

Research paper

Mite allergoids coupled to nonoxidized mannan from *Saccharomyces cerevisiae* efficiently target canine dendritic cells for novel allergy immunotherapy in veterinary medicine



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A B S T R A C T

We have recently reported that grass pollen allergoids conjugated with nonoxidized mannan of *Saccharomyces cerevisiae* using glutaraldehyde results in a novel hypoallergenic mannan-allergen complex with improved properties for allergen vaccination. Using this approach, human dendritic cells show a better allergen uptake and cytokine profile production (higher IL-10/IL-4 ratio) for therapeutic purposes. Here we aim to address whether a similar approach can be extended to dogs using canine dendritic cells. Six healthy Spanish Greyhound dogs were used as blood donors to obtain canine dendritic cells (DC) derived from peripheral blood monocytes. Allergens from *Dermatophagoides farinae* mite were polymerized and conjugated with nonoxidized mannan. Nuclear magnetic resonance (NMR), gel electrophoresis (SDS-PAGE), immunoblotting and IgE-ELISA inhibition studies were conducted to evaluate the main characteristics of the allergoid obtained. Mannan-allergen conjugate and controls were assayed *in vitro* for canine DC uptake and production of IL-4 and IL-10. The results indicate that the conjugation of *D. farinae* allergens with nonoxidized mannan was feasible using glutaraldehyde. The resulting product was a polymerized structure showing a high molecular weight as detected by NMR and SDS-PAGE analysis. The mannan-allergen conjugate was hypoallergenic with a reduced reactivity with specific dog IgE. An increase in both allergen uptake and IL-10/IL-4 ratio was obtained when canine DCs were incubated with the mannan-allergen conjugate, as compared with the control allergen preparations (unmodified *D. farinae* allergens and oxidized mannan-allergen conjugate). We conclude that hypoallergenic *D. farinae* allergens coupled to nonoxidized mannan is a novel allergen preparation suitable for canine allergy immunotherapy targeting dendritic cells.

1. Introduction

Allergen-specific immunotherapy (ASIT) is considered the best long term treatment to manage environmental allergies, such as allergic rhinitis, asthma and atopic dermatitis in human and veterinary medicine (Akdis, 2012; Jutel et al., 2013; Loewenstein and Mueller, 2009). In humans, ASIT is mostly used for treating respiratory and hymenoptera venom allergies, while in dogs its indication is mainly focused

on atopic dermatitis (Keppel et al., 2008; Marsella, 2010; Shida et al., 2004; Willemse et al., 2009). In all cases, these conditions are dependent on IgE-mediated Th2-type responses, which are down-regulated by ASIT in an allergen-specific manner.

ASIT reduces the clinical signs and symptoms of canine atopic dermatitis by shifting the allergic Th2-type allergen specific response towards a Th1-type and/or Treg responses (Keppel et al., 2008). A repetitive administration of the offending allergen to the allergic dog is

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required for several years, and clinical amelioration is not usually seen until six-to nine months after initiation. At least 1 year of treatment should be given before discarding immunotherapy as a potential long-term management option (Rael and Lockey, 2014). Taking this into account, together with the fact that the administration of conventional ASIT is time-consuming, different approaches have been considered to improve it. The use of rush immunotherapy protocols (Mueller et al., 2004), or novel adjuvants to enhance the Th1-type response (Jassies-van der Lee et al., 2014; Mueller et al., 2005), have been already described in canine atopic dermatitis.

Hypoallergenic, glutaraldehyde-modified allergen extracts (polymerized allergoids) are increasingly being used in human medicine, as their safety profile allows for convenient rush and cluster initiation protocols (Pfaar et al., 2010; Subiza et al., 2008). Recently, we have shown that polymerized allergoids derived from grass pollen allergens coupled to nonoxidized mannan from *Saccharomyces cerevisiae* target human dendritic cells (DCs) (Sirvent et al., 2016). These cells are the main initiators of the immune responses by capturing, processing and presenting the antigens to naïve T cells (Wilson and Villadangos, 2005). DCs are engaged in the polarization of naïve T cells toward the different functional T cell phenotypes through cell-to-cell cognate and cytokine signals, which are dependent on the immunomodulating features related to the antigen (Apostolopoulos et al., 2013; Liu, 2001; Steinman et al., 1999). Grass allergens coupled to native mannan, besides being better captured by human DCs, increase IL-10 and decrease IL-4 production by these cells (Sirvent et al., 2016). This favors the induction of Treg and impairs Th2 cells, a main action mechanism of ASIT (Palomares et al., 2014).

