

# Biodegradable PLGA Particles for Improved Systemic and Mucosal Treatment of Type I Allergy

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The goal of treatment for numerous diseases is the induction of an appropriate, or the modification of an ongoing, immune response. Therapy for type I allergy also has this goal, whereby the application of specific immunotherapy (SIT) (desensitization or hyposensitization therapy) has remained nearly unchanged since Noon [1] invented the procedure in 1911. In SIT, allergens are applied subcutaneously in increasing doses aiming to counterbalance the Th2-dominated response with IgE-mediated effects. The Th2-promoting adjuvant aluminium hydroxide (alum) is often used for allergen formulation, which may cause several well-known side effects during the treatment, such as the initial boost of IgE [2], sensitization against new allergens [3], and reactions at the injection site [4].

Immunotherapies in general have improved over the past decades, often focusing on producing active substances with a lower risk for side effects. However, some active substances per se are unable to evoke efficient immune responses because of their small molecular size [5,6]. In the allergy field, various techniques have been used to develop hypoallergens [7–11]. Another major concern is that novel sensitizations might be induced when hypoallergens are applied in the context of Th2-promoting adjuvants. Therefore, improved and optimized adjuvants, immune-stimulating substances or motifs, or delivery vehicles are needed.

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This work was supported by the Austrian Science Fund SFB F018 #8, #12 and #7, and a TransKoop grant of the Viennese Fund for the Promotion of Economy (WWFF).

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For the adjuvant effect, three principal mechanisms occur: (1) the antigen is deposited and delivered; (2) antigens are targeted through vehicle delivery systems to cells specialized for antigen uptake and presentation, including macrophages and dendritic cells; and (3) the adjuvant acts as an immunostimulant [5].

Many of the vaccine delivery systems have particulate characteristics. Examples include emulsions such as MF59, a squalene microemulsion [12]; microparticles made of different materials [13–16]; immune-stimulating complexes [17]; and liposomes [18]. These particulate features allow an enhanced uptake by antigen-presenting cells (APCs). Moreover, the transport to secondary lymph organs is facilitated and together these events support an immune response to simultaneously applied antigen [19]. In the past decades, an amazingly high number of novel drug and antigen delivery systems in the form of micro- or nanoparticles have been developed, many of them interesting for allergen immunotherapy [20], and various natural and synthetic polymers have been explored for their suitability for particle production. Among these numerous materials, the polymer of lactic acid and the copolymer of lactic and glycolic acid, poly(D,L-lactico-glycolic) acid (PLGA), have been studied extensively because of their biocompatibility and biodegradability [21], and the Food and Drug Administration has approved their use in humans.

## The features of PLGA particles

### *Biodegradability*

PLGA particles can be prepared using different well-established techniques, such as spray freeze-drying [22], coacervation, (double) emulsion–solvent evaporation technique [23], or salting-out [24]. Jain [25] reviewed various traditional and novel techniques, such as in situ microencapsulation, for preparing different drug-loaded PLGA devices. The advantage of PLGA is that it is completely biodegradable; it can be broken down through nonenzymatic hydrolytic cleavage when the PLGA device comes into contact with artificial or biologic fluids [26]. PLGA degrades in two stages [27]. During the first process the ester bonds are broken randomly through hydrolysis, and oligomers and monomers are generated. This breakdown leads to a polymer molecular weight decrease. Thereafter, during erosion, microspheres lose mass and the polymer breakdown may be increased through autocatalysis by acidic products [28].

