



Targeting antigens to murine and human M-cells with *Aleuria aurantia* lectin-functionalized microparticles

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Abstract

Neuraminidases act as a virulence factors for several pathogens that invade the human body through Peyer's patch M-cells. Because of the structural similarity of *Aleuria aurantia* lectin (AAL) to neuraminidases, we hypothesized that AAL might also target human M-cells. In an in vitro human M-cell co-culture model significantly more particles were transported across the epithelium when microparticles were functionalized with AAL versus those that were not. Moreover, high concentrations of AAL induced no detectable cytotoxic effects on the related intestinal epithelial cell cultures, epithelial Caco2- and HT29-MTX-E12-cells. Upon incubation with AAL, PBMCs of allergic volunteers proliferated in response to AAL and secreted the cytokines, IL-2, IFN- γ , IL-10 and IL-5 in a concentration-dependent manner, indicating immune-stimulatory properties of the lectin. We conclude that AAL-coated microparticles may have the potential to target entrapped antigens to human M-cells for oral vaccination.

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1. Introduction

Many pathogens invade the body by exploiting the mucosal routes such as the gastro-intestinal (G/I) tract. Higher vertebrates have the ability to mount an adaptive immune response to pathogens without compromising the integrity of the mucosal barrier [1]. For this reason Peyer's patch "membranous" or "microfold" cells, termed M-cells, have been evolved. These cells are able to transport antigens from the lumen across the follicle-associated epithelium (FAE) to the

underlying lymphoid cells, thereby stimulating specific mucosal immune responses [2]. M-cells are characterized by their poorly organized brush border, high endocytic activity and basolateral lymphocyte-containing pocket [3]. They are a differentiated epithelial cell subset comprising ~5% of the cells within the human FAE and they perform antigen sampling for presentation to the organized lymphoid tissues beneath [4,5]. Such organized mucosal lymphoid tissues are located in the nasal cavity, pharynx, bronchia, small intestine, colon and rectum [6]. M-cells are preferentially exploited for invasion by many pathogens. Examples include the enteric pathogens are *Vibrio cholerae* [7], *Salmonella typhimurium* [8], *Yersinia pseudotuberculosis* [9] and reoviruses [10]. Also non-enteric pathogens like influenza virus A mainly enter the body by targeting M-cells of another mucosal immunity induction site, human nasopharyngeal tonsils [11]. Thus, extensive studies

Abbreviations: AAL, *Aleuria aurantia* lectin; WGA, wheat germ agglutinin; M-cells, membranous cells; BP, birch pollen extract; HSA, human serum albumin

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have been performed in vaccine research to find specific surface markers for targeting M-cells. However, there are considerable species and site related variations [12–14]. Murine M-cells express α -L-fucose, but for other species, no specific surface markers for M-cells have yet been consistently defined, even thorough claims have been made for sialylated Lewis antigen A in man [15]. Other investigations attempting to identify human M-cell surface receptors include analysis of the gene expression patterns in human M-like cells [16].

The *Aleuria aurantia* lectin (AAL) has α -L-fucose specificity and exhibits structural similarity to neuraminidases from different pathogens that invade via M-cells [17]. AAL is derived from the edible orange peel mushroom *A. aurantia*. Our hypothesis is that AAL might be a means for targeting M-cells. In a murine model we have shown by indirect methods that this may be the case since feedings of microparticles loaded with birch pollen proteins induced allergen-specific Th1-responses only when they were coated with AAL [18]. Arising from the immune responses detected in mice, it seems that we may have succeeded in harnessing the sampling function of M-cells in this model.

The aims of this study were to examine whether this principle would hold in a human system. We demonstrate here that AAL-functionalized microparticles: (1) are non-toxic, (2) can target Peyer's patches *ex vivo*, (3) are effectively transported by M-cells of human origin, and (4) harbor immune-stimulatory properties.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice, that were 18–20 weeks old, were obtained from Harlan UK, Blackthorn, England. Mice were euthanized by cervical dislocation and all experiments were performed post-mortem.

