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Research paper

Protamine nanoparticles with CpG-oligodeoxynucleotide prevent an allergen-induced Th2-response in BALB/c mice

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ABSTRACT

The currently applied immunotherapy of type I allergy with aluminum hydroxide (alum) as adjuvant elicits – among other side effects – an initial IgE-boost. In contrast, CpG-oligodeoxynucleotides (ODNs) drive the immune response toward Th1. The biodegradable material protamine can spontaneously form nanoparticles together with such ODNs. Our aim was to investigate the immune response induced by protamine-based nanoparticles (proticles) with CpG-ODN as an allergen delivery system.

Proticles complexed with Ara h 2 extracted from raw peanuts as model allergen were injected subcutaneously into naïve BALB/c mice. Ara h 2-specific antibodies were analyzed by ELISA and rat basophilic leukemia (RBL) cell assay. Cytokine levels were investigated in supernatants of stimulated splenocytes. The *in vivo* distribution after subcutaneous injection was examined via fluorescence imaging. BMDCs were stimulated with proticles, and expression of stimulation and maturation markers as well as cytokines in supernatants was investigated.

A favorable increase in Ara h 2-specific IgG2a antibodies was found after immunization with proticles-Ara h 2, whereas Ara h 2-specific IgE was not detectable. Accordingly, the ratio of IL-5/IFN- γ was low in this group. Granuloma formation was completely absent at injection sites of proticles. The distribution of Ara h 2 after subcutaneous injection was markedly decelerated when complexed to proticles. Stimulation of BMDCs with proticles-Ara h 2 caused upregulation of CD11c and CD80 as well as an increased IL-6 production.

Our data suggest that biodegradable protamine-based nanoparticles with CpG-ODN counteract the Th2-dominated immune response induced by an allergen and therefore are suitable as novel carrier system for immunotherapy of allergy.

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Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); Alum, aluminum hydroxide; BMDCs, bone marrow derived dendritic cells; BSA, bovine serum albumin; CpG, cytosine phosphate guanine; DCs, dendritic cells; DMSO, dimethyl sulfoxide; EDTA, ethylene-diamine tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; FDA, food and drug administration; IFN, interferon; IL, interleukin; ODNs, oligodeoxynucleotides; o.n., overnight; PBS, phosphate-buffered saline; RBL, rat basophilic leukemia; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; STDV, standard deviation; TBST, tris-buffered saline with 0.05% Tween®; Th, T helper; TLR, toll-like receptor; TNF, tumor necrosis factor.

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1. Introduction

Immunotherapy of allergy is routinely performed subcutaneously with aluminum hydroxide (alum) as adjuvant. However, alum triggers various side effects like swelling, indurations, erythemas, cutaneous nodules, and granulomas at the injection site [1]. It also favors a Th2-response by inducing undesired production of IgE [2,3]. Therefore, other adjuvants or delivery systems need to be established [4–6]. Currently, several novel carrier systems, like polymeric nanoparticles (reviewed in [7,8]), or alternative adjuvants, like Toll-like receptor targeting substances (reviewed in [9]), and therapeutic strategies (reviewed in [10]) are under investigation. Our group could already show the efficacy and safety

of PLGA-particles for treatment of allergy in several mouse models [6,11,12].

Another alternative adjuvant system could be protamine-based nanoparticles (proticles) with the TLR-9 ligand CpG-oligodeoxynucleotides (ODNs). Protamines are arginine-rich peptides of approximately 4 kDa, which occur naturally in sperm, and can be isolated from mature fish testicles or milt (usually salmon) or produced synthetically. Protamine is biodegradable and has been used for decades in human medication, e.g. for reversal of heparin activity during surgery [13] or as insulin-additive [14].

Protamine can spontaneously assemble into nanoparticles with DNA or RNA via electrostatic interaction. CpG-ODNs are DNA sequences containing CpG motives, which consist of repetitive sequences of cytosines and guanines phosphorylated at the phosphodiester backbone. Such CpG motives are characteristic for microbial DNA and are recognized by Toll-like receptor-9 (TLR-9), a receptor expressed on plasmacytoid dendritic cells (pDCs) and B-cells in humans. CpG-ODNs induce a Th1-biased immune response. Therefore, they were already used in experimental studies [15] and more recently in clinical trials for treatment of allergy and asthma [16,17]. CpG-ODNs were already successfully combined with protamine before [18–20]. Protamine has already been assessed for vaccination in a BALB/c mouse model of bee venom allergy [21]. However, this working group used protamine as an additional substance for their PLGA-based microparticles together with CpG and bee venom allergen phospholipase A2.

The objective of the present work was to test for the first time the immunological properties of protamine-based nanoparticles in complex with CpG-ODN and the model allergen Ara h 2 from peanut in an *in vivo* model.

2. Materials and methods

2.1. Allergens Ara h 2 and Bet v 1

Natural Ara h 2 was purified from 20 g raw, unshelled peanuts as previously described [22]. Briefly, peanuts were extracted for 2 h in 100 ml buffer (20 mM Tris, pH 8.2) under constant agitation at room temperature (RT). Crude peanut extract (PE) was pre-cleared by centrifugation at 3000g for 15 min at RT, and the supernatant centrifuged at 10,000g for 30 min at RT and filtered using a paper filter (Whatman, Maidstone, United Kingdom). PE was loaded onto a 5 ml anion exchange column (Q Sepharose Fast Flow, GE Healthcare, Buckinghamshire, UK) using the ÄKTA FPLC system. Bound protein was eluted by a three-step gradient of elution buffer (20 mM Tris, 1 M NaCl, pH 8.2): 1–20% over 20 column volumes (CV); 21–50% over 15 CV; 51–100% over 5 CV. After SDS-PAGE analysis, Ara h 2 containing fractions were pooled, dialyzed against distilled water overnight (o.n.), and the sample snap frozen and lyophilized. Recombinant birch pollen allergen Bet v 1a (Biomay, Vienna, Austria) was used as irrelevant control allergen.