### *Biocompatibility*

PLGA is not only biodegradable but also highly biocompatible because the resulting monomeric units, lactic and glycolic acid, are physiologically occurring substances in the human organism and can be easily metabolized through the Krebs cycle into carbon dioxide and water [29]. Therefore, this material has a long history of use in humans as resorbable surgery sutures, bone fixation mate-

rial, artificial skin, tracheal replacement, vascular grafts, dental materials, and implants [30]. Furthermore, PLGA polymers are commercially available as clinical products for delivering gonadotropin-releasing hormone analog for treating prostate cancer and endometriosis; human growth hormone for treating growth deficiencies; and doxycycline hyclate for treating chronic adult periodontitis [21]. Clinical trials are underway to test their application with paclitaxel in treating solid tumors, with vapreotide in treating esophageal bleeding varices, and with plasmid DNA encoding for human papillomavirus (HPV) epitopes to cure HPV-associated anal dysplasia and cervical intraepithelial neoplasia [31,32]. Thus, PLGA particles have been used as resorbable and inert carriers for small drug molecules of various classes, such as anticancer agents, antihypertensive agents, and immunomodulators [21]; for macromolecules like peptides, proteins, and hormones; and for protein subunits and DNA [33].

### *Antigen delivery*

PLGA polymers are broadly used because, in addition to their biocompatibility and biodegradability, their use as delivery devices has several advantages. First, the bioerosion rates and thereby the release kinetics can be tailored to the needs of the respective application [34]. This material is able to deposit the entrapped antigen and release it in a delayed continuous or pulsatile manner [35], depending on the particle size [5] and several surface characteristics, thereby mimicking booster applications [36,37] and allowing reduction of numerous injections. Second, the adjuvancy of PLGA is of major interest for vaccines in any field of medicine [38] because it allows reduction or even removal of further adjuvants. Third, the ability of PLGA particles to elicit cellular T-cell responses in addition to antibody responses [39–41] makes them an intriguing weapon also against intracellular infections (caused by bacteria, viruses, or parasites) or cancer. Fourth, they are able to protect the entrapped antigen from enzymatic breakdown and can therefore also be used for oral applications [42]. Along this line, studies have shown that PLGA can evoke systemic and mucosal immune responses [43,44]. Fifth, depending on their size, their surface charge, hydrophobicity, and administration routes, different cells of the immune system can be targeted [45]. For instance, after subcutaneous immunization, microspheres with a diameter of 1 to 10  $\mu\text{m}$  are phagocytosed by macrophages that are recruited to the injection site, and thereby intracellular antigen delivery and subsequent enhanced antibody response is guaranteed. In contrast, particles larger than 10  $\mu\text{m}$  remain as deposit on the injection site and release the antigen in a sustained manner. After intravenous application, particles approximately 200 nm in diameter are phagocytosed by Kupffer cells and end up in the lysosomal part of liver tissue [46]. Applied orally, microspheres smaller than 5  $\mu\text{m}$  are effectively taken up by Peyer's patches and are brought to mesenteric lymph nodes and spleen by macrophages, whereas particles from 5 to 10  $\mu\text{m}$  remain in Peyer's patches for up to 35 days, and microparticles larger than 10  $\mu\text{m}$  were not absorbed at any point in the gastrointestinal tract (GIT) [47].

On the cellular level, the particle size, surface properties, antigen type and loading, and release kinetics of PLGA particles may also influence the outcome of parenteral immunizations [48]. For example, particles smaller than 10  $\mu\text{m}$  can be directly taken up by APCs and result in major histocompatibility complex (MHC) II-restricted or cytotoxic T-lymphocyte (CTL) responses [49]. These small PLGA particles deliver the encapsulated antigen to APCs through uptake in either phagolysosomes or cytosol [33,50]. The first mechanism leads to presentation of antigen epitopes on MHC II. The CTL responses mirror the second mechanism, in which antigens can be processed and presented like endogenous antigens on MHC I. This feature is important because alum, for instance, is not capable of eliciting such  $\text{CD8}^+$  responses. PLGA particles larger than 10  $\mu\text{m}$  may also release the antigen extracellularly, thereby stimulating a secondary immune response through interaction with circulating or B-cell-bound immunoglobulins [48]. Whether B cells can also phagocytose microparticles and process and present the encapsulated antigen is not certain. Apart from the size of the particles, a high antigen load could be responsible for the induction of T helper 2 (Th2) responses with simultaneous poor affinity maturation during the secondary response [51]. Antigen release during an initial burst, and thereafter in pulses, can be advantageous when memory cells display longevity, whereas a continuous release may be necessary to restimulate memory cells [52].