2.2. Allergic individuals

All allergic human subjects had a history of hayfever during early spring, a positive skin prick reaction (wheal >5 mm) to birch pollen extract (Soluprick; ALK, Hørsholm, Denmark), and a radioallergosorbent test to birch pollen >3 (median, 24.9 kU/ml; RAST/CAP; Pharmacia, Uppsala, Sweden). None had received immunotherapy before.

2.3. *In vitro* toxicology assay

The human enterocyte-like cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, USA), whereas the mucus-secreting HT29-MTX-E12-cells (E12) [19,20] were kindly donated by Professor Per Artursson (Department of Pharmacy, University of Uppsala, Sweden). Lactate dehydrogenase assay (LDH) was used for measuring membrane integrity of Caco-2- and E12-cells,

and performed according manufacture's instruction (Sigma, St. Louis, MO, USA). The assay is based on the reduction of NAD^+ by the action of LDH. Briefly, cells were grown for 5–6 days in 96-well microtiter plates and incubated with AAL, wheat germ agglutinin (WGA; in the concentration range of 0–1 mg/ml) or 1% (v/v) Triton X-100 as control, diluted in DMEM without FCS and phenol red, at 37 °C/5% CO_2 . After 45 min, 60 μl /well were transferred in a new microtiter plates and 60 μl of freshly prepared LDH-solution containing the enzyme and the substrate were added for another 30 min. Reaction was stopped with 1N HCl and optical density measured at 492 nm. Measurements were performed in triplicate.

2.4. Binding of AAL-microparticles to murine Peyer's patches

Immediately after mice death, small intestine were removed and perfused with 5% CO_2 /95% O_2 -saturated Krebs-buffer of following mM composition: NaCl 112, NaHCO_3 25, KCl 5, CaCl_2 2.5, KH_2PO_4 1.2, MgSO_4 1.3, D-glucose 12. The duodenum was opened by a longitudinal cut and softly rinsed with Krebs-buffer. For covalent coupling of proteins to microparticles, the surface carboxylic groups of microparticles were activated by carbodiimide/*N*-hydroxysuccinimide and subsequently incubated with AAL or HSA, as previously described [21]. The tissues were incubated with AAL-fluospheres in Krebs-buffer or control-fluospheres for 20 min. Afterwards tissue were washed and fixed in 4% paraformaldehyde. Peyer's patches and adjacent parts were cut out, mounted, sealed and stored at 4 °C in the dark. All images were obtained using a Nikon Eclipse E400 microscope and a Nikon Digital Camera DXM1200.

2.5. Cellular uptake of AAL-fluospheres by human M-like cells

Polarized Caco-2-cells were grown on transwell filter inserts in inverted orientation for 21 days and co-cultured with human Raji B-cells for 3 days as described elsewhere [22,23]. Carboxy-modified fluospheres 0.01% (w/v) (2 μm , Molecular Probes, Leiden, NL) or same fluospheres functionalized with AAL were incubated for 60 min on the apical sides of Caco-2/Raji-B co-cultures at 37 °C under gentle agitation. Thus, for cellular uptake, particles had to compete against gravitation. After thoroughly washing the membranes with PBS, fluospheres from basolateral side as well as of the membranes were collected and dissolved in 0.1N NaOH. Fluorescence was measured at 485/530 nm. As control, cellular uptake-study was performed simultaneously with mono-cultures of polarized Caco-2 cells ($n=6$ for fluospheres and AAL-fluospheres, respectively).

2.6. Stimulation of PBMC

PBMC were isolated from peripheral blood of allergic patients by Ficoll-Hypaque density gradient centrifugation

(Pharmacia) and cultivated in serum-free Ultra Culture Medium (BioWhittaker, Walkersville, MD) supplemented with 2 mM L-glutamine and 2×10^{-5} M 2-ME. PBMC ($2 \times 10^5/200 \mu\text{l}$) were stimulated with birch pollen-loaded microparticles functionalized either with AAL or human serum albumin (HSA) in triplicates in 96-well plates (Nunc; Nunc) in the concentration range of 50–400 μg microparticle/well. Both types of microparticles contained approximately 40 μg birch pollen proteins/mg. After 6 days, cell-free culture supernatants were harvested and analyzed for IL-2, IL-5, IL-10 and IFN- γ production by ELISA [24].