2.2. Nanoparticle preparation and characterization

Protamine-based nanoparticles were prepared in aqueous solution by merging equal volumes of the components to induce self-assembly, as previously described by Lochman et al. [23]. Stock solutions of CpG-ODN (Invivogen ODN 1826, eubio, Vienna, Austria), protamine (Grade IV; Sigma Aldrich, Steinheim, Germany), and Ara h 2 or Bet v 1 were diluted in MilliQ water to final concentration in dispersion of 100 µg/ml CpG-ODN, 300 µg/ml protamine, and 100 µg/ml allergen.

The hydrodynamic diameter and zeta potential of the particles were determined by photon correlation spectroscopy (PCS) using a zetasizer Nano series ZS (Malvern Instruments, Herrenberg,

Germany). Scattered light was detected at an angle of 173° for size measurements and 12.8° for zeta measurements, respectively. Proticles consisting of protamine and CpG-ODN were 215 nm in diameter (monomodal size distribution: polydispersity 0.07; $n = 3$) with zeta potential of $+23.9 \text{ mV} \pm 2.5 \text{ mV}$ ($n = 3$). Proticles complexed with Ara h 2 were 600 nm to 1300 nm. Particles complexed with Alexa-labeled-Ara h 2 exhibited a larger diameter than with unlabeled Ara h 2. Zeta potential was approximately -7 mV for proticles containing unlabeled Ara h 2 and $+18 \text{ mV}$ with Alexa-labeled Ara h 2.

2.3. Immunization of mice

Female BALB/c mice (Charles River Laboratories, Sulzfeld, Germany), aged 8 weeks, were treated according to European Community rules of animal care (permission of the Austrian Ministry of Science, BMWF-66.009/0172-II/3b/2011). Mice were immunized subcutaneously ($n = 8/\text{group}$) with 100 µl of either PBS (PBS), dispersion of protamine-based nanoparticles with 10 µg CpG (proticles), dispersion of nanoparticles with 10 µg CpG and 10 µg Ara h 2 (proticles-Ara h 2), 10 µg Ara h 2 in PBS (Ara h 2), or 10 µg Ara h 2 adsorbed to 90 µl alum (Serva, Heidelberg, Germany; alum-Ara h 2). Immunizations were performed on days 7, 21, 35, and 49, and blood was drawn on days 0, 14, 28, 42, 56, and 70.

2.4. Antibody detection in sera by ELISA

Detection of Ara h 2-specific antibodies was performed as described previously [24]. Briefly, microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with Ara h 2 (0.5 µg per well in 100 µl 50 mM NaHCO₃, pH 9.6) o.n., 4 °C, and blocked with TBST/1% BSA (RT, 2 h). Serum samples were diluted 1:10 for IgE and 1:100 for IgG1 and IgG2a detection in TBST/0.1% BSA and incubated o.n. at 4 °C. Isotype standard antibodies (BD Pharmingen, Schwenchat, Austria) were used for dilution series (starting concentration 500 ng/ml, dilution steps 1:2). Isotype-specific rat-anti-mouse antibodies (BD) were used 1:700 in TBST/0.1% BSA (RT, 2 h) and peroxidase-labeled anti-rat antibody (GE Healthcare, Buckinghamshire, UK) 1:2000 (RT, 2 h). Detection was done with ABTS (Sigma, Vienna, Austria), and optical density was determined in a microplate reader (SpectraMax Plus, CA, USA) at 405–490 nm.

2.5. Rat basophilic leukemia assay

RBL assay was performed as described previously [25]. Briefly, RBL-2H3 cells (generously provided by Dr. Arnulf Hartl, Institute of Biochemistry, University of Salzburg, Austria) were cultured in RPMI medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, 1% penicillin/streptomycin, and 100 µM 2-mercaptoethanol at 37 °C and 5% CO₂. RBL cells (4×10^4 cells/well in 100 µl medium) were seeded in a 96-well round-bottom plate (96 MicroWell, Nunclon, Nunc, Roskilde, Denmark) and incubated overnight at 37 °C and 5% CO₂. Cells were passively sensitized with pooled sera from mice (diluted 1:20) for 2 h at 37 °C and 5% CO₂. Afterward, cells were washed twice with Tyrode's buffer (137 mM NaCl, 2.68 mM KCl, 1.4 mM CaCl₂, 1.7 mM MgCl₂, 5 mM D-glucose, 10 mM HEPES, 0.42 mM NaH₂PO₄-H₂O, 12 mM NaHCO₃) at pH 7.2, supplemented with 0.1% BSA (Sigma). For stimulation, Ara h 2 (0.3 µg in 100 µl/well), codfish extract as irrelevant control allergen (0.3 µg in 100 µl/well), phorbol-myristate-acetate as positive control (PMA, 0.1 µg in 100 µl/well; Sigma), or Tyrode's buffer as negative control were added for 30 min at 37 °C and 5% CO₂.

2.6. Measurement of body temperature

On day 70 (sacrifice), mice were challenged intravenously with Ara h 2 (50 µl of 1 mg/ml in 0.9% NaCl) and immediately examined for body temperature changes by a hand-held thermometer over a period of 20 min.

2.7. Intradermal skin tests

Evans blue (100 µl of 5 mg/ml NaCl 0.9%; Merck, Darmstadt, Germany) was injected into the tail vein of mice. 30 µl of Ara h 2 (5 µg/ml PBS), 30 µl of codfish extract (50 µg/ml PBS) as control allergen, mast cell degranulation compound 48/80 (20 µg/ml PBS; Sigma) as positive control, and PBS as negative control were administered intradermally into the shaved abdominal skin. After 20 min, mice were sacrificed and the diameter of the color reaction measured inside the abdominal skin. The color intensity was determined using a hand-held reflection densitometer (Vipdens, Brixen, Italy). Skin reactivity indices per group were calculated using the following formula: mean of diameter of skin reactivity X mean of densitometrical signal intensity.

Skin of injection sites was checked macroscopically for granulomas.

2.8. Stimulation of splenocytes

Spleen cell preparation and stimulation were performed as described previously [24]. For stimulation, 100 µl of Ara h 2 (40 µg/ml), codfish extract (400 µg/ml), Con A (Sigma; 5 µg/ml) as positive control, or medium were added. Measurement of IL-4, IL-5, IL-10, IL-13, and IFN- γ was performed by ELISA (BenderMed Systems, Vienna, Austria) in supernatants of stimulated splenocytes (diluted 1:2), according to manufacturer's instructions.