The advantageous features and flexible preparation that allows PLGA particles to be tailored to respective needs led to their application as carriers for several protein antigens [43,53–63] and plasmid DNA [44]. They were capable of stimulating systemic and mucosal immunity [43] on not only the humoral but also the cellular level. They displayed their function through different application routes, such as peroral, intranasal, intramuscular, intratracheal, intradermal, intraduodenal, and intraperitoneal [64–70].

### **Use of PLGA in the treatment of allergy: first steps**

Apart from early published studies on the entrapment of ovalbumin (OVA) or bovine serum albumin as model antigens [37,43,71–78], Sharif and colleagues [79,80] in 1994 were the first to attempt to use PLGA particles as carriers for allergens and in 1995 to encapsulate mite allergen extract. This group prepared the microparticles by double emulsion technique, rendering a mean size of 1  $\mu\text{m}$ , and characterized the effect of the encapsulation process on the integrity and activity of the allergens. When the mite extract was released again, the proteins showed small alterations in the isoelectric focusing pattern. However, these changes only slightly affected the capability to inhibit IgE-binding to the extract or IgG-binding to mite allergen Der p 1. The authors therefore suggested that the encapsulation and release processes do not adversely affect the mite proteins, making them suitable for hyposensitization therapy.

Approximately 6 years later the idea of allergen in PLGA particles resurfaced. The food allergen  $\beta$ -lactoglobulin (BLG) from milk was entrapped in PLGA

[62,81] to induce tolerance in mice through a single feeding of encapsulated antigen. Five days after feedings, mice were immunized intraperitoneally with BLG and OVA adsorbed to alum. Evaluated on day 28, anti-BLG IgE was suppressed in a concentration-dependent manner in serum and intestinal contents of mice pretreated with different amounts of BLG encapsulated in PLGA microspheres (mean diameter of 7.8  $\mu\text{m}$ ). These feedings showed that 0.5  $\mu\text{g}$  of entrapped BLG was enough to reach the same suppression as 5 mg of soluble BLG. In these studies, 0.5  $\mu\text{g}$  of PLGA-BLG per gram of mouse reduced IgE in serum and intestinal secretions (2.39 and 1.48 titres, respectively) compared with gavage with unloaded particles or free BLG (approximately 3.6 and 2.2 titres, respectively). This suppression was specific for BLG because no changes were seen regarding OVA-specific IgE. The delayed-type hypersensitivity, evaluated as thickness measurements of the footpad after intradermal administration, was reduced in the BLG-particle group treated with 0.5  $\mu\text{g}$  per gram of mouse and in the group treated with free BLG (5 mg per gram of mouse). This high dose of soluble antigen was required because the dose was shown to be essential for tolerance induction [82]. In these studies, the greatest benefit of PLGA particles was the drastically reduced dose of allergen necessary to induce tolerance.

### **PLGA for parenteral or oral applications of allergens: immunogenicity**

Two working groups independently encapsulated *Olea europaea* pollen extract [83], the major olive pollen allergen Ole e 1 [84], through double-emulsion method. The main focus of these studies was the in vitro characterization of this delivery system with the prospect of using it for either parenteral [83] or oral immunization [84]. In the latter study, the size of the particles ranged from 0.5 to 2  $\mu\text{m}$ , thereby making them suitable for optimal uptake by Peyer's patches. A protein load of 6 to 7  $\mu\text{g}/\text{mg}$  polymer was reached. The release pattern of allergen from these particle preparations showed a biphasic shape with an initial burst (around 47% of entrapped allergens were released within the first hour) followed by a continuous slow release (74% of the encapsulated amount were released after 42 days). Entrapment of fluorescence-labeled allergen showed that Ole e 1 was distributed close to the surface of the microparticles, possibly causing the initial burst release. The authors discussed this scheme as optimal for mucosal application because of the limited exposure time of particles to the mucosal surface and the high dose required to prime an immune response. In addition to these in vitro experiments, intraperitoneal immunization was performed [85]. The main antibody type induced by PLGA-allergen was the Th1 isotype IgG2a, whereas the predominant antibodies were IgE and IgG1 in the group immunized with Ole e 1 adsorbed to alum. Accordingly, splenocytes from PLGA-immunized mice stimulated with Ole e 1 mainly produced interferon (INF)- $\gamma$ , in contrast to mice immunized with allergen plus alum, which synthesized high levels of interleukin (IL)-4.