2.7. Data analysis and statistics

Data are presented as mean values $\pm$ S.E.M. Statistical analyses were performed using the Student's *t*-test to compare mean values between the Gaussian-distributed groups (software SPSS Vers.11.5 for Windows). In each case, *p* values <0.05 were considered statistically significant.

3. Results

3.1. AAL-microparticle bind specific to Peyer's patches

One of the aims was to demonstrate that the generated microparticles were suitable for specific targeting to Peyer's patches *ex vivo*. Murine Peyer's patch can be easily discerned

from the adjacent crypts by their lack of microvilli (Fig. 1). In the Peyer's patches a large number of fluorescein-labeled AAL-microparticles were localized (a, c) whereas hardly any uncoated microparticles could be detected in the adjacent parts of the Peyer's patches (b, d).

3.2. AAL induces no cytotoxicity in mucus-secreting E12-cells and enterocyte-like Caco-2 cells

AAL was tested for the potential to induce cytotoxic effects on human mucus-secreting E12-cells and Caco-2 cells *in vitro*. The cell viability of Caco-2 cells and E12-cells was unaffected by incubation with AAL (Fig. 2). Even the highest concentration of 1 mg/ml AAL caused only 1.1 ± 0.3 and $2.5 \pm 1.2\%$ cell death in Caco-2 and E12, respectively. The control lectin WGA impaired the viability of the cells to a greater extent. Addition of 1 mg/ml WGA resulted in 14.7 ± 0.8 and $8.1 \pm 2.6\%$ cell death, respectively.

3.3. AAL-microparticle bind to human M-like cells *in vitro*

We circumvented the obstacle to obtain fresh human Peyer's patches for binding-studies, by testing our AAL-microparticles in an *in vitro* M-cell like co-culture model of human origin [16,19]. In this model, M-cells differentiate from enterocyte-like Caco2-cells upon co-culture with

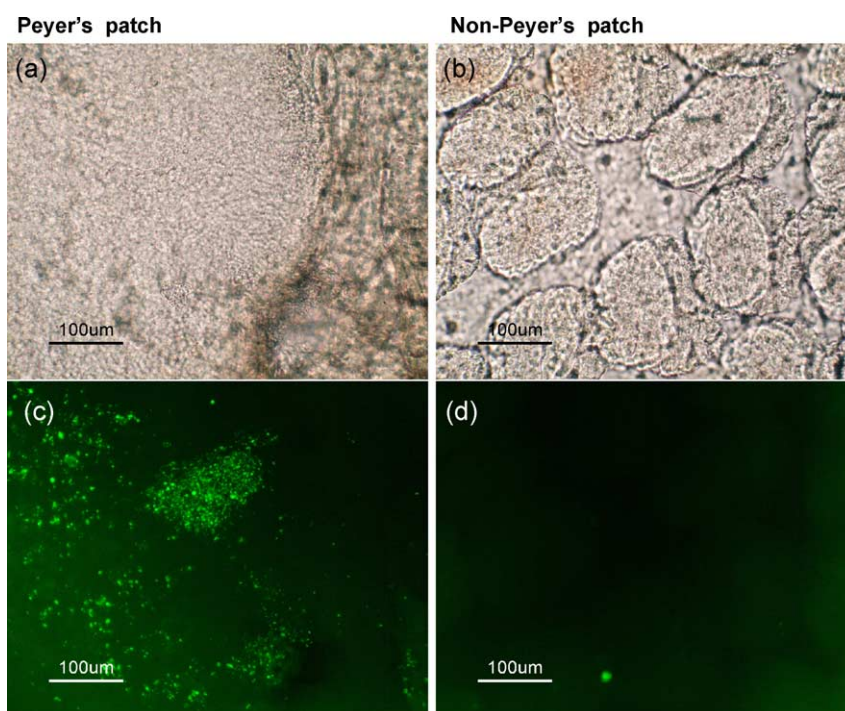


Fig. 1. Binding of AAL-microparticles to murine Peyer's patches. Fluorescence-labeled microparticles functionalized with AAL were incubated with murine small intestinal segments of mice. After washing, intestines were fixed and images taken under light (a, b) and fluorescence microscopy (c, d). AAL-microparticles bound specifically to murine Peyer's patches (a, c), whereas nearly no microparticles could be observed in adjacent parts (b, d).

