canaliculi ($\text{pH} < 4,2$) protoniert und in die aktive Form, ein Sulfenamid, umgewandelt werden. Anschließend wird kompetitiv die H^+/K^+ -ATPase antagonisiert. Dadurch wird weniger Magensäure produziert und Nahrungsmittelproteine können folglich schlechter verdaut werden. Eine orale Sensibilisierung ist somit möglich (16).

2 Beitrag des Autors zur Publikation

Im Rahmen der vorliegenden Diplomarbeit konnte zu der im Anhang befindlichen Publikation „M-cell specific targeting with Neuraminidase-functionalized microparticles for oral immunotherapy of food allergy“, eingereicht zur Veröffentlichung im Journal of Allergy and Clinical Immunology, von mir folgendermaßen zum Abschnitt „proof of concept study – Oral immunotherapy of OVA food allergic mice with NA-functionalized, OVA- loaded MPs“ beigetragen werden:

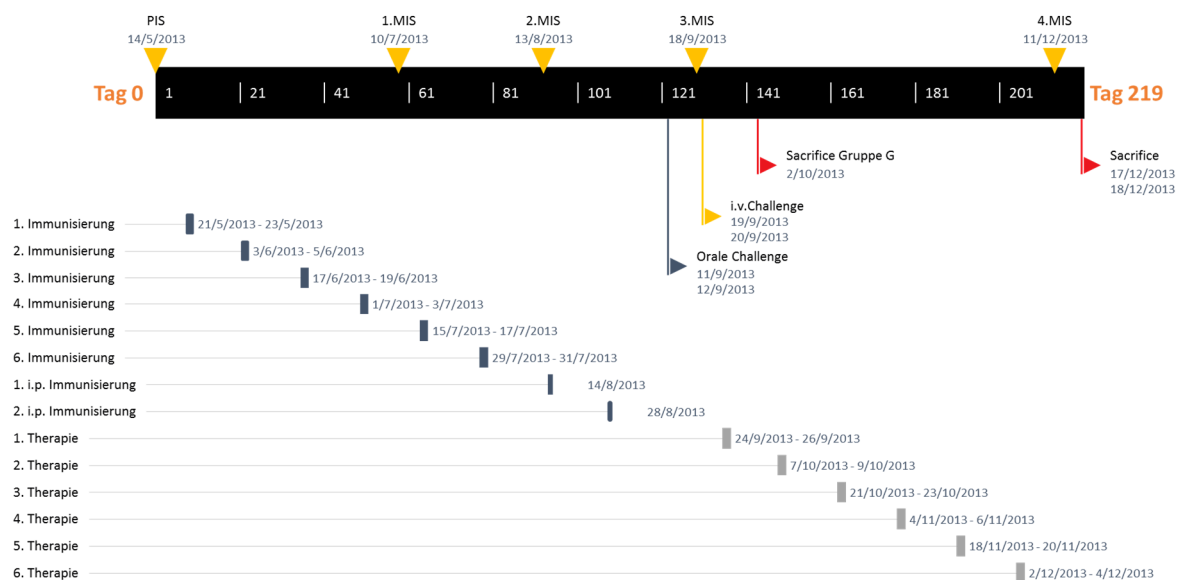


Abbildung 3: Zeitachse der durchgeführten Versuche.

63 weibliche Balb/c Mäuse wurden am 23.04.2013 ins AKH Wien zum Institut für Pathophysiologie und Allergieforschung geliefert. Die Mäuse waren bei ihrer Ankunft im Institut 6 bis 8 Wochen alt und wurden 2 Wochen zur Akklimatisierung im Tierstall unter konventioneller Haltung ohne Behandlung gehalten.

Vor Beginn der Sensibilisierung wurde den Mäusen am 14.05.2013 Blut abgenommen. Diese Präimmunisierungs-Sera wurden benötigt, um die unspezifischen Hintergrundspiegel von IgE mit den späteren Blutentnahmen vergleichen zu können. Alle Mäuse, ausgenommen Gruppe i (naive Mäuse), wurde nach dem oben beschriebenen Zeitprotokoll (Abb. 3) immunisiert.

Die erste Immunisierung erfolgte am 21.05.2013. Ich arbeitete ab der vierten Immunisierung durchgehend bis zum Ende der Studie mit und war in alle Entscheidungen bezüglich des weiteren Vorgehens eingebunden.

Sowohl die Überprüfung des Fortschreitens der Immunisierung, als auch in späterer Folge die Therapie unterlagen meiner Mitverantwortung. Weiters war ich erheblich an der Planung und Durchführung des Sacrifice aller Mausgruppen beteiligt. Ebenso unterlag die Analytik aller Proben durch verschiedenste ELISA Methoden meiner Planung und Durchführung. Die Auswertung erfolgte mittels GraphPad Prism 5 software und Excel. Im Rahmen der Erstellung der Publikation habe ich eine erste Version, des diese Arbeiten betreffenden Teil des Methoden Kapitels, verfasst.

3 Publikation:

M-cell specific targeting with Neuraminidase-functionalized microparticles for oral immunotherapy of food allergy

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Abstract

Background: We demonstrated that *Aleuria aurantia* lectin (AAL)-coated poly (D,L-lactide-co-glycolide) microparticles (MPs) specifically targeted mucosal M-cells and beneficially influenced IgE-mediated allergy.

Objective: Due to structural similarities with AAL, we analysed *Vibrio cholerae* Neuraminidase (NA) as a novel M-cell-specific binding substance and evaluated the safety and therapeutic efficacy of NA-coated, allergen-loaded MPs for oral immunotherapy of food allergy.

Materials and methods: NA alone and the NA-functionalized MPs were characterized for their suitability for oral application by digestion experiments, cell ELISA and immunofluorescence stainings with Caco-2 cells and in a human M-cell co-culture model. We analysed the safety of the coated MPs *in vitro* by stimulation of naïve splenocytes and *in vivo* in naïve and allergic BALB/c mice. As a proof of concept the therapeutic efficacy was evaluated in a mouse food allergy model.

Results: NA revealed high binding specificity to α -L fucose and monosialoganglioside 1 (GM1) and enhanced transepithelial uptake in a M-cell co-culture model. Mouse splenocytes released significantly more IL10 and IFN γ after stimulation with NA and NA-coated MPs. Safety testing in naïve animals indicated a higher production of OVA-specific IgA after 6 cycles of oral NA-coated MP feeding, while in allergic mice this regimen induced higher IFN γ and IL10 levels. Treatment with OVA-loaded NA-MPs attenuated anaphylactic responses as determined by rectal body temperature measurements and was associated with higher levels of IL10 and regulatory T cells compared to controls.

Conclusion: Our data suggest NA-coated, allergen-loaded MPs to be a safe and efficient novel treatment option in a mouse food allergy model.

Capsule summary

Oral application of allergen-loaded, NA-coated PLGA-MPs beneficially modulates an ongoing Th2 immune response and attenuates anaphylaxis.

Clinical implication

Oral immunotherapy with NA-coated PLGA-MPs might represent a promising novel treatment option of food allergy.

Keywords

Neuraminidase, M-cell, oral immunotherapy, PLGA-microparticles, food allergy

Abbreviations

AAL	Aleuria aurantia lectin
FAE	Follicle associated epithelium
FS	Fluospheres
GALT	Gut associated lymphoid tissue
GM1	Monosialoganglioside 1
i.g.	Intragastric
i.p.	Intraperitoneal
i.v.	Intravenous
M-cell	Membranous or Microfold cells
MP	Microparticles
NA	Neuraminidase
OVA	Ovalbumin
PLGA	Poly (D,L-lactic-co-glycolic) acid
RT	room temperature
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SIT	Specific immunotherapy
TEER	Transepithelial electrical resistance
TCT	N,N',N''-triacetylchitotriose

3.1 Introduction

Oral immunotherapy has since long time been of special interest for researchers worldwide because of its advantages over subcutaneous injections of currently applied specific immunotherapy (SIT). It is not only less invasive, but is also associated with a better compliance of patients.¹ Oral immunotherapy can elicit both, systemic as well as mucosal immune responses, and is, thus, especially of interest as a treatment option in food allergy. The design of oral immunotherapeutics is challenging due to the harsh milieu of the gastrointestinal tract. Drugs have to fulfill several criteria making them suitable for oral application, such as digestion stability and better bioavailability by overcoming the first pass metabolism.²

One promising strategy is the use of poly (D-L-lactic-co-glycolic) acid (PLGA) particles as a carrier system. They can protect encapsulated proteins against gastrointestinal degradation,³ are fully biocompatible⁴ and feature immunogenic properties by driving the immune system towards a Th1 response.⁴⁻⁶ The efficacy of PLGA particulate carriers can even be enhanced by specifically targeting M-cells (microfold or membranous cells) in the intestine by surface modification of the particles.