## PLGA for parenteral and oral allergen applications: therapeutic potential

The true therapeutic potential of PLGA particles was shown for the first time in mice with the major birch pollen (BP) allergen Bet v 1 [86,87] and BP extract [88–91]. When Bet v 1 entrapped in PLGA particles (PLGA–Bet v 1) was compared with Bet v 1 adsorbed to alum, similar levels of allergen-specific IgG1 titres were achieved. The most important difference between the two protocols was the different sensitization potential: particle-treated mice did not develop type I skin sensitivity, whereas 100% of animals in the alum-treated group showed a positive result. In allergic mice, an ongoing Th2-dominated response could be redirected to a Th1-biased profile with a single subcutaneous shot of PLGA–Bet v 1. Accordingly, spleen cell cultures of the PLGA–Bet v 1 group produced high levels of IFN- $\gamma$  and some IL-10 on stimulation with Bet v 1, but only low levels of IL-4. The group treated with alum showed even higher amounts of these cytokines but, unfortunately, also an increase of IL-4.

To evaluate the safety of the PLGA polymer *in vivo*, we subcutaneously applied 100  $\mu$ L nanoparticle solution and 100  $\mu$ L of alum without any additional proteins to naïve mice. As can be seen in Fig. 1, no granuloma formation occurred at the site of the PLGA particle shot but the administration of alum induced a granuloma after 12 to 24 hours, which was clearly visible and could be confirmed in histologic examinations of the immunization sites. These two

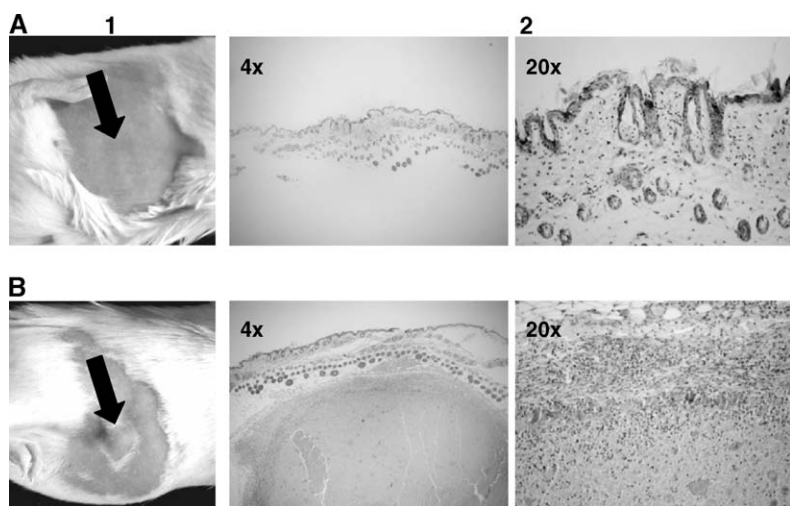


Fig. 1. PLGA nanoparticles are readily degraded *in vivo* and do not induce inflammation in mice. Empty PLGA particles (A, 100  $\mu$ L) were applied subcutaneously in untreated mice ( $n=6$ ). At the injection sites (arrow) no symptoms of inflammation were induced (1) and no granuloma formation or infiltration with inflammatory cells was seen in histology (2, magnification 4 $\times$  and 20 $\times$ ). In contrast, alum (B, 100  $\mu$ L) applied in the same mice induced granuloma formation after 12 to 24 hours, which remained two more months until biopsies were taken (1, photograph of shaved mouse skin; 2, histologic analysis 4 $\times$  and 20 $\times$ ). Representative tests are shown.

solutions were not only applied in mice but (with informed consent) also in healthy volunteers. PLGA nanoparticles were also clearly readily degradable and did not induce any inflammation, whereas alum led to classical inflammatory symptoms and to granulomas, which were visualized through ultrasonic skin measurement (Fig. 2).