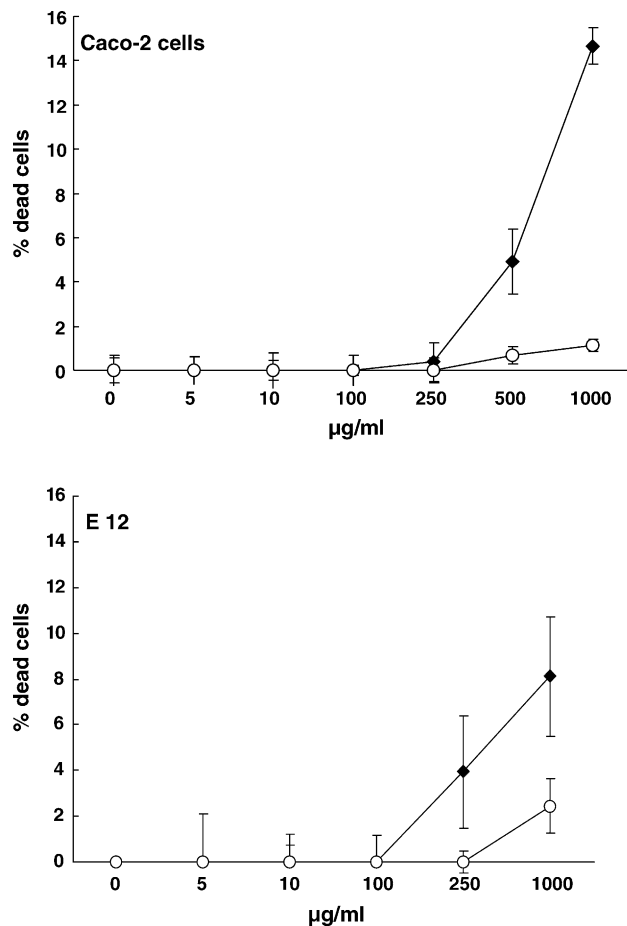


Fig. 2. Influence of AAL and WGA on cell viability. Caco2- and E12-cells were grown for 5 days in 96 well plates, followed by incubation with various concentrations of AAL (○) or WGA (◆). Subsequently, LDH-leakages of cell membranes were measured as indicator of cell viability. At the highest concentration, AAL impaired the viability of human enterocyte-like Caco2- and mucus-secreting E12-cells to 1.14 ± 0.29 % and 2.45 ± 1.21 %, respectively. In contrast, incubation with 1 mg/ml WGA resulted in toxic cell lysis to 14.66 ± 0.8 % and 8.12 ± 2.6 %, respectively. Data represent mean $\pm$ S.E.M. of three experiments.

Raji-B cells derived from a human Burkitt lymphoma. In this experiment a significantly increased cellular uptake and transcytosis of microparticles was achieved when they were coated with AAL, as compared to non-functionalized microparticles. Even in the absence of a human M-cell-specific marker, the conversion to M-cell like cells was proven at a functional level by a significantly enhanced particle uptake in the filter-grown co-cultures versus mono-cultures (Fig. 3; fluospheres: $p = 0.0286$, AAL-fluospheres: $p = 0.0485$). Importantly, there was a significant increase in cellular uptake of fluospheres functionalized with AAL compared to non-functionalized ones in both, the mono-culture ($p = 0.0179$) as well as in the co-culture model ($p = 0.0149$). The co-cultures therefore took up more AAL-coated than uncoated particles and in each case, the levels of uptake were increased over the uptake seen in respective mono-cultures.