2.9. Stimulation of BMDCs

BMDCs were generated from femoral and tibial bone marrow cells of naïve BALB/c mice. Briefly, bone marrow cells (2.5×10^6 cells/ml) were cultured in RPMI-1640 medium containing 10% fetal calf serum, glutamine, antibiotics (all from Life Technologies, Vienna, Austria) and 20 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (Peprotech, Vienna, Austria). Fresh culture medium was added after 3 and 6 days of culture. After 9 days of culture, suspension and adherent cells (detached by treatment with 2 mM EDTA in PBS) were pooled and resuspended.

Binding/uptake of particles: BMDCs were seeded at 5×10^5 cells/ml. Proticles with unlabeled or labeled Ara h 2 (DyeLight 488, Pierce, Rockford, USA) were added (30 µg Ara h 2/500 µl proticle solution), and fluorescence examined after 2, 5, and 22 h after washing (Axio Observer.Z1 inverted microscope/AxioVision software, Zeiss, Jena, Germany).

Stimulation markers and cytokines: BMDCs (1×10^6 cells/500 µl/well) were cultured in the presence or absence of stimulants for 24 h, 48 h, or 72 h. Stimulants/well: LPS (0.5 µg), proticles-Ara h 2 (5 µg Ara h 2/50 µl proticles), proticles (50 µl), Ara h 2 (5 µg), CpG (5 µg), protamine (15 µg), Bet v 1 (5 µg) as irrelevant control allergen, or proticles-Bet v 1 (5 µg Bet v 1/50 µl proticles). Cell supernatants were stored at -20°C until cytokine analysis (Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix, eBioscience, Vienna, Austria) by flow cytometry (FACS Calibur, BD Biosciences, Vienna). Cells were triple-stained with anti-mouse CD11c-Alexa Fluor[®] 647, anti-mouse CD80-PE, anti-mouse CD86-FITC (eBioscience, Vienna, Austria), or isotype controls (according to manufacturer's instructions) in combination with 0.125 µg/sample anti-mouse

CD16/32 (Fc-Block, eBioscience). Flow cytometry data were analyzed using the Cyflog software (CyFlo Ltd, Turku, Finland).

2.10. Biodistribution

Ara h 2 was labeled with Alexa Fluor[®] 647 (Invitrogen, Lofer, Austria) according to manufacturer's instructions. Briefly, Alexa Fluor[®] 647 (1 mg/100 µl DMSO) was added drop-wise to Ara h 2 (2 mg/200 µl 0.1 M sodium bicarbonate puffer, pH 8.3). After desalting (illustra MicroSpin G-25 Columns; GE Healthcare, Buckinghamshire, UK), eluates were dialyzed against distilled water.

For biodistribution, 10 µg labeled Ara h 2 in 100 µl PBS (soluble: Ara h 2*, or complexed to proticles: proticles-Ara h 2*) were injected subcutaneously into flanks of naïve BALB/c mice. Animals under isoflurane-anesthesia were scanned in real-time by optical fluorescence imaging (MaestroTM2.2, In vivo Fluorescence Imaging System, CRI, Inc., Woburn, MA; deep red filter from 730–950 nm, 10 nm steps) at 1, 15, 40, 60, 180, 240, 300, 360 min, 20 h, and 24 h after application.

2.11. Statistical analysis

Antibody and cytokine levels were analyzed by One-way ANOVA with Newman-Keuls as post hoc test, and body temperature changes with Wilcoxon signed rank test (values before vs. after i.v. challenge). Geometric mean values of fluorescence histograms were compared by Student's *t*-test (two tailed). Data analysis was done by GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). *p* < 0.05 was considered statistically significant.

3. Results

3.1. Specific antibody levels in sera

Allergen-specific IgG2a antibodies were highest in the group treated with proticles-Ara h 2 (Fig. 1A). IgG1 also showed a significant increase in the proticles-Ara h 2 group; however, levels were significantly lower than in groups immunized with Ara h 2 or alum-Ara h 2 (Fig. 1B). In comparison with Ara h 2 and alum-Ara h 2-treatment, proticles-Ara h 2 induced significantly less allergen-specific IgE (Fig. 1C). This was confirmed using RBL cells, which can bind serum IgE-antibodies via FcεRI. Crosslinking by a specific allergen then leads to a release of beta-hexosaminidase from RBL cells. In our experiments, Ara h 2 incubation of RBL cells sensitized with sera containing high levels of specific IgE indeed rendered high beta-hexosaminidase release, whereas no release was seen using sera from proticle-Ara h 2 group harboring low or no specific IgE (Fig. 1D). There was no release when irrelevant allergen codfish was used for stimulation.

3.2. Cytokine levels in stimulated splenocytes

Levels of IFN- γ secreted by Ara h 2-stimulated splenocytes were comparable in all groups (Fig. 2A). However, IL-5 levels were highest in splenocytes of animals immunized with Ara h 2 or alum-Ara h 2 (Fig. 2B). This resulted in a favorable low IL-5/IFN- γ ratio in the group treated with proticles-Ara h 2 (0.4), in contrast to treatment with Ara h 2 (7.5) and alum-Ara h 2 (1.6).

3.3. Body temperature measurement

Anaphylaxis (statistically significant drop of body temperature within 20 min after i.v. challenge with Ara h 2) was found in animals immunized with Ara h 2 or alum-Ara h 2, but not in groups