To estimate the putative effects of PLGA in humans, we isolated human peripheral blood mononuclear cells in patients allergic to BP and stimulated them with PLGA nanoparticles with or without entrapped Bet v 1, with Bet v 1 alone or Bet v 1 in alum. The stimulation indices shown in Fig. 3 indicate that empty nanospheres were already able to induce cell proliferation, which was further underlined by the levels of IFN- $\gamma$  detected in the supernatants of these cultures (Fig. 4). In a prophylactic setting with empty PLGA microspheres, Jilek and colleagues [92] also saw an effect of plain particles. They treated mice with plain microspheres or microspheres filled with plasmid DNA encoding for the whole bee venom phospholipase A2. The particle preparations were able to induce a transient IgG2a-response during a subsequent sensitization and to evoke IL-10 production in stimulated splenocytes. Although in our in vivo mouse model the plain nanospheres had no effect on the allergen-specific immune response (in neither the prophylactic nor the therapeutic protocol) [86], our in

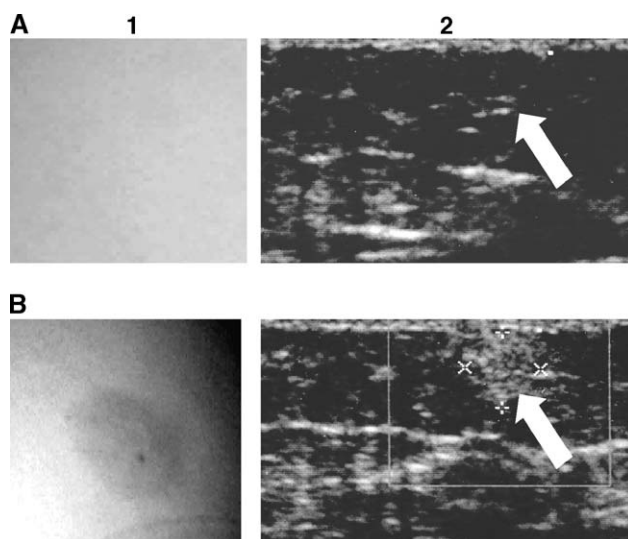


Fig. 2. PLGA nanoparticles are biocompatible after subcutaneous application in humans ( $n=2$ ). Empty PLGA nanoparticles (A, 100  $\mu$ L) were injected popliteal subcutaneously. Neither inflammatory symptoms at the skin surface (1) nor subcutaneous granuloma formation, evaluated through ultrasonic measurement (2), could be observed. However, the application of alum (B, 100  $\mu$ L) induced itching, swelling, redness, and heat at the application site after 12 to 24 hours (1). After 14 days, granulomas were apparent in ultrasonic scans (2) and were still present after several months. Arrows indicate site of injection.

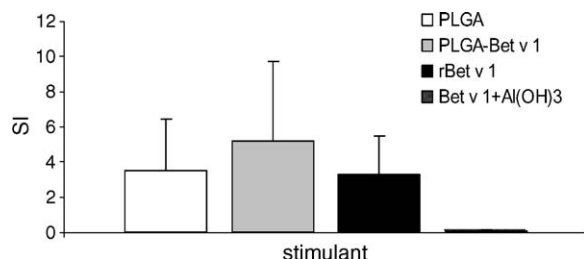


Fig. 3. PLGA nanoparticles induce T-cell proliferation in PBMC of patients allergic to BP ( $n=3$ ). Peripheral blood mononuclear cells from patients allergic to BP were isolated and  $2 \times 10^5$  cells per 200  $\mu$ L were stimulated with either plain PLGA nanoparticles (PLGA, 100  $\mu$ L), PLGA filled with rBet v 1 (PLGA-Bet v 1, 5.85  $\mu$ g Bet v 1 per 100  $\mu$ L suspension), recombinant Bet v 1 (rBet v 1, 5.85  $\mu$ g/100  $\mu$ L), or Bet v 1 adsorbed to alum (Bet v 1 + Al(OH)<sub>3</sub>, 5.85  $\mu$ g/100  $\mu$ L suspension). The empty and filled particles induced a proliferation of cells that was at least as high as with the recombinant allergen. In contrast, alum seemed to severely damage the cells because no proliferation could be induced at all. Proliferation was measured as <sup>3</sup>H-thymidine incorporation, and mean values + SD of stimulation indices (SI) are given. No statistical analysis was performed because of the small patient number. SI represents counts per minutes with stimulant divided by counts per minutes with medium.