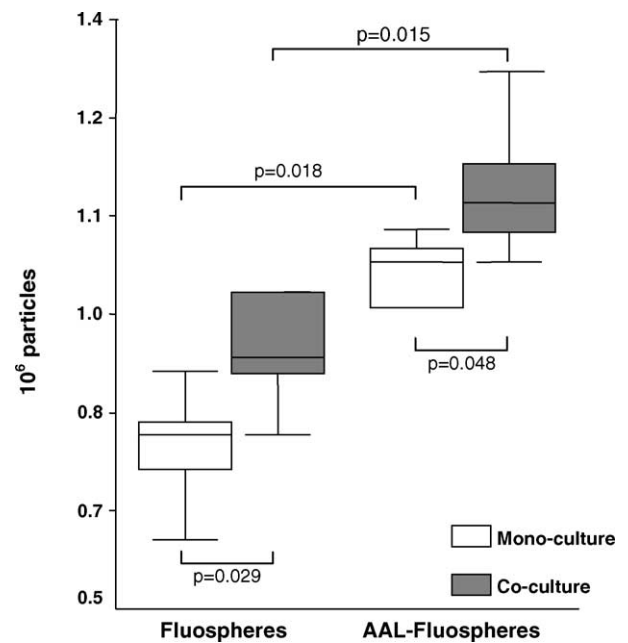


Fig. 3. Cellular uptake of microparticles in the presence and absence of AAL-functionalization. Caco-2 cells were grown in mono-culture (white panels) or in co-culture with Raji-cells (grey panels). Microparticles were incubated for one hour with the cell cultures. After rigorous washing, fluospheres- and AAL-fluospheres of cellular and basolateral compartments were dissolved in 0.1N NaOH and detected fluorimetrically at 485/535 nm. In the co-cultures, significantly more uncoated particles were absorbed and transcytosed than in the mono-cultures. Additional functionalization of the microparticles with AAL resulted in a significant increase in particle uptake compared to non-functionalized microparticles. Data from six experiments are presented as mean $\pm$ S.E.M.

3.4. AAL-microparticles-induced cytokine production by PBMCs of allergic individuals

To evaluate the effects of allergen-loaded microparticles functionalized with either AAL or HSA (control) on the cytokine production from allergic patients, PBMC from three birch pollen-allergic individuals were stimulated with different concentrations of both types of coated microparticles and analyzed for the secretion of IL-2, IL-5, IFN- γ and IL-10. As shown in Fig. 4, AAL-functionalized microparticles induced a dose-dependent cytokine production whereas particles coated with HSA did not. AAL-coated microparticles stimulated significant production of Th1-cytokines (IL-2, IFN- γ), Th2-type (IL-5) and immunomodulating cytokines (IL-10). Large amounts of IFN- γ (1.2–6.4 ng/ml) and IL-2 (14–46 ng/ml) were detected, whereas IL-10 (8–23 pg/ml) and IL-5 (37–51 pg/ml) were found in smaller quantities in the supernatants of PBMCs derived from allergic individuals.

4. Discussion

We generated AAL-microparticles that may be applicable not only for murine proof-of-concept studies for targeted oral vaccine delivery to M-cells in Peyer's patches, but also for