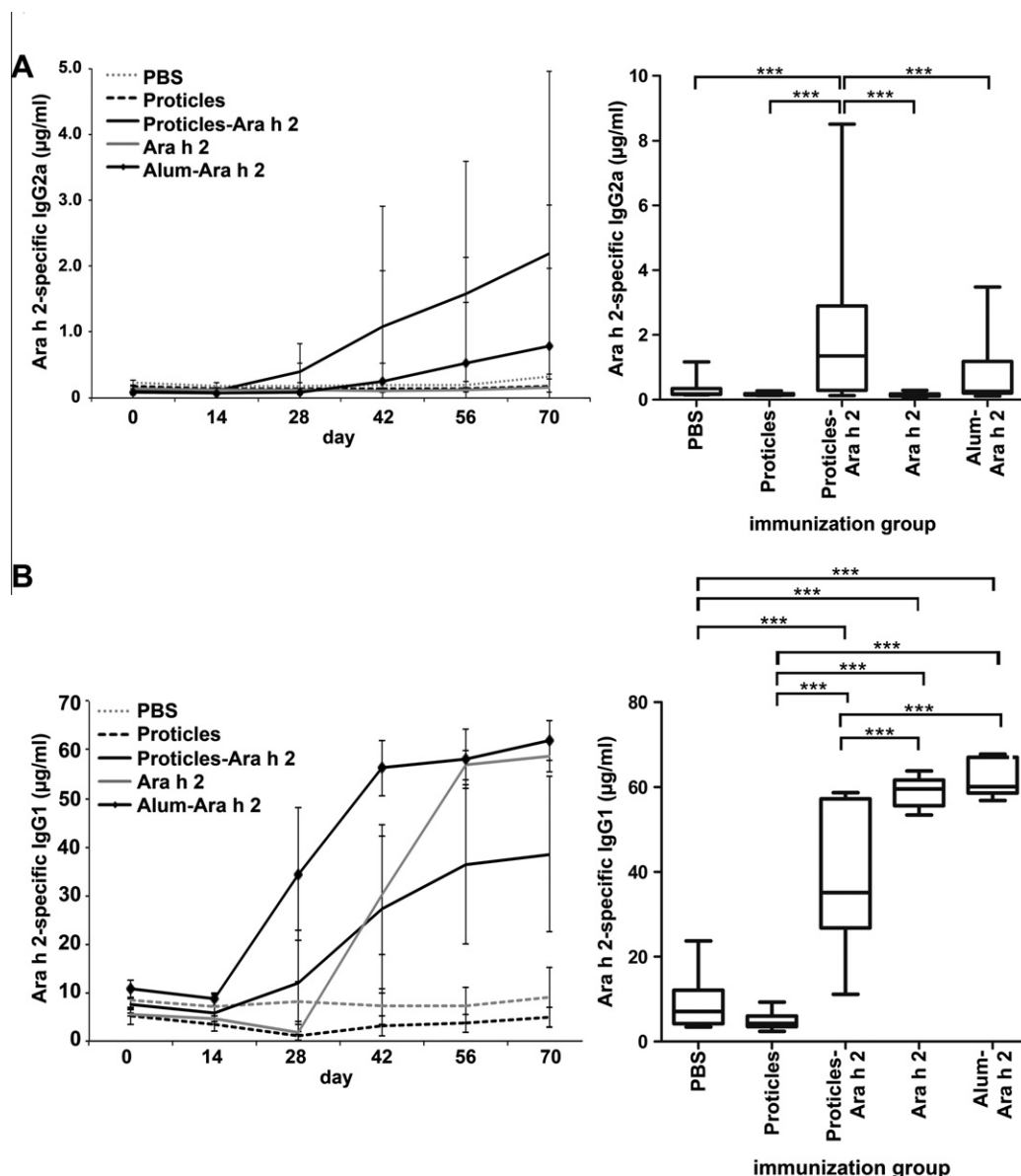


Fig. 1. Allergen-specific antibody levels in immunized mice. The highest Ara h 2-specific IgG2a antibody levels (A) were observed after treatment with proticle-Ara h 2. Of allergen-specific IgG1, only moderate levels were induced by proticles-Ara h 2 (B). In contrast to immunization with Ara h 2 or alum-Ara h 2, proticles-Ara h 2 did not induce significant levels of Ara h 2-specific IgE (C). Left panels: means of antibody levels during immunization, right panels: antibody levels at 70 day-time point. Means of triplicate determinations $\pm$ standard deviation from 2 independent experiments for individual mouse sera are shown (** $p < 0.001$). In RBL assay, high beta-hexosaminidase release was seen after stimulation with Ara h 2 in groups immunized with alum-Ara h 2 or Ara h 2, but not with proticles-Ara h 2 (D). Due to usage of pooled sera, no statistical comparison was performed for RBL-results.

treated with proticles-Ara h 2, PBS or empty proticles (PBS: 0.35 ± 0.38 , $p = 0.84$; proticles: 0.29 ± 0.65 , $p = 0.69$; proticles-Ara h 2: 1.83 ± 0.69 , $p = 0.16$; Ara h 2: 2.98 ± 0.33 , $p = 0.0078$; alum-Ara h 2: 2.31 ± 0.61 , $p = 0.0156$). Data are given in $^{\circ}\text{C}$ (mean $\pm$ STDEV before minus after i.v. challenge).

3.4. Reactivity to Ara h 2 in intradermal skin tests, and granuloma formation

Type I skin test reactivity to Ara h 2 was found to a lower extent in the proticles-Ara h 2 group, as compared to Ara h 2 or alum-Ara h 2 treatments (Fig. 3). No reaction to Ara h 2 was seen in control groups (PBS or empty proticles). Granulomas were absent in animals treated with proticles or proticles-Ara h 2, whereas 100% of

animals injected alum-Ara h 2 showed granulomas at injection sites.

3.5. Proticle uptake, stimulation markers and cytokines in BMDCs

BMDCs were incubated with proticles complexed with either labeled or unlabeled Ara h 2. We observed an efficient uptake of particles within 2 h of incubation and a decline of the fluorescence signal within 5 h, which was nearly gone after 22 h (Fig. 4).

For assessment of maturation markers and cytokines, BMDCs were stimulated with medium, proticles-Ara h 2, Ara h 2, CpG (Fig. 5), proticles, LPS, protamine, proticles-Bet v 1 or Bet v 1 (data not shown). Proticles-Ara h 2-stimulation caused maturation of BMDCs by significantly increasing CD11c after 48 and 72 h, and CD80 after 24 and 48 h (Fig. 5).