We and others have investigated an oral immunotherapy approach based on M-cell specific targeting with lectin functionalized PLGA microparticles (MPs).⁷⁻¹¹ M-cells are located within the follicle associated epithelium (FAE) overlying the gut associated lymphoid tissue (GALT).¹²⁻¹³ These cells are responsible for the uptake and transport of intact particulate structures to the mucosa associated lymphatic tissue,¹⁴ rendering them a perfect entry for allergens in oral immunotherapy. M-cells are rich of specific carbohydrate residues, such as α -L fucose and monosialoganglioside (GM 1).¹⁵⁻¹⁶ Therefore, lectins, which selectively bind to the glycocalyx of epithelial cells may serve as M-cell specific targeters.¹⁴ In our recent work, we reported Aleuria aurantia lectin (AAL), a protein derived from the edible orange peel mushroom *Aleuria aurantia*, to feature α -L fucose specificity.¹⁷ Functionalization of PLGA particles with AAL increased the transepithelial transport via M-cells and the induction of a Th1 dominated immune response in human peripheral blood mononuclear cells of allergic individuals.¹⁷ In mouse studies, oral application of birch pollen sensitized mice with allergen-loaded, AAL-functionalized PLGA-MPs shifted a Th2 biased immunity to a prominent Th1 response indicated by high levels of IgG2a, IFN γ and of the T-regulatory cytokine IL10.⁷

These studies confirmed the potential of M-cell targeting via lectin-functionalized MPs as novel oral immunotherapy for type I allergy.

In the current study, we aimed to enhance the M-cell specific targeting capacity by the use of NA as specific targeting ligand. Neuraminidase of *Vibrio cholerae* has structural similarities with AAL,¹¹ and is an important pathogenicity factor for the bacterium as it cleaves off sialic acid from the bacterial contact site of M-cells.¹⁸ Therefore, we evaluated NA for its *in vitro* and *in vivo* properties as M-cell targeter and its safety and suitability as biorecognitive ligand for PLGA-MPs for oral immunotherapy in food allergy.

3.2 Materials and methods

3.2.1 Characterization of NA as a targeting substance

Neuraminidase from *Vibrio cholerae* (Sigma-Aldrich, St. Louis, MO, USA) was tested for digestion stability in simulated gastric fluid (SGF) experiments, as described recently.¹⁹ The digestion was stopped after 60, 120 and 180 min. Thereafter, NA was further digested in simulated intestinal fluid (SIF) experiments with 3.2 mg/ml pancreatin in a ratio of 1:5 (Kreon 10.000IE, Abbott Products GmbH, North Chicago, IL, USA). After 5, 10, 15, 30 or 45 min digestion was stopped by non-reducing SDS-PAGE puffer and cooking, according to a modified protocol.²⁰ Protein integrity was evaluated by SDS-PAGE using Coomassie brilliant blue staining and silverstaining.

Binding of the targeter NA to intestinal epithelial cells 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), which exhibit an intestinal phenotype, were incubated with increasing concentrations of FITC-NA (16, 32, 64 or 128 μg) for 30 min. For determination of background staining, cells were incubated with or without a biotin-labeled IgG followed by FITC-Avidin (Sigma-Aldrich). Acquisitions were performed using a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA) and analysis was done with FlowJo 9.3.3 software (Tree Star, Ashland, OR, USA).

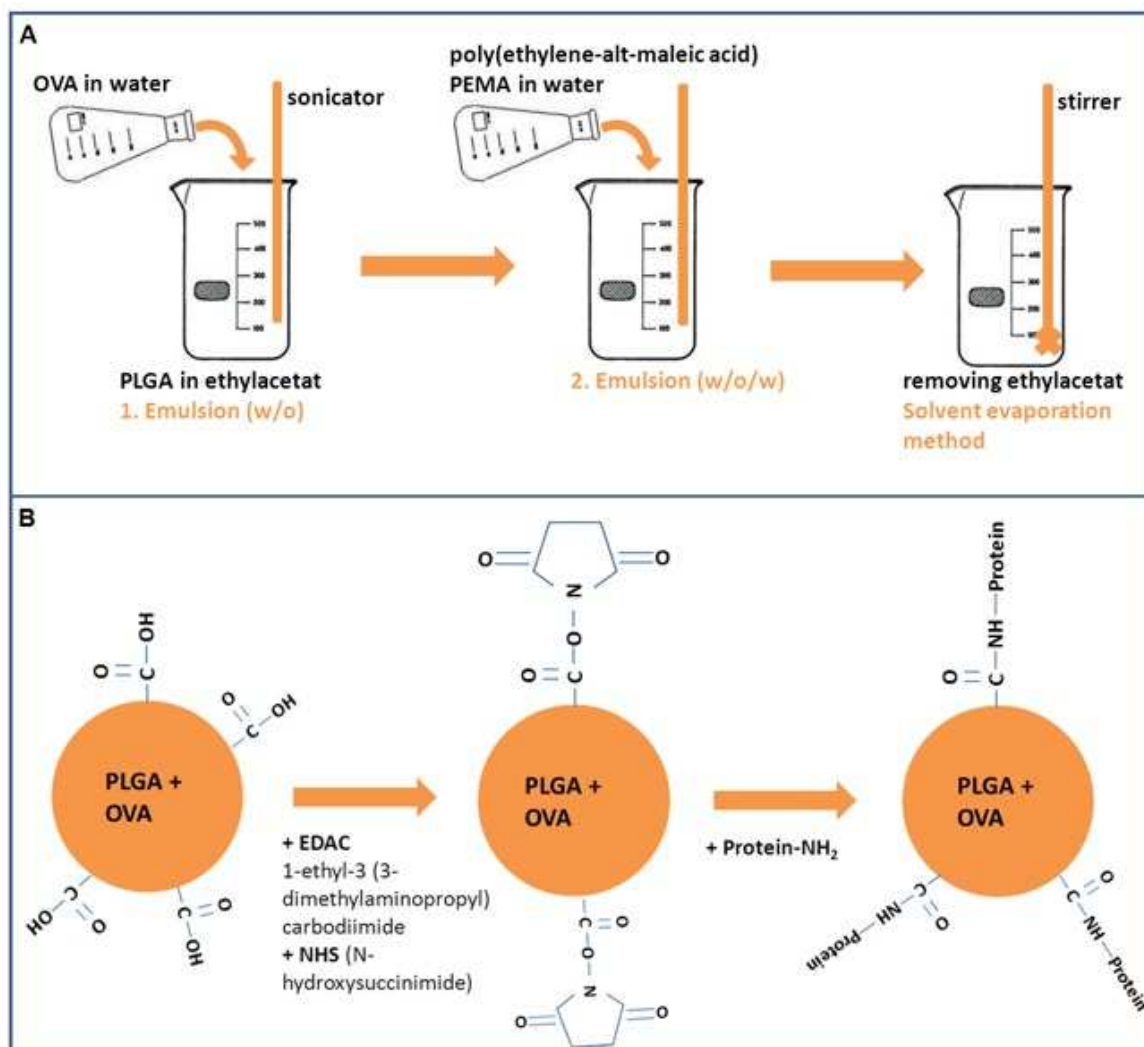
To characterize the binding partners of NA on Caco-2 cells, inhibition experiments were performed. Caco-2 cells were grown for 14 days until formation of a monolayer. Cells were washed with PBS and incubated with 2% bovine serum albumin in PBS for 30 min. Eighty-five microliter of biotin-NA (288 $\mu\text{g}/\text{ml}$) were pre-incubated with 85 μl of increasing concentrations of α -L fucose, N,N',N''-triacetylchitotriose (TCT; both 79, 158, 318 μM) or with glycosylated or deglycosylated GM1 from bovine brain (GM1; all from Sigma-Aldrich; 0.5, 5, 10, 25 and 50 $\mu\text{g}/\text{ml}$) for 60 min before incubating with Caco-2 cells for 60 min. FITC-Avidin (200 pmol/well) was added for 30 min. Fluorescence intensity was measured at 485/530 nm, values were normalized and calculated as percentage of binding of non-inhibited targeting substances. Three independent sets of experiments were performed.