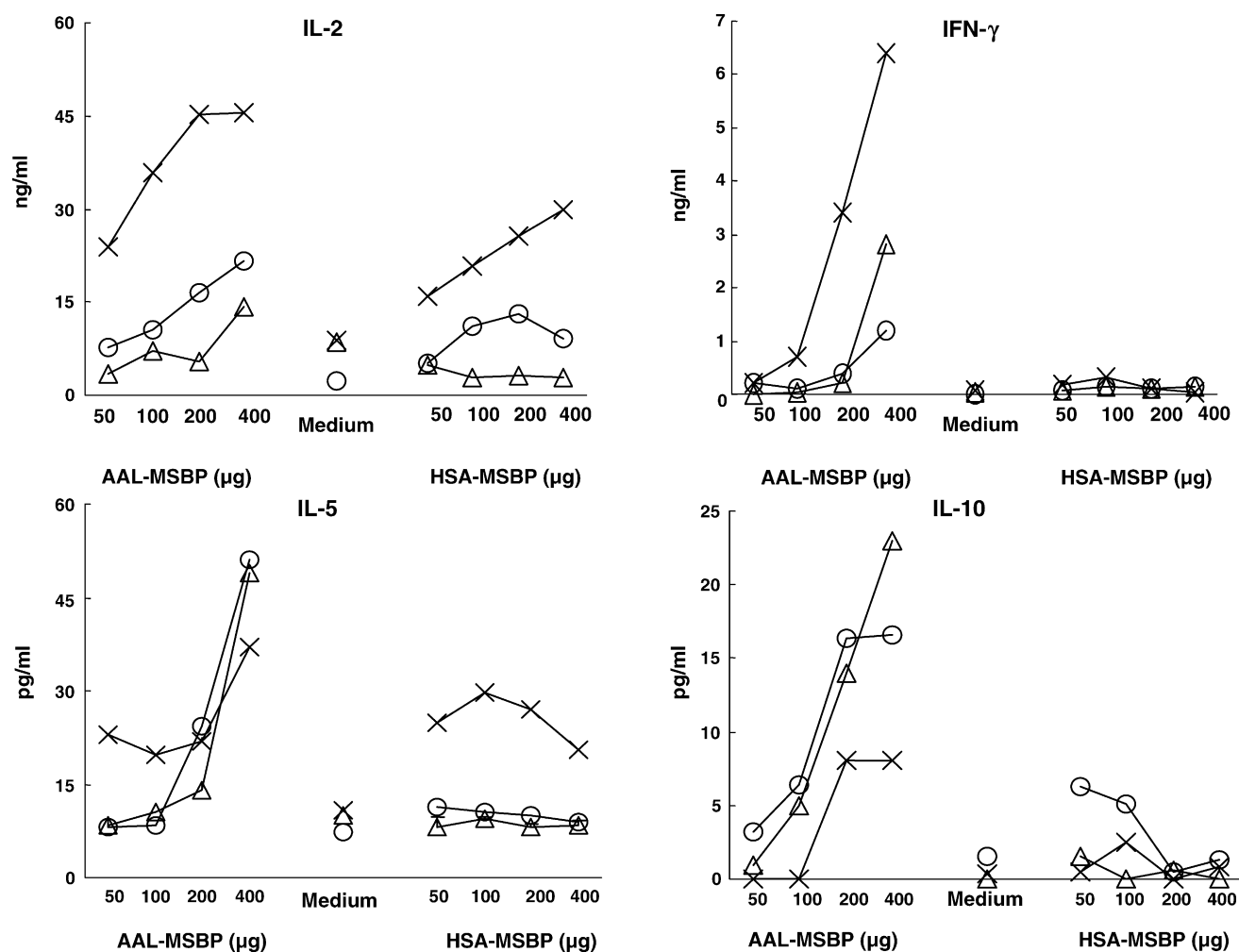


Fig. 4. Cytokine production upon stimulation with allergen-loaded microparticles. PBMC from three birch pollen-allergic individuals (Δ , $\circ$, $\times$) were cultured with birch pollen-loaded microparticles functionalized with either AAL (AAL-MSBP) or HSA (HSA-MSBP) in the concentration range of 50–400 μ g microparticle per well for 6 days. Subsequently, cell-free supernatants were analyzed for IL-2, IL-5, IL-10 and IFN- γ . Incubation of allergen-loaded microparticles (40 μ g birch pollen proteins/mg microparticles) resulted in a concentration-dependent induction of all cytokines only when microparticles were coated with AAL.

oral human vaccination. The biggest advantage of oral vaccines is believed to be the induction of mucosal immunity in addition to systemic immunity [25], since lymphocytes activated in a certain organ system tend to home back to their site of activation [26]. In practice, the bioavailability of oral vaccine and peptide particulate formulations is lower than for parenteral routes, primarily because of insufficient particle uptake across epithelial barriers including Peyer's patches. Lectins, which are naturally occurring proteins with affinity for sugar residues, bind specifically to oligosaccharide moieties on the surface of intestinal cells and have been investigated as agents of bioadhesion in the gut [21,27]. For mucosal drug targeting in humans these bioadhesins have to be suitable for conjugation to delivery systems, be non-toxic, stable in the gut and compatible with other gut luminal contents such as food digesta and commensal flora. Finally, the bioadhesins should enhance intestinal absorption by the targeted cells. AAL seems to combine all of these beneficial properties.