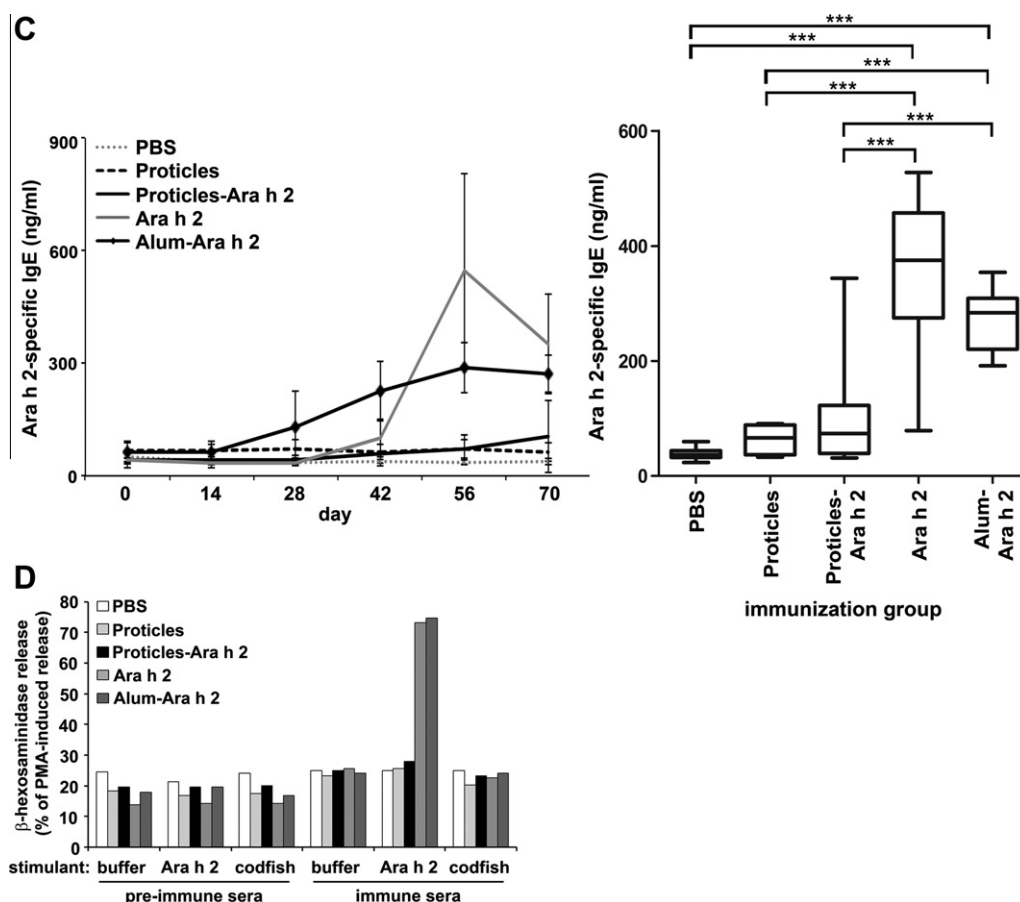


Fig. 1. (continued)

Positive controls LPS and/or CpG induced significant secretion of IL-13, IL-1 α , IL-22, IL-5, IL-6, IL-10, IL-27, IFN- γ , and TNF- α compared to medium (data not shown), whereas with other stimulants, relevant levels were only detected for TNF- α , IL-6 and IL-10 (Fig. 6). Proticle-Ara h 2-stimulation triggered significant secretion of IL-6 in BMDCs over medium.

3.6. Biodistribution of Ara h 2 complexed to proticles

The subcutaneous distribution of proticles-Ara h 2 was followed *in vivo* in real-time imaging using Alexa Fluor® 647-labeled Ara h 2 either applied in solution or complexed to proticles. A decreased intensity of fluorescence signal was already observed 1–6 h after application of Ara h 2, whereas a sustained intense signal could be found after 24 h with Ara h 2-complexed proticles (Fig. 7).

4. Discussion

Protamine is used since decades in medical formulations and holds the “generally recognized as safe” status of the FDA [19]. Protamine nanoparticles (proticles) represent a new drug delivery system for oligonucleotides [23,26]. This biodegradable material was used here in complex with CpG-ODNs, which induce a Th1-biased immune response [20]. The major allergen Ara h 2 from peanut served as model allergen and was non-covalently attached by ionic interactions to preformed proticles. The allergen Ara h 2 was selected as it is among the most potent food allergens, and a safe specific immunotherapy is urgently needed. Furthermore, this protein was methodologically a good candidate as it efficiently

attached to proticles in our hands (data not shown). As peanut proteins increase their allergenic potential during food processing, specifically by heat treatment, we used Ara h 2 derived from raw peanut in the present work.

We injected proticles with or without allergen subcutaneously into female BALB/c mice and compared this treatment to PBS, Ara h 2 alone, or Ara h 2 in suspension with alum. Immunization with proticles-Ara h 2 led to a significant increase in allergen-specific IgG2a already after the second shot, whereas IgG1 levels were only moderate, and allergen-specific IgE was not induced in significant amounts by proticle-Ara h 2-treatment. Accordingly, the IL-5/IFN- γ ratio was very low in this group. These humoral and cellular results collectively suggest that immunization with Ara h 2 complexed to proticles with CpG-ODN can effectively prevent a Th2-dominated response induced by an allergen. Moreover, anaphylactic symptoms (body temperature drop) by Ara h 2-challenge could be avoided in the animals treated with proticle-Ara h 2. Nevertheless, positive intradermal skin test reactions were observed in this group, possibly due to Ara h 2-specific IgG1-antibodies, which are anaphylactogenic in mice [27,28], but not in humans.

Importantly, and in contrast to alum, protamine-based particles proved their biodegradability, as they caused no granuloma formation upon injection.