3.2.2 Preparation of functionalized particles

OVA-loaded PLGA microparticles were prepared by the water-in-oil-in-water solvent-evaporation technique as indicated in supplementary figure 1. Briefly, 400 μ L aqueous solution of OVA (500 mg/mL; Sigma-Aldrich, Lot SLBD2312V and 076K7045) or FITC-OVA were emulsified with 400 mg PLGA in ethyl acetate by sonication for 2 min. After adding 8 mL aqueous solution of PEMA (0.5% in w/v), the emulsion was sonicated again 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 in order to remove the residual ethyl acetate. The microparticles were resuspended in 20 mM HEPES/NaOH-buffer pH 7.0 after removal of non-encapsulated OVA by thorough washings with the same buffer.

The PLGA content was determined gravimetrically after lyophilization. The particle size distribution was determined using a Malvern Mastersizer 2000 laser particle size analyzer (Malvern Instruments, Malvern, UK).

NA was covalently coupled to the surface of PLGA-microparticles using a modified carbodiimide method.²¹ Briefly, 5 mL PLGA-MP suspension in 20 mM HEPES/NaOH pH 7.0 was activated for 2 h at room temperature by adding 5 mL of a freshly prepared solution of 1400 mg 1-ethyl-3(3-dimethylaminopropyl) carbodiimide and 59 mg N-hydroxysuccinimide in the same buffer. In order to remove the excessive reagents, the MPs were washed three times with 20 mM HEPES/NaOH pH 8. After resuspending the MPs in 5 mL of the same buffer, 260 μ L aqueous solution of NA (2.45mg/mL, Sigma-Aldrich, Lot 110M4103V) were added followed by end-over-end incubation overnight at room temperature. Non-reacted binding sites were saturated by incubation with 3 mL glycine solution (200 mg/mL in the same buffer) for 5 h at room temperature. Finally, the MPs were washed three times by centrifugation to remove excessive reagents, resuspended in 5 mL isotonic 20 mM HEPES/NaOH pH 7.4, and stored at -80 °C until use.



Supplementary Figure 1: Preparation (A) and functionalization (B) of OVA-loaded PLGA-MPs.

The concentration of modified PLGA-particles was determined gravimetrically after lyophilisation. The particle size distribution was determined using a Malvern Mastersizer 3000 laser particle size analyzer (Malvern Instruments, Malvern, UK). As a reference, non-modified PLGA microparticles were treated as above but adding solely buffer instead of the EDAC, NHS, NA and glycine.

Covalent coupling of NA to carboxylate-modified fluospheres (FS) (Invitrogen, Carlsbad, CA, USA) was done according to manufacturer's instructions.

3.2.3 Characterization of NA-coated or uncoated MPs as allergen delivery systems

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

The presence of the targeter at 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 3h 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 NA was detected using a NA-specific polyclonal mouse serum, followed by an anti-mouse IgG horseradish-peroxidase-labeled antibody (Abcam, Cambridge, UK). TMB (BD Biosciences) was used as substrate for ELISA, Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was used for Western Blot.

To rule out a strong endotoxin contamination of the preparations, lipopolysaccharide (LPS) levels of NA and NA-coated MPs were determined using Endosafe – PTS cartridges (Charles River Laboratories, Wilmington, MA, USA) according to manufacturer`s instructions.

To test whether NA-coated fluospheres (FS) bind to intestinal cells, Caco-2 cells were grown on 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. Cells were stained with α -tubulin antibody followed by an anti-mouse IgG Alexa Fluor 568 antibody (Life Technologies, Carlsbad, CA, USA) for detection. The nucleus was visualized with DAPI (Life Technologies) staining. Acquisitions were done using a Zeiss Axioplan 2 (Carl Zeiss AG, Oberkochen, Germany).

Internalization of functionalized particles was tested by incubating uncoated and NA-coated MPs loaded with FITC-OVA with Caco-2 cells for 2 h at 37°C. After fixation, cells were permeabilized and unspecific binding was blocked. The cytoskeleton was visualized using a Phalloidin antibody (Invitrogen) followed by DAPI nucleus staining. Analysis was done with a LSM700 confocal microscope (Carl Zeiss AG).

3.2.4 M-cell like co-culture model

For co-culture experiments, Caco-2 cells (5×10^5 cells/filter) were seeded in an inverted orientation on transwell filters (Sigma-Aldrich, pore size $3 \mu\text{m}$) and cultured for 21 days upon a transepithelial resistance (TEER) of greater than $300 \Omega/\text{cm}^2$. For generation of M-cells, Raji-B cells, a Burkitt lymphoma cell-line (LGC Standards, Teddington, UK), were added for 4 days to the basolateral side of the epithelial layer.²² Tight junction integrity was confirmed by TEER measurements and M-cell formation was verified by measurement of alkaline phosphatase activity. After 1 h incubation with phenolred-free RPMI, NA-coated or uncoated MPs (Plain MPs) containing FITC-OVA were added to the apical side of the epithelial layer for 120 min. Microparticles transported through the epithelium against gravitation were collected from the basolateral side, centrifuged and dissolved in 0.1 M NaOH. Protein concentrations were determined using a NanoDrop ND-1000 Spectrophotometer (peqlab Biotechnology GmbH, Erlangen, Germany). Statistics were calculated from 3 wells per condition.

3.2.5 Animals

Healthy female BALB/cAnNCrl mice (6-8 weeks) 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. Animals were treated according to European Union rules for animal care and with approval by the local ethics committee and the Austrian Federal Ministry of Science and Research (permission number GZ BMWF-66.009/0051-II/10b/2008 and BMWF-66.009/0070-II/3b/2012).

3.2.6 Intestinal uptake of gavaged particles

To investigate whether orally applied particles are taken up in the intestine, mice were fed with NA-functionalized and plain FS. After 1 h, mice were sacrificed and the intestines were harvested and embedded in Tissue Tek (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands). Sectioned tissue was fixed and stained with a Phalloidin antibody and DAPI. Acquisitions were done on a LSM700 confocal microscope.

3.2.7 Animal treatment protocols

To evaluate the safety of orally applied MPs, naïve mice (n=8) were fed every other week for 6 times with plain or NA-coated MPs containing 200 µg OVA on 3 consecutive days. Serum was collected after each MP application (Fig. 3C). Rectal body temperature measurements were done before and up to 72 h after a single gavage of plain or NA-functionalized MPs (200 µg OVA) using a Thermalert TH-5 thermometer (Physitemp Instruments Inc., Clifton, NJ, USA). Mouse mast cell protease-1 (mMCP-1) levels were detected using the mouse MCPT-1 Ready-set-go ELISA Kit (eBioscience). Sera taken after the last oral application were diluted 1:100 and concentrations were calculated according to a standard curve.