vitro results with splenocytes indicate that they may additionally act through antigen-independent immune modulation.

Because of better patient compliance and convenience, multiple efforts have been made to construct oral vaccines for the treatment of type I allergy. PLGA microparticles are able to protect an encapsulated antigen from acidic and proteolytic degradation in the gut [42,87,91]. Therefore, they could also be used for oral administration of antigens [93]. Orally applied PLGA particles are readily absorbed by Peyer's patches, where they are taken up by so-called "membranous

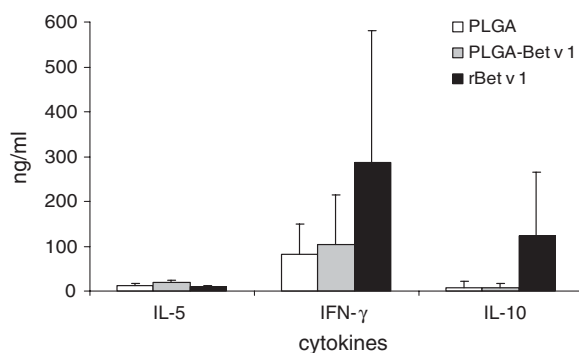


Fig. 4. In patients allergic to BP, the stimulation of peripheral blood mononuclear cells with PLGA particles mainly induces IFN- $\gamma$  production. The examination of the supernatants of the stimulated cells in enzyme-linked immunosorbent assay showed that PLGA particles, even when plain, were able to induce a Th1-biased cytokine pattern, dominated by high amounts of IFN- $\gamma$  and very low levels of IL-5 and IL-10 (stimulated as described in legend of Fig. 3). Mean values + SD are given.

cells,” transected, and transported to the lymphoid tissue where they encounter APCs and induce antibody responses [94].

To examine whether PLGA particles can also be used for oral application of allergens, we used BP extract for encapsulation in microspheres [88]. These microparticles had a mean diameter of 4.8  $\mu\text{m}$ , allowing uptake by cells of the GIT. An average load of 40  $\mu\text{g}$  BP protein per milligram of microparticles was achieved, with 60% of this protein actually still capable of binding a specific monoclonal antibody after 2 hours of simulated gastric digestion. In this study, we especially aimed at targeting the microparticles to specific sites of the intestine to prolong their persistence and thereby enhance antigen delivery. For targeting the sialic residues on enterocytes, wheat germ agglutinin (WGA) was coupled to microspheres. Feeding these BP allergen-loaded and WGA-functionalized PLGA to naïve mice induced an antigen-specific IgG response in serum. In a subsequent study, the targeting was directed toward the intestinal immune induction sites, the Peyer’s patches covered by membranous cells. These cells express  $\alpha$ -L-fucose moieties, which can be targeted by the lectin from *Aleuria aurantia* (AAL), the edible orange peel mushroom [91]. In an immunization protocol, feedings of naïve mice with AAL-coated PLGA induced mainly IgG2a