To investigate whether dendritic cells participate in the uptake of proticles – and therefore in the observed immune response – we incubated BMDCs of naïve BALB/c mice with proticles complexed with DyeLight-labeled Ara h 2. Particle-uptake occurred within 2 h, and the fluorescence signal was hardly detectable after 22 h, indicating an efficient uptake and degradation of proticles-Ara h 2 by BMDCs. Incubation of BMDCs with CpG as well as proticles

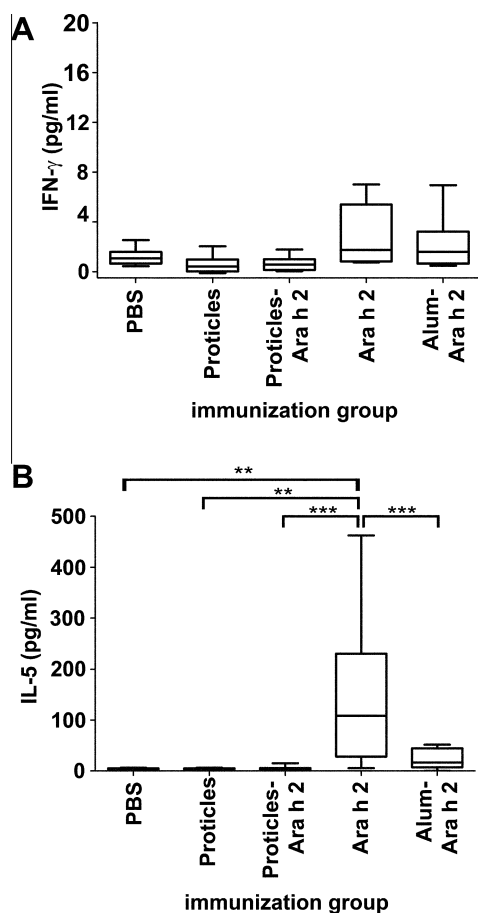


Fig. 2. Cytokine levels in stimulated splenocytes. The splenocytes of differently immunized mice were stimulated with Ara h 2 for 72 h and cytokine release measured in supernatants by ELISA. IFN- γ levels were not significantly different between groups (A), whereas IL-5 was significantly elevated in splenocytes of mice immunized with Ara h 2 only (B) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

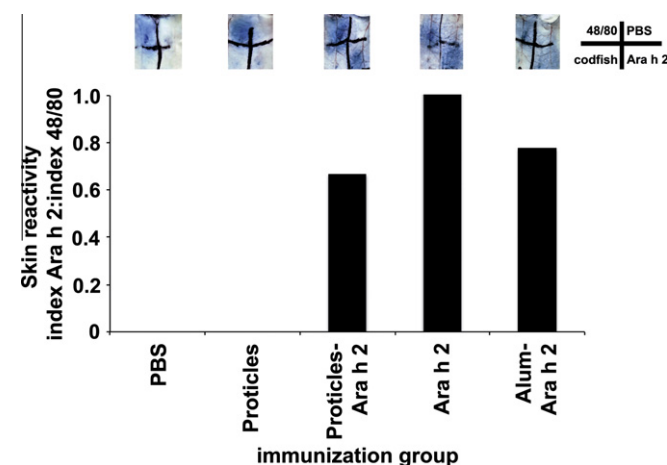


Fig. 3. Reactivity in intradermal skin testing. Immunized mice were tested intradermally with either PBS (negative control), 48/80 (positive control), Ara h 2 (relevant allergen) or codfish (irrelevant allergen). Representative skin tests of every group are shown on top. Skin test reactivity indices per group were calculated as described in the methods section. Indices for Ara h 2 reactivity in relation to indices for 48/80 reactivity are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

complexed with CpG resulted in the upregulation of surface molecules CD11c, CD80, and CD86, as previously shown for human

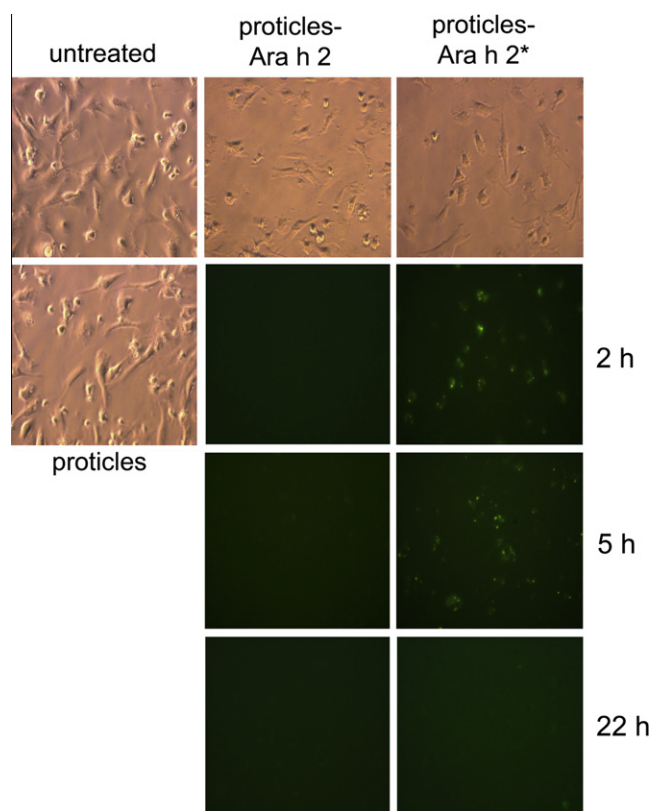


Fig. 4. Binding/uptake of proticles by BMDCs. Differentiated BMDCs were incubated with proticles-Ara h 2 without labeling (proticles-Ara h 2) or with fluorescence-labeled Ara h 2 (proticles-Ara h 2*). Binding was evaluated by fluorescence microscopy after incubation for 2 h, 5 h, and 22 h after extensive washing. Representative results of two independent experiments are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

plasmacytoid dendritic cells [18]. Importantly, also proticles-Ara h 2 induced CD80-upregulation, indicating the potential of these particles to stimulate DCs. However, proticles without Ara h 2 induced higher CD80 upregulation and in addition also CD86. This higher expression rate of co-stimulatory molecules by empty proticles might be due to the fact that CpG-ODN molecules could be more accessible as compared to when Ara h 2 is complexed in addition or that empty particles are smaller in size. Accordingly, also cytokine release was higher after stimulation with empty proticles than with proticles-Ara h 2. Nevertheless, BMDCs showed a significant release of IL-6 also after stimulation with proticles-Ara h 2. IL-6 is known to be responsible for B-cell differentiation and induction of final maturation of B-cells into antibody-producing cells [29,30]. It was shown that the expression of DCs surface markers does not necessarily correlate with release of cytokines like IL-6, IL-10, or IL-12p70, or with the impact of DCs on Ig-production by B-cells [31]. Accordingly, although expression of surface markers and release of cytokine IL-6 did not correlate, immunization with proticles-Ara h 2 induced a strong immune response in our mouse model. Our BMDC experiments indicate that the observed immune response induced by proticles-Ara h 2 may involve DCs.