To test the safety of functionalized allergen-loaded MPs in allergic mice, BALB/c mice (n=8) were first injected with OVA (2 µg OVA adsorbed to 1.3 µg aluminum hydroxide) intraperitoneally (i.p.) 5 times in 2 weeks intervals. Two weeks after the last immunization, animals were orally challenged twice with 50 mg OVA with a 3 days interval to induce a strong local allergic response (Fig. 4A), adapted from.²³ Three weeks after oral challenge, animals received either uncoated (Plain MPs) or NA-coated PLGA-MPs loaded with OVA (200 µg OVA per gavage) intragastrically (i.g.) on 3 consecutive days for 6 treatment cycles in 2 weeks intervals. Naïve control animals were left untreated for the entire period. Thereafter, mice were again orally challenged with 50 mg OVA and sacrificed. (Fig. 4A)

The therapeutic potential of NA-functionalized, allergen-loaded particles was investigated in a murine food allergy model. The oral sensitization protocol was based on a previously established immunization regimen.¹⁹ BALB/c mice were fed twice every other week with 200 µg OVA under concomitant gastric acid suppression for 6 treatment cycles. To induce a sustained immune response, mice were injected i.p. twice with OVA (2 µg OVA adsorbed to 1.3 µg aluminum hydroxide) in a 2 week interval. Two weeks thereafter, mice were orally challenged with 50 mg OVA on 2 consecutive days. One week later an intravenous (i.v.) challenge using 50 µg OVA was performed and rectal body temperature was measured using a Thermalert TH-5 thermometer (Physitemp Instruments Inc.) before and 15 as well as 30 min after challenge. One group was sacrificed at this timepoint for control. Four days after the systemic challenge, the treatment protocol was initiated. Mice received oral therapy on 3 consecutive days for 6 cycles in 2 week intervals. Allergic mice were either treated with NA-coated (NA-MPs) or uncoated MPs (Plain MPs) loaded with OVA (n=7). For

control purposes one group was orally treated with OVA only (OVA i.g.). Another group did not receive any treatment (no therapy) and another group was left naïve for the entire sensitization and treatment period (naïve). Two weeks after the last treatment cycle, mice were again i.v. challenged with 50 µg OVA and rectal body temperature was measured before and after 15 and 20 min. Thereafter, mice were immediately sacrificed. (Fig. 5A)

3.2.8 Spleen cell stimulation experiments

Spleen cells of mice were prepared as described previously.¹⁹ Spleen cells (5×10^5 cells/well) were stimulated either with OVA, NA, NA-coated or uncoated MPs for 72 h. Concanavalin A (ConA, Sigma-Aldrich) was used as a positive control and medium alone as negative control (ctrl). Supernatants were harvested and analyzed for 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 dilution series after subtraction of blank levels.

3.2.9 OVA-specific IgG1, IgG2a, IgA and IgE ELISA

Sera were screened for OVA-specific IgG1, IgG2a, IgA and IgE by ELISA, as described recently.¹⁹ Sera were diluted 1:200 for IgG1, IgG2a and IgA and 1:20 for IgE. Rat anti-mouse IgG2a, IgG1, 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. Sera before the first immunization were defined as background levels and were subtracted from sera taken after immunizations. Concentrations were calculated according to a standard curve.

3.2.10 Total and OVA-specific IgA and IgE in intestinal lavages

After sacrifice, intestines were removed under sterile conditions and flushed with 2 ml ice-cold PBS and protease inhibitor (Roche, Rotkreuz, Switzerland). Intestinal lavage fluid was screened for mucosal IgA and IgE. 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 being followed by Streptavidin-HRP (eBioscience). The color reaction was developed with TMB (BD Biosciences) and measured at 450-630 nm. OVA-specific IgA was measured in intestinal lavage fluid as described above for serum OVA-specific IgA except that the samples were used undiluted. Measurements of OVA-specific IgE were performed on undiluted samples using a rat anti-mouse IgE antibody (0.1 µg/well, BD Biosciences) being followed by anti-rat IgG HRP (GE Healthcare). The color reaction was developed with TMB (BD Biosciences) and measured at 450-630 nm.

3.2.11 Flow cytometry analysis of regulatory T cells

Spleen cells were stained for CD4+CD25+Foxp3+ T-regulatory cells with the mouse regulatory T-cell staining kit (eBioscience), according to manufacturer's protocol. Absolute numbers of CD4+ T-cells and CD4+CD25+Foxp3+ T-cells were calculated per spleen. Acquisitions were performed using a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA) and analysis was done with FlowJo 9.3.3 software.

3.2.12 Statistical analysis

Data of inhibition experiments were statistically compared with the GraphPad Prism 5 software using the two-fold ANOVA and Bonferroni post-test. Co-culture results were statistically compared using student's t test. Fluorescent intensity results of different FS and cytokine measurements of *in vitro* experiments as well as antibody concentration, comparison of body temperature, cytokine levels and regulatory T cell evaluation of *in vivo* studies were analyzed by one-way ANOVA and Tukey's multiple comparison test. A *P*-value <0.05 was considered statistically significant.

3.3 Results

3.3.1 Characterization of the targeter NA and NA-functionalized particles

The first experiments focused on characterization of NA as novel targeting agent.

3.3.1.1 In vitro characterization of NA

To evaluate its digestion stability and, thus, its suitability for oral application, NA was subjected to simulated gastric and intestinal digestion experiments. In SGF experiments (Fig. 1A) the protein remained stable for up to 180 min under simulated physiological gastric digestion conditions. Protein bands of NA ranged from 20 to 90 kD under denaturizing conditions. Furthermore, the targeter remained stable after subjection to an additional intestinal digestion step for up to 45 min (data not shown).

The binding of NA to intestinal epithelial cells was assessed by flow cytometry (Fig. 1B). FITC-labeled NA revealed an increasing binding intensity to the colon carcinoma cell line Caco-2, which mimic an intestinal phenotype, in a dose dependent manner (Fig. 1B; FITC-NA 16µg: left panel, blue line (MFI 9.38), FITC-NA 32µg: left panel, orange line (MFI 12.8), FITC-NA 64µg: left panel, light green line (MFI 23.3), FITC-NA 128µg: left panel, dark green line (MFI 33.2) versus MFI control (red line) 10.8).

As a next step, we analyzed the specific binding partners of NA on Caco-2 cells. In competition experiments (Fig. 1C), the binding of NA to Caco-2 cells could be inhibited by α -L fucose in a dose dependent manner (mean inhibition with 318 µM α -L fucose: 40%). The negative control substance TCT did not reveal significant reduction of NA binding to Caco-2 cells (mean inhibition 18%). Additionally, NA was determined to bind to GM1 (mean inhibition: 25.4%), however only if GM1 was glycosylated (data not shown).

3.3.1.2 Preparation and characterization of NA-functionalized microparticles

In a subsequent step, OVA-loaded MPs were prepared by the double emulsion technique. The diameter of the hardened particles was 3.9 µm at the mean and the allergen-content was 164.2 ± 9.3 µg OVA/ mg particles. As the H-type of PLGA was used as a matrix, which contains free carboxylate end groups, amine-containing ligands were bound to the superficial protruding polymer chains via amide bonds. For

that purpose, the carboxylates were activated with carbodiimide to yield an active ester intermediate that readily reacts with primary amines to yield a covalently linked ligand. According to the results of a modified bicinchonic acid assay for proteins using plain particles as a control, $86.2 \pm 6.8 \mu\text{g}$ NA were bound to the surface of one mg particles. Any changes in the diameter due to NA-coupling were not observed.

The efficient coupling the targeter NA to the surface of MPs was evaluated qualitatively by ELISA and Western Blot using murine polyclonal anti-NA antibodies. In all assays, the biorecognitive ligand NA was detectable on the MPs (data not shown).

As the encapsulation of antigens into polymer particulate carriers should protect the antigen from enzymatic digestion, the NA-coated or uncoated OVA-loaded PLGA-MPs (Plain MPs) were tested for protein degradation in SGF experiments. SDS-PAGE analysis revealed that OVA (45 kDa) encapsulated in functionalized or uncoated MPs remained stable up to 120 min in SGF which represents the average gastric transit time (Fig. 2A). In contrast, unprotected OVA proteins were degraded within 30 min. Furthermore, PLGA-encapsulated OVA was protected from intestinal digestion for up to 45 min (data not shown). LPS measurements revealed a 19-fold reduction of LPS content compared to unbound NA by particle preparation and coupling procedure during OVA-loaded MP production, ruling out a high endotoxin contamination (data not shown).