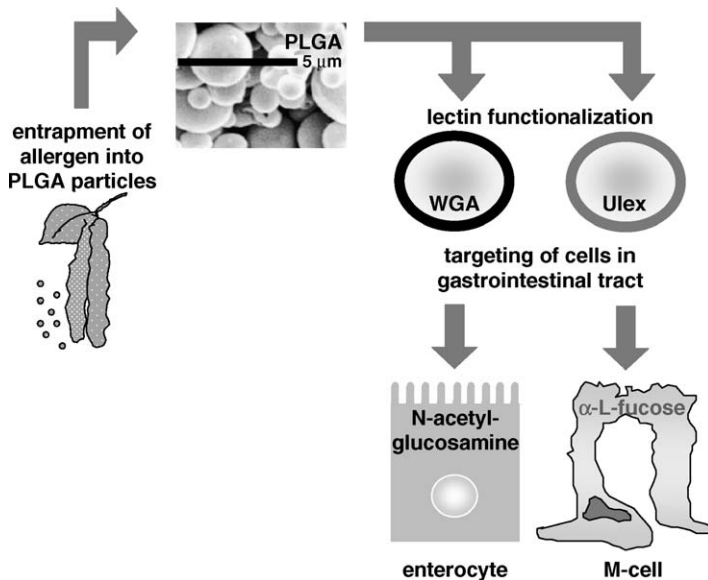


Fig. 5. For the oral application, PLGA particles are targeted through lectins to the sugar coats of the intestinal cells. Allergen can be entrapped in PLGA microparticles through the spray-drying technique [88]. A heterologous size distribution among the particles guarantees a release pattern, which mimics booster applications of the allergen. To target particles to the enterocytes, wheat germ agglutinin (WGA) can be attached through covalent binding. To target membranous cells (M-cells), particles can be directed to  $\alpha$ -L-fucose residues on their surface through lectins (eg, *Aleuria aurantia* lectin of the edible mushroom orange cup). The result is a Th1-biased immune response similar to that of bacterial and viral pathogens, which use M-cells for their invasion.

[90]. These preparations, compared with WGA-functionalized microspheres (Fig. 5), were further examined for their therapeutic capacity through oral treatments of BP-sensitized mice [91]. Here, the membranous cell-targeted PLGA induced higher IgG2a titers than WGA or noncoated particles. The efficacy of this targeting approach was also reflected in the higher proliferation capacity of BP-stimulated splenocytes of these mice and by the fact that IFN- $\gamma$  was only found in splenocyte supernatants from mice treated with AAL-functionalized PLGA targeted to membranous cells. This efficacy indicates that targeting of the intestinal immune induction sites is necessary to (1) avoid immune ignorance and (2) to modulate an ongoing Th2-type response toward Th1.

The differentiation of membranous cells in vitro is possible in a coculture model of CaCo-2 cells with Raji-lymphoma B-cells [95]. When the transport of the PLGA microspheres across this epithelium was studied, an increased cellular uptake and transcytosis was observed even against gravitation with AAL-grafted PLGA [89]. There is structural similarity of AAL to the neuraminidases of several pathogens such as *Salmonella typhimurium* [96], which invade humans through membranous cells.

### Ongoing studies and future prospective

Clinical studies with PLGA-entrapped allergens will prove whether these experimental concepts can be translated into practical application. Further approaches are presently designed to target APCs, such as dendritic cells of the immune system, with PLGA and encapsulated peptides [97–104]. Furthermore, several growth factors [57,105,106] and cytokines have been prepared in PLGA and additional hydrogels and scaffolds, such as transforming growth factor  $\beta$  [107], IL-2 [108], granulocyte-monocyte colony-stimulating factor [109], and IFN- $\alpha$  [110]. Although the initial ideas for these approaches were focused on treating other diseases, the preliminary results may also serve as prototypes for incorporation of other cytokines in treating allergic disorders.

### Summary

Numerous studies with different antigens and our experiments indicate that PLGA represents a controlled release system for allergens, which allows reduction of necessary booster shots in parenteral immunizations. In the treatment of type I allergy, ongoing Th2 responses can be modified by PLGA-entrapped allergens, at least in the murine model and probably also in humans, as we conclude from our in vitro studies. Furthermore, this material is highly biocompatible and biodegradable because it does not evoke granuloma formation or inflammatory symptoms in mice and humans after subcutaneous application. It protects the entrapped antigen from degradation at the mucosal sites. Therefore, nanospheres at the parenteral route and microspheres targeted to membranous

cells in oral applications can be realistic perspectives for counter-balancing an allergic immune response in the near future.

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