Addressing the distribution characteristics of proticles-Ara h 2, we measured the biodistribution over 24 h. The addition of Alexa-labeling to Ara h 2 changed the zeta potential of the proticles; however, in our study, the zeta potential is measured in MQ-water, and the modest difference between -7 mV and $+18$ mV will presumably be further minimized after application

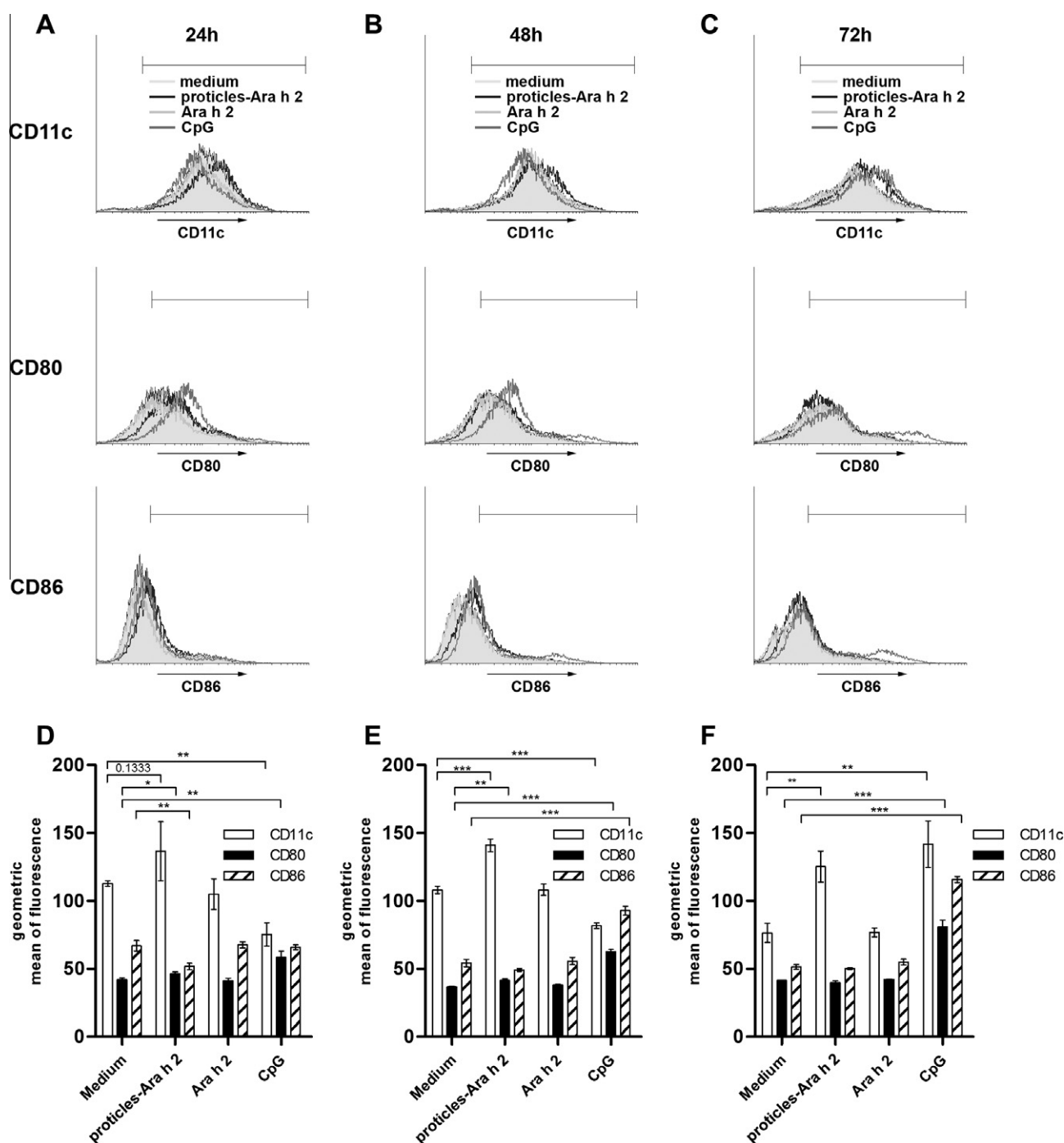


Fig. 5. Regulation of surface markers in stimulated BMDCs. Expression of CD11c, CD80, and CD86 was analyzed by flow cytometry after stimulation for 24 h (A and D), 48 h (B and E), and 72 h (C and F) in differentiated BMDCs. Histogram plots for stimulation with medium, proticle-Ara h 2, Ara h 2, or CpG are shown (A–C). Means of geometric means of biological triplicates $\pm$ STDV are given in D–F. Asterisks mark statistically significant different regulation of surface markers compared to medium (* p < 0.05, ** p < 0.01, *** p < 0.001).

in vivo due to adsorption of proteins and ions; therefore, a more or less neutral zeta potential for both unlabeled and labeled particles can be expected under physiological conditions. When Ara h 2 was complexed within proticles, the fluorescence intensity at the injection site remained nearly unchanged until the end of the observation period. In contrast, soluble Ara h 2 showed diffusion already between 1 and 6 h. These results suggest that antigen release from proticles occurs much slower as compared to soluble antigen, indicating the need of fewer injections for successful immunotherapy. However, it has to be considered that labeling of the allergen might

influence interaction with cells and the behavior at the injection site. Therefore, depot formation of the allergen needs to be reaffirmed in future biodistribution experiments comparing head-to-head the soluble and the proticle-complexed allergen.