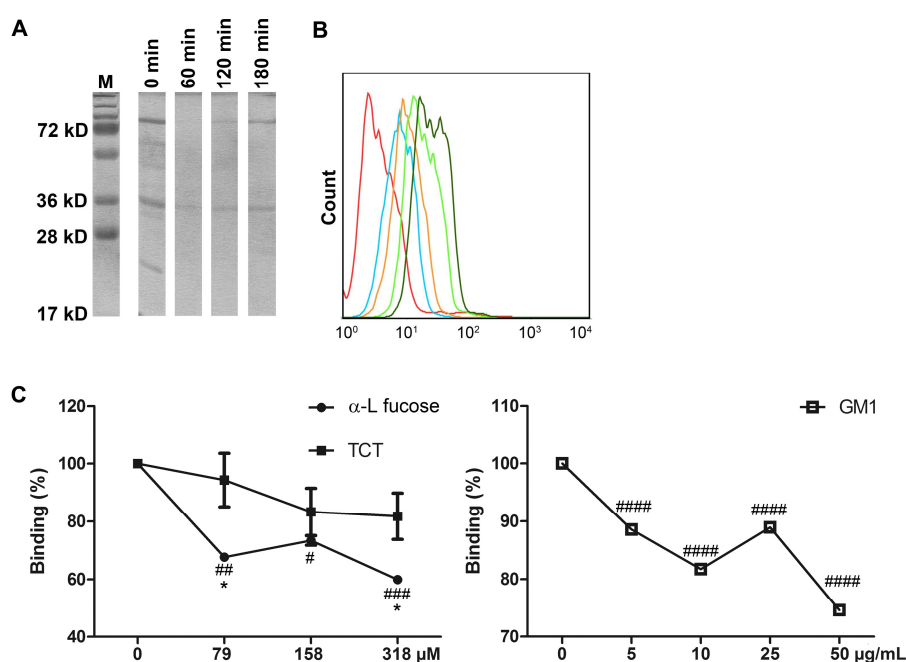


Figure 1: Characterization of NA as a targeter.

3.3.1.3 Binding properties of NA-functionalized particles to epithelial cells

To visualize the binding and uptake of uncoated or NA-coated MPs by intestinal epithelial cells confocal microscopy evaluations were performed using Caco-2 cells. These assays indicated an enhanced binding of NA-coated particles to intestinal cells compared to uncoated particles (Fig. 2B, upper right panel). Additionally, OVA-loaded MPs were readily taken up into Caco-2 cells (Fig. 2B, lower panel). Moreover, NA-coated FS were efficiently taken up into the submucosal intestinal tissue 1 h after oral MP feeding to naïve mice (Fig. 2C).

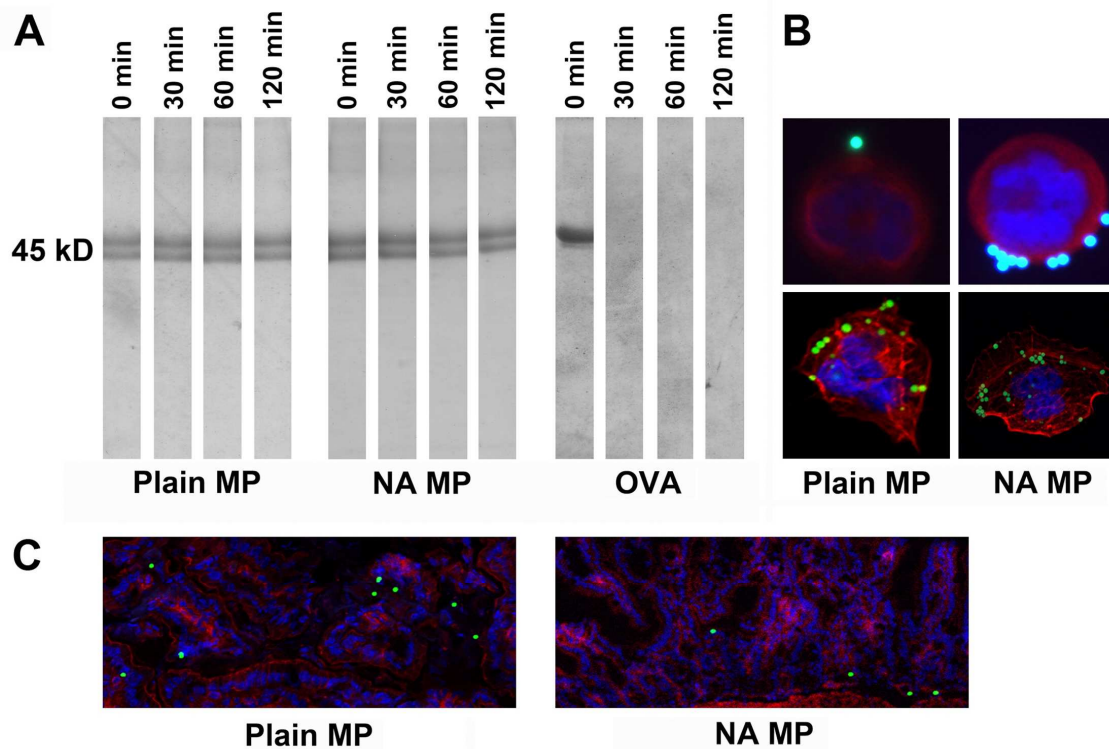


Figure 2: Characterization of NA-functionalized MPs.

The binding of NA-functionalized MPs to M-cells was confirmed in an *in vitro* co-culture model of human origin. M-cells were generated from Caco-2 cells by addition of the Burkitt lymphoma cell line Raji-B cells.²¹ The transepithelial uptake of NA-MPs was significantly enhanced in co-culture compared to monoculture of Caco-2 cells representing the intestinal epithelium ($P=0.003$). Moreover, higher protein transport through M-cells was detected in the upper compartment of transwell filters using NA-

coated MPs as a delivery system compared to uncoated control MPs ($P=0.003$) (Table 1).

MP preparation	Monoculture ($\mu\text{g/ml}$) mean$\pm$SD	Coculture ($\mu\text{g/ml}$) mean$\pm$SD	P-value
Plain MP	30 $\pm$ 26	127 $\pm$ 61	n.s.
NA-MP	73 $\pm$ 76	357 $\pm$ 15	0.003
P-value	n.s.	0.003	

Table 1: Protein concentrations determined in the upper compartment of the transwell system after incubation with MPs containing 150 μg OVA/well indicating transepithelial protein shuttling by MP preparations under different culturing conditions.

3.3.2 In vitro and in vivo safety assessment of NA-coated OVA-loaded MPs

3.3.2.1 Immunomodulatory effect of NA-coated MPs in naïve animals

The *per se* immunogenicity of the targeter NA and of functionalized or uncoated PLGA-MPs loaded with OVA as allergen was analyzed by stimulating spleen cells of naïve BALB/c animals. The cytokines IL4, IL10 and IFN γ were determined in spleen cell supernatants by ELISA (Fig. 3A). In contrast to the positive control ConA, the IL4 titers of all other stimulants were beneath the detection limit of the ELISA kit (data not shown). Incubation of naïve splenocytes with NA induced a significantly higher production of IFN γ and the T-regulatory cytokine IL10 compared to medium alone (ctrl). When MPs were used for stimulation, only NA-MPs significantly enhanced expression of IFN γ and IL10 when compared to uncoated MPs (Fig. 3A).

In addition to the *in vitro* studies we evaluated the *in vivo* impact of OVA-loaded uncoated (Plain MPs) and NA-coated MPs on the activation of mast cells. Measuring mMCP1 levels in serum of naïve mice after single gavage of MPs any mast cell mediator release triggered by MPs (Fig 3B) was observed as the levels were below those of naïve animals.