Our data clearly demonstrate that AAL does not harm intestinal cells and might be safe and suitable for application also in humans. Further AAL is capable of detecting α -linked fucosyl units regardless of the structure of the aglycone. It has been successfully employed to examine fucoglycoproteins non-reactive with the lectins from *Lotus tetragonolobus* and *Ulex europaeus* [28]. This feature is important, since murine M-cells can be targeted almost exclusively by their α -L-fucose residues [12–14,29]. Indeed, AAL-microparticles bound to murine Peyer's patches, but not to the adjacent parts using fresh isolated small intestine. Encapsulated antigens may, therefore, be directly delivered to immunocompetent cells that underlie the Peyer's patches enabling site-directed investigations in the intestine. This explains our previous observation where antigen-loaded microparticles were able to achieve an effective immune response in mice only when targeted to M-cells by AAL [18].

The six 4-stranded β -sheet motifs of AAL and its close structural similarity to *S. typhimurium* LT2 neuraminidase has been revealed recently by two independent groups by crystallographic studies [30,31]. Extensive searches in literature, protein and structural databases (PDB, SCOP) showed that the six 4-stranded β -sheet motifs are very common for neuraminidases. Some of the neuraminidases from pathogens like influenza virus [32] and paramyxoviruses [33] are known to be required for invading the host through the mucosa. Moreover, nearly all pathogens that invade humans through M-cells possess this enzyme. Therefore, it is tempting to speculate that neuraminidases-like structures in general might be a prerequisite for invasion of the human host by this route. Our hypothesis was that AAL might structurally mimic neuraminidases, target an antigen to M-cells and facilitate its entrance to the human body. As a consequence, we performed in vitro transport studies where M-like cells of human origin were generated by co-culturing with B-cells [22,34], demonstrating the significantly enhanced uptake of AAL-conjugated particles versus unconjugated ones. From these data, it seemed likely that AAL functionally imitates neuraminidases through its structure. While co-culture remains controversial in respect of the human M-cell phenotype, it is gaining more acceptances due to the characteristics being demonstrated by a number of research groups over time [22,23,34–36]. These include particle and pathogen uptake and gene expression changes [16,22,23,34]. In this study the colon cancer cells Caco-2 were used that also express α -L-fucose [37]. Indeed, the binding of AAL to these cells could be inhibited specifically and in a dose-dependent manner by α -L-fucose (data not shown). However, we have to admit that the question whether the enzymatic similarity of AAL plays a role for its targeting capacity remains still open.

Finally, we demonstrated that AAL itself had the capability to induce secretion of IL-2, INF- γ , IL-10 and IL-5 from PBMCs of birch pollen-allergic subjects in a concentration-dependent manner. The fact, that all analyzed cytokines were induced upon AAL-stimulation of cells from allergic individuals shows that AAL was able to impact Th1/Th2 or regulatory T-cells. The resulting, non-biased immune response should be beneficial, since an imbalance between Th1 or Th2 may result in autoimmune diseases or allergy. It has been recently shown that not only IL-10 [38], but also IL-2 play important roles in immunomodulation and induction of tolerance [39]. In general soluble antigens induce tolerance [40] whereas particulates that are efficiently taken up by M-cells, result in immunization. Failure in presenting antigens in a particulate form may therefore induce tolerance [41]. This might also be the case, when soluble antigens are taken up by M-cells.

Taken together, AAL-microparticles bind to murine Peyer's patches and human M-like cells, are non-toxic and immunostimulatory. Due to the structural similarity of AAL and various neuraminidases of pathogens invading the body through M-cells, we suggest that AAL might facilitate the entrance of vaccines via this route and promote specific

immune responses. Thus, we propose that AAL-microparticles may be suitable vehicles for oral immunotherapy, e.g. in type I allergy.

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