Currently, there exist several approaches for improving the efficiency and safety of allergen-specific immunotherapy (SIT) (reviewed in [10]), like the targeting of T-cells for induction of tolerance, alternative routes for application, using recombinant allergens and applying immune modifier substances for fusion with allergen or combined treatment with SIT. Our study made

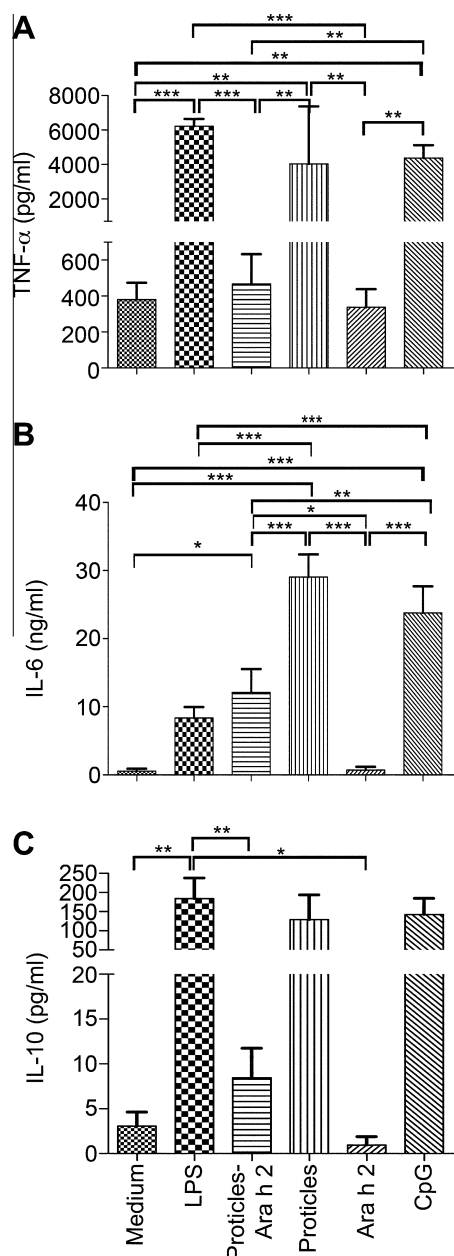


Fig. 6. Cytokine release from stimulated BMDCs. Supernatants were harvested from the cells analyzed by FACS (Fig. 5). Elevated levels of TNF- α (A), IL-6 (B), and IL-10 (C) were found with the different stimuli compared to medium after 72 h stimulation. Mean values of biological triplicates + STDV are shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

use of coupling an adjuvant (in our case CpG-ODN) to an allergen. CpG was already used as coupling partner for ragweed allergen Amb a 1 and was successful in a human clinical study, as the treatment substantially reduced symptoms of allergic rhinitis during the ragweed season [17]. However, a previous study suggested that the immunostimulatory effects of CpG-ODN could be markedly increased by complexing to protamine nanoparticles. Following this concept, the present study provides the proof of concept for favorable delivery, and immune modulation by these biodegradable allergen carriers being co-complexed with CpG-ODN as adjuvant. The protamine nanoparticle-complexed allergy vaccine fulfills several demands of SIT: (1) individual allergens can be complexed to proticles for component-resolved immunotherapy; (2) the pathological Th2-response to allergen is suppressed; (3) due to the depot effect, the number of injections might be reduced, lowering

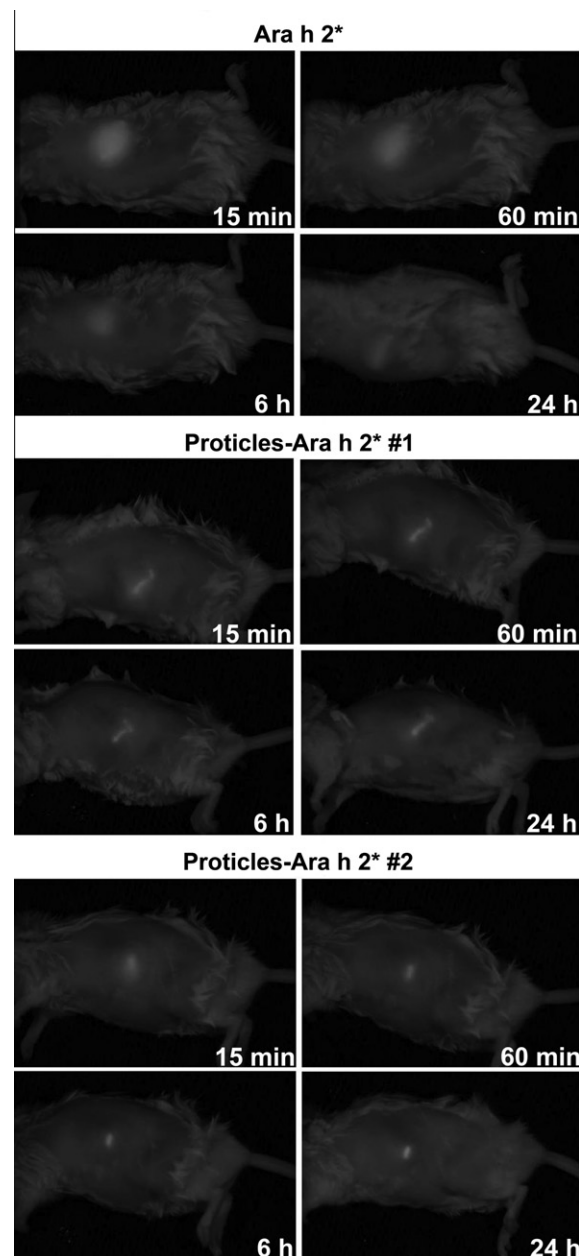


Fig. 7. Biodistribution of Ara h 2 complexed to proticles. Ara h 2 was labeled with Alexa Fluor® 647 and applied subcutaneously into the flank of BALB/c mice, either in solution (Ara h 2*, $n = 1$) or complexed to proticles (proticles-Ara h 2*, $n = 2$). A fast *in vivo* distribution (decreasing fluorescence signal) was observed for soluble Ara h 2* already at 1–6 h after application. Only a minimal loss of fluorescence signal was found up to 24 h for proticles-Ara h 2*, indicating a favorable longer retention time at the injection site of proticles-Ara h 2. Representative results of two independent experiments are shown.

treatment costs and enhancing patients' compliance; (4) simultaneously, the carrier material protamine is completely biodegradable minimizing the risk for local granuloma formation.

In summary, protamine-based nanoparticles with CpG-ODN may present an interesting biodegradable carrier system for the treatment of allergic diseases.

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