For further *in vivo* safety evaluation of the oral application of OVA-loaded uncoated or NA-coated MPs, naïve BALB/c mice received 6 cycles of the previously established oral treatment protocol (Fig. 3C, left panel).⁷ During the application of MPs to naïve animals neither adverse clinical symptoms nor any systemic response to NA-coated MPs with the targeter being of bacterial origin was observed. Repeated measurements of core body temperature after gavages of uncoated or NA-coated MPs did not show elevation of body temperature for a prolonged period of measurements (up to 72h after gavage; data not shown). After six cycles of oral MP application no induction of OVA-specific IgE antibodies was observed with responses being below the background levels determined in naïve mice (Fig. 3C, middle panel). However, OVA-specific serum IgA levels were induced being significantly elevated in mice receiving NA-coated MPs compared to those fed with uncoated MPs as well as to naïve animals (Fig. 3C, right panel). Measurements of spleen cell supernatants after stimulation with the encapsulated antigen OVA did not result in detectable levels of IL4, IFN γ or IL10 in all three groups (data not shown).

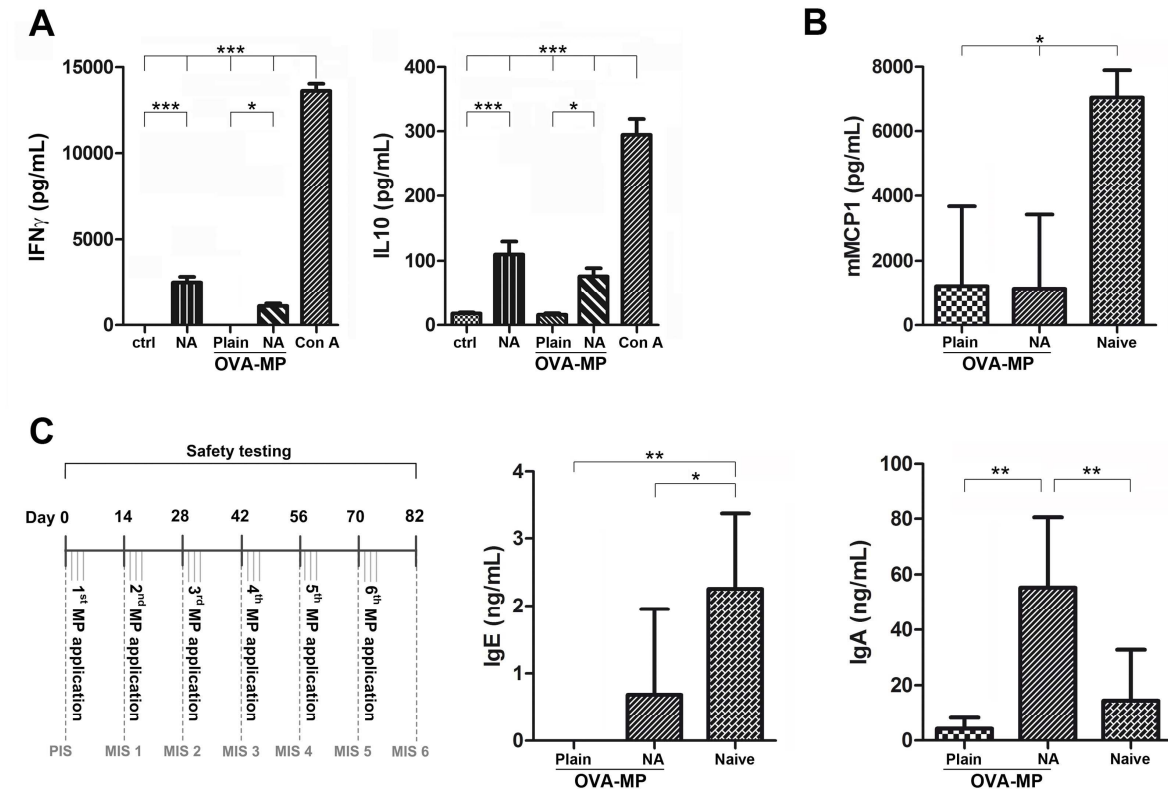


Figure 3: Safety assessment and immunomodulatory properties of NA and NA-MPs in naive mice.

3.3.2.2 Safety assessment of NA-functionalized MPs in OVA allergic mice

The safety of oral administration of NA-functionalized, OVA-loaded MPs in allergic organisms was evaluated in a third *in vivo* immunization protocol using OVA-allergic mice (Fig. 4A). After i.p. sensitization, significantly higher titers of OVA-specific IgE, IgG1, IgG2a and IgA were elicited in allergic mice as compared to naïve animals (data not shown). After 6 cycles of oral MP application a marginal decrease of OVA-specific IgE (Fig. 4B, black bars) and IgG1 (data not shown) serum levels was observed accompanied with a slight increase of OVA-specific IgA (Fig. 4C, black bars) and IgG2a (data not shown) titers compared to the levels measured after sensitization (dotted bars), however without reaching statistical significance. Additionally, spleen cell response after stimulation with OVA was evaluated after 6 oral MP administration cycles to assess antigen specific T-cell responses. Significantly higher levels of the Th1 cytokine IFN γ were found after oral application of NA-functionalized, OVA-loaded

MPs compared to animals being fed with uncoated MP (Fig. 4D). Additionally, the group receiving NA-coated, OVA-loaded MPs showed significantly higher IL10 levels compared with the group fed uncoated MPs and naïve animals (Fig. 4E).

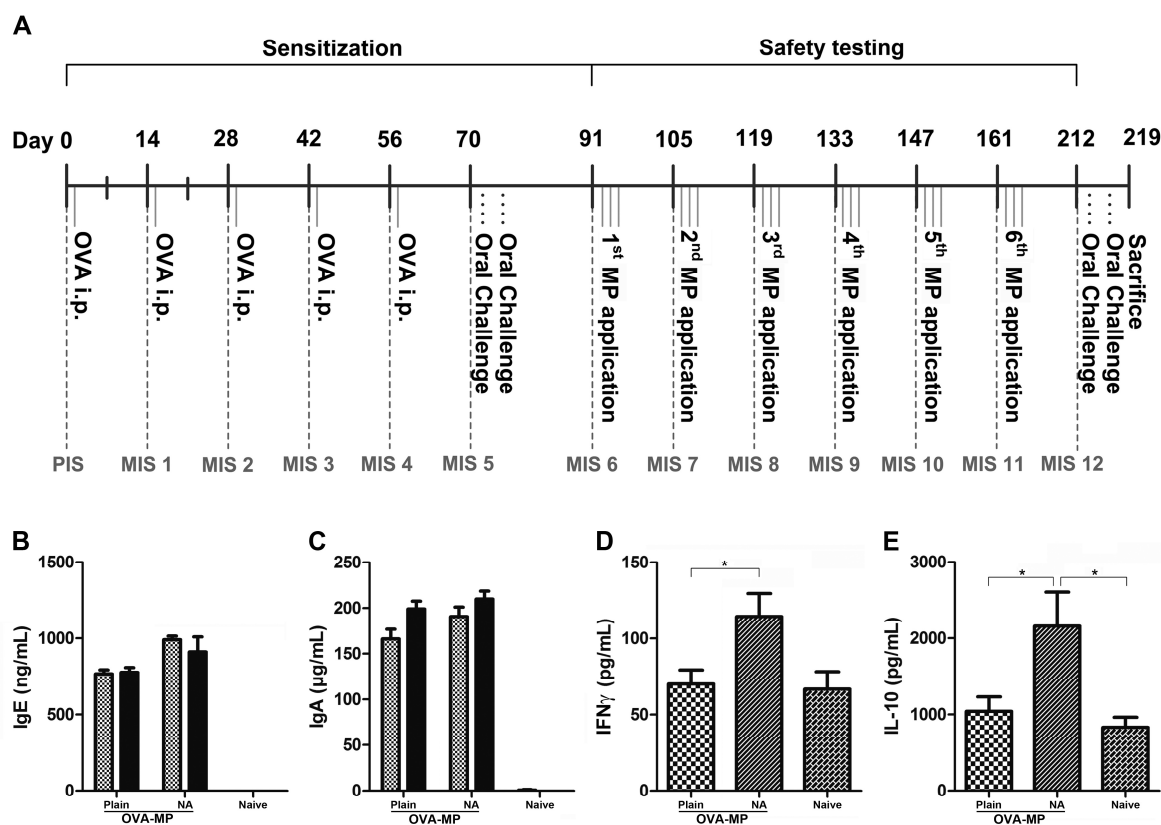


Figure 4: Safety assessment of NA-MP gavages in allergic animals.

3.3.3 Proof of concept study - Oral immunotherapy of OVA food allergic mice with NA-functionalized, OVA-loaded MPs

After repeated oral immunizations with OVA under concomitant acid suppression a strong IgE-mediated immune response was detected. To induce a sustained systemic response, mice were further injected twice with OVA i.p. (Fig 5A). Thereafter, mice were subjected to oral and systemic challenges and core body temperature was measured to evaluate the anaphylactic response. In all animals a pronounced drop of body temperature (Fig 5B, left panel) was observed accompanied with severe clinical signs of anaphylaxis. After sensitization, mice were treated with OVA-loaded, uncoated or NA-coated MP. Control groups either received OVA i.g., which simulated standard oral immunotherapy, were sacrificed after sensitization, were left untreated or remained naïve for the entire study period. Due to the severe anaphylactic response after sensitization we performed a stringent follow-up for more than 1 h after each oral immunotherapy. No clinical signs of an adverse responses were observed following oral MP application. After 6 cycles of oral immunotherapy the systemic allergen challenge was repeated to re-evaluate the allergic response. Following an immediate decline of body temperature in all animals we observed an earlier recovery from anaphylaxis indicated by no further significant drop of body temperature 15 min after the challenge only in the groups being treated with NA-MP or OVA i.g. All other groups revealed a further significant decline of core body temperature (Fig 5B, right panel). Measuring serum antibody titers, we observed marginal increases of OVA-specific IgE (Fig 5C), IgG1 and IgG2a (data not shown) during the treatment period in immunized animals without reaching statistical significance. Only the control group receiving OVA i.g. revealed a significant increase of specific IgA serum levels (data not shown). In the intestine we found significantly elevated levels of total IgA in mice treated with uncoated or NA-coated MP as well as in animals receiving OVA i.g. (Fig 5D). In the latter group also elevated titers of OVA-specific IgA were determined (Fig 5E), which was accompanied by higher levels of OVA-specific IgE (data not shown). When supernatants of OVA-stimulated splenocytes of mice taken after immunotherapy were screened for cytokine production no significant differences in IL4 and IFN γ levels were observed (data not shown), but the levels of the regulatory cytokine IL10 in animals receiving NA-MPs were significantly elevated compared to the control (Fig 5F).

Flow cytometric analysis of regulatory T-cell numbers revealed significantly elevated counts in mice treated with uncoated or NA-coated MPs with levels being restored to those measured in naïve animals by MP treatment (Table 2).

Group	% of CD25⁺ Foxp3⁺ cells of CD4⁺ cells mean $\pm$SD (95% CI)	Absolute counts of CD4⁺CD25⁺Foxp3⁺ cells mean $\pm$SD (95% CI)	p-value
Plain MP	11.01 $\pm$ 2.11 (9.07-12.96)	346.7 $\pm$ 86.66 (266.6-426.9)	<0.05
NA-MP	10.59 $\pm$ 0.88 (9.78-11.40)	350.0 $\pm$ 55.65 (299.4-402.3)	<0.05
OVA ig.	8.22 $\pm$ 2.89 (5.54-10.90)	265.9 $\pm$ 113.4 (161.0-370.7)	
No therapy	7.62 $\pm$ 1.26 (6.46-8.79)	274.0 $\pm$ 48.20 (229.4-318.6)	*
Naïve	10.56 $\pm$ 1.75 (8.94-12.18)	375.1 $\pm$ 68.71 (311.6-438.7)	

Table 2: Flow cytometry analysis of regulatory T cell counts in spleens of mice after oral immunotherapy. P-values indicate statistically significantly elevated regulatory T cell counts compared to the control group receiving no therapy (*).

3.4 Discussion

A number of lectins have been investigated for their suitability as M-cell specific targeters. However, some of them are toxic and susceptible to proteolytic degradation in the gastrointestinal tract.²

Neuraminidase, also termed sialidase, is encoded within a pathogenicity island of the *Vibrio cholerae* genome,²⁵ and removes sialic acid from gangliosides to yield the cholera toxin receptor GM1.^{18, 25} Our *in vitro* data support the assumption that NA binds, similar to the lectin AAL, via α -L fucose but also via GM1 to intestinal epithelial cells. As both, α -L fucose and GM1 are expressed on M-cells,²⁶⁻²⁷ NA might represent a promising M-cell specific targeting substance. In this study we have investigated the suitability, safety and functionality of NA as a novel M-cell targeter on PLGA-MPs to be used for oral immunotherapy of food allergy.

Indeed, the binding of NA alone and NA-MPs to intestinal cells was proven by *in vitro* and *in vivo* experiments. Additionally, M-cell specific binding of NA-MPs was confirmed in co-culture experiments. As NA hydrolyzes complex gangliosides on intestinal epithelial cells to yield GM1,²⁹ high local concentrations of GM1 are achieved.²⁴ Thereby, NA may disclose its own binding partner on M-cells, making its targeting even more efficient, which is a clear advantage to the structurally similar AAL, which we have focused on in recent studies. Furthermore, NA is part of the mucinase complex, which enhances the pathogenicity of *Vibrio cholerae* by degrading the mucin layer in the gastrointestinal tract. Based on this mechanism, NA coated particulate carriers might have improved access to intestinal cells, particularly to M-cells, which are protected by only a thin mucus layer.²⁹

However, as NA is part of the pathogenicity island of *Vibrio cholerae*, the safety of an oral application of PLGA-MPs functionalized with NA had to be analyzed in depth. By investigating the immunomodulatory capacity of NA in naïve animals, we observed an induction of the Th1 cytokine IFN γ and of the T-regulatory cytokine IL10, indicating a beneficial effect in cases, where a Th2 biased immune response is prevails. Additionally, repeated gavages of NA-grafted, OVA-loaded MPs did not induce mast cell responses or allergen specific IgE in naïve mice. But the question remained, whether feedings with NA-MPs are safe enough in an organism that is already allergic to an antigen. Indeed, no enhancement of the IgE mediated OVA allergic response could be detected in OVA-allergic animals fed with NA-functionalized, OVA-loaded

MPs. On the contrary, also in allergic animals Th1 and T-regulatory responses were induced.

To confirm the efficacy of NA-functionalized MPs for oral allergy immunotherapy, PLGA-MPs were loaded with OVA and functionalized with the targeter. By entrapment of OVA with NA-functionalized PLGA-MP, the allergen was protected against gastric and intestinal degradation, compared to non-encapsulated OVA, which is degraded by gastric enzymes within 30 minutes.^{19, 30} This allows the intact allergen to bind to M-cells and to be taken up by antigen presenting cells residing underneath the M-cells. Thereby, an allergen specific immune response can be induced, which might contribute to the counterbalance of a Th2 immunity.

This hypothesis was confirmed by another study, where feedings of allergic mice with AAL functionalized, birch-pollen loaded PLGA-MPs beneficially altered the allergic immune response of animals by the induction of IL10, IFN γ and IgG2a.⁷ In line with these results, oral therapy with NA-functionalized, OVA-loaded MPs significantly increased the T-regulatory cytokine IL10, whereas the serological antibody titers were not significantly altered after oral treatment compared with the levels after sensitization. Interestingly, oral treatment with NA-MPs ameliorated anaphylaxis upon allergen challenge. In the current study, gavages of NA-functionalized MP were further compared with current treatment strategies. Oral immunotherapy (OVA i.g.) revealed similar antibody responses in comparison with feedings of NA-functionalized, OVA-loaded MPs, but did neither stimulate Th1 nor T-regulatory cytokine induction in the food allergy model.

In conclusion, we propose that NA of *Vibrio cholerae* represents a suitable, highly efficient and safe M-cell specific targeting ligand due to its α -L fucose and GM1-binding specificity. The immunomodulatory characteristics of NA may be additionally beneficial for surface modification of PLGA-MPs for oral immunotherapy of food allergy. Coating of MPs with NA increases the uptake via M-cells, enabling encapsulated and protected antigens to be directed to the mucosal immune induction sites. In an *in vivo* model NA-functionalized, OVA loaded MPs were proven as a suitable novel concept for oral immunotherapy of food allergy. Due to the induction of IL10 a Th2 environment could sufficiently be counterbalanced, as also indicated by an amelioration of anaphylaxis, which additionally supports suitability of NA-grafted MPs for allergy treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary information to figure legends:

Figure 1. Characterization of NA as a targeter. NA remained stable up to 180 min in SGF experiments (A) and bound to Caco-2 cells in a dose-dependent manner (B; FITC-NA 16µg: left panel, blue line, FITC-NA 32µg: left panel, orange line, FITC-NA 64µg: left panel, light green line, FITC-NA 128µg: left panel, dark green line versus MFI control (red line)). GM1 and α -L fucose were identified as binding partners as they inhibited NA binding to Caco-2 cells in a dose dependent manner (C). Data is mean + standard error of mean (SEM). * P / $\#P$ <0.05, ** P / $\#P$ <0.01, ### P <0.001, #### P <0.0001.

Figure 2. Characterization of NA-functionalized MPs. The allergen OVA, which was encapsulated in uncoated (Plain-MPs) or NA-MPs, remained stable against digestion up to 120 min in SGF (A). The binding of MPs to Caco-2 (B) and to murine intestinal cells (C) was confirmed by immunofluorescence.

Figure 3. Safety assessment and immunomodulatory properties of NA and NA-MPs in naïve mice. NA and NA-MPs induced significantly higher levels of IFN γ and IL10 (A) but did not influence mast cell responses as determined by mMCP1-levels in serum after single oral application (B). Repeated oral administration of NA-MPs in naïve mice induced no OVA specific IgE but IgA expression (C). Data are mean + SEM, * P <0.05, ** P <0.01, *** P <0.001.

Figure 4. Safety assessment of NA-MP gavages in allergic animals. Sensitization and treatment protocol is depicted in (A). Sera taken after sensitization (dotted bars) and after repeated oral MPs application (black bars) were screened for OVA specific IgE (B) and IgA (C), where no significant differences were observed. Spleen cells of mice fed with NA-MPs produced significantly more IFN γ (D) and IL10 (E) after OVA stimulation. Data are mean + SEM, * P <0.05.

Figure 5. Oral immunotherapy of food allergic mice with OVA-loaded MPs. After oral sensitization and oral as well as systemic allergen challenge, mice were orally treated with plain- or NA-, OVA-loaded MPs or OVA alone or were left untreated (A). Anaphylaxis, measured as a decline of rectal body temperature (B) before (left panel) and after oral treatment (right panel), was ameliorated only in mice treated with NA-

MPs (red line) or fed OVA alone (orange line) compared to all other groups (Plain-MP group (light grey), no oral treatment (black line)). No significant differences in IgE production were observed after oral treatment (C). Intestinal total IgA (D) and OVA specific IgA (E) were determined after sacrifice by ELISA. IL10 production of OVA stimulated spleen cells of allergic mice was significantly higher only in the NA-MP treated group (F) Data are mean + SEM, * $P < 0.05$, *** $P < 0.01$.

Supplementary Figure 1. Preparation of functionalized OVA-loaded PLGA-MP.

PLGA-MPs were loaded with OVA as model allergen using the double emulsion technique (A). PLGA-MPs were functionalized with targeting substances after activation of carboxyl groups using EDAC (B).

4 Abstract

4.1 Deutsch

Als alternative Möglichkeit zur spezifischen Immuntherapie sollte in dieser Arbeit die orale Immuntherapie von Nahrungsmittelallergien untersucht werden. Dazu wurden Neuraminidase-dotierte Mikropartikel, die eine hohe Affinität zu Fucose-Resten an der Oberfläche von intestinalen M-Zellen besitzen, aus dem biokompatiblen und biodegradierbaren Poly (D,L-lactide-co-glycolide) hergestellt und mit Ovalbumin als Allergen beladen. In Anlehnung an ein etabliertes Immunisierungsprotokoll wurden diese Mikropartikel sensibilisierten und nicht sensibilisierten Mäusen verabreicht. In definierten Zeitabständen wurden Zytokinspiegel und Immunglobulingehalt der Sera bestimmt und zusätzlich die Körpertemperatur gemessen. Entsprechend den Ergebnissen zeigten oral immunisierte Mäuse nur geringe anaphylaktische Reaktionen und signifikant erhöhte Interferon γ und Interleukin-10 Spiegel. Insgesamt könnten Neuraminidase-dotierte, Allergen-hältige Mikropartikel eine sichere und wirksame Alternative in der Therapie von Nahrungsmittelallergien darstellen.

4.2 Englisch

In an effort to elucidate the utility of oral immunotherapy for treatment of egg allergy, Neuraminidase-coated microparticles loaded with egg albumin prepared from poly (D-L-lactic-co-glycolic) acid were orally applied to sensitized and non-sensitized mice according to an established immunization protocol. At certain time points sera were analyzed for cytokine and immunoglobulin levels. Additionally, body temperature was determined. According to the results, orally immunized mice showed strongly reduced anaphylactic reactions as well as significantly higher IFN γ and IL 10 levels. All in all, Neuraminidase-coated, allergen-loaded microparticles might be a safe and efficient novel treatment option in food allergy.

5 Zusammenfassung

In früheren Studien konnte gezeigt werden, dass Aleuria aurantia lectin (AAL)-funktionalisierte Poly (D, L-lactide-co-glycolide)- Mikropartikel (MP) an M (membranöse) - Zellen des Darmtraktes binden und eine IgE-induzierte Allergie positiv beeinflussen.

Aufgrund der strukturellen Ähnlichkeit mit AAL sollte nun Neuraminidase (NA) aus *Vibrio cholerae* auf seine Eignung als M-Zell bindender targeter untersucht werden. Für einen möglichen Einsatz zur oralen Immuntherapie von Nahrungsmittelallergien wurde die Sicherheit und therapeutische Wirksamkeit von Allergen-beladenen, NA-beschichteten Mikropartikeln untersucht.

Um die Tauglichkeit von NA und NA-funktionalisierte Mikropartikeln zur oralen Applikation festzustellen, wurden Verdauungs-Experimente, Zell ELISA und Immunfluoreszenz Färbungen an Caco-2 Zellen sowie Bindungsstudien an humanen M- Zellen durchgeführt. Die Sicherheit von funktionalisierten Mikropartikeln wurde einerseits in vitro, durch die Stimulierung von Milzzellen von naiven Mäusen und andererseits in vivo, in naiven und allergischen BALB/c Mäusen analysiert. Die therapeutische Wirksamkeit der MP bei Nahrungsmittelallergie wurde im Mausmodell charakterisiert.

NA zeigte eine hohe Bindungsrate an α -L-Fucose und Monosialoganglioside 1 (GM1) und förderte die transepitheliale Aufnahme in einem M-Zell-Co-Kultur Modell. Nach Stimulation mit NA oder NA-beschichteten MP exprimierten murine Milzzellen signifikant mehr IL10 und IFN γ . Nach sechs Zyklen oraler Gaben von mit NA-funktionalisierten MP zeigten Sicherheitstests bei naiven Tieren erhöhte Spiegel von OVA - spezifischem IgA. Bei allergischen Mäusen wurden signifikant höhere IFN γ und IL10-Spiegel nach Verabreichung festgestellt. Nahrungsmittelallergische Mäuse, die mit OVA beladenen NA-MP therapiert wurden, zeigten eine geringere anaphylaktische Reaktion, die sich im geringeren Abfall der rektal gemessenen Körpertemperatur äußerte. Dies ging im Vergleich zu den Kontrollgruppen mit den höheren Konzentrationen an IL10 und regulatorischen T Zellen einher.

Insgesamt deuten die Ergebnisse darauf hin, dass mit NA beschichtete und mit Allergen beladene Mikropartikel eine effiziente und sichere, neue Therapieform für Nahrungsmittelallergie in einem Mausmodell sein könnten.

6 Literaturverzeichnis

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