The above referenced study was performed in human DCs (Sirvent et al., 2016). Thus, it was unclear whether the functional properties of the allergoid coupled to mannan could be extended to canine DCs. Therefore, we decided to assess the effect of allergoid coupled to mannan in dogs. Instead of grass allergens, such assessment was performed with the mite species *D. farinae*, the main offending allergen in canine atopic dermatitis (Nuttall et al., 2001a; Youn et al., 2002).

Herein we describe a new polymerized allergoid from *D. farinae* coupled to nonoxidized mannan derived from *S. cerevisiae*. Such an allergoid-mannan complex shows a reduced reactivity with specific IgE from atopic dogs and is better captured by canine DCs than mannan-free allergen preparations. Moreover, this allergoid-mannan complex induces a higher IL-10 and lower IL-4 production by canine DCs as compared with the control counterparts.

2. Materials and methods

2.1. Animals and blood collection (buffy-coats)

Six Spanish Greyhound dogs (mean age 6 years old; 22–30 kg) were used. All them were healthy blood donors from a veterinary transfusion center (Centro Transfusión Veterinario, Madrid, Spain) negative for the presence of antibodies to *Leishmania*, *Ehrlichia*, *Borrelia*, *Anaplasma* and *Filaria*. The buffy-coats, normally discarded after processing the whole blood to obtain plasma and erythrocytes, were used in the present study. Therefore, no special permissions were required. Blood collection was carried out in strict accordance with European legislation for animal research (2010/63/EU).

2.2. *D. farinae* allergen and yeast mannan

D. farinae allergen freeze-dried extracts derived from pure mite cultures were from Immunotek (Madrid, Spain). Total protein content was measured by the Bradford assay using serum albumin as standard (Bio-Rad Laboratories, Madrid, Spain).

Mannan from *S. cerevisiae* (Lesaffre Ibérica, Madrid, Spain) was purified as described (Manzano et al., 2016). Briefly, mannan was extracted from yeast in hot citrate buffer (0.02 M; pH 7.0) during 90 min,

precipitated with ethanol and dialyzed against distilled water. Mannan was precipitated in the presence of cetavlon (Sigma-Aldrich, Madrid, Spain) (50% v/v) after several hours in a shaker by adding 2% borate sodium (pH 8.8). The precipitate was recovered by centrifugation and washed twice with 2% acetic acid in ethanol plus a final wash with 100% ethanol. Once re-dissolved and dialyzed in distilled water, it was applied to a DEAE-Sephadex A-50 column equilibrated with 0.02 M Tris-HCl buffer (pH 7.5). A linear gradient from 0 to 0.5 M NaCl was used. Mannan-containing fractions were collected and pooled, dialyzed extensively against distilled water and lyophilized in aliquots until used.

2.3. Allergen-mannan conjugation

Allergens from *D. farinae* were polymerized and conjugated with nonoxidized mannan with glutaraldehyde as we described for grass pollen (Manzano et al., 2016). Briefly, glutaraldehyde was added to a solution containing a mixture of the allergen and native mannan in PBS. Reaction was performed during 6 h at 4 °C in continuous stirring and stopped with glycine, followed by tangential flow filtration with distilled water (membrane cut off, 100 kDa) to remove free mannan and molecules below that size. Polymerized allergen-mannan conjugates (PM) were recovered in the concentrated retentate (> 100 kDa fraction) that was further lyophilized until use.

Monosaccharides were measured by gas chromatography as described (Manzano et al., 2016). Briefly, The samples (1 mg) containing polysaccharides were first hydrolyzed with 3 M trifluoroacetic acid (121 °C, 1 h). The released monosaccharides were then converted into their corresponding alditol acetates by reduction with NaBH₄ (Sigma) and subsequent acetylation. Identification and quantification were performed by gas-liquid chromatography on a 6890A instrument (Agilent Technologies, Santa Clara, California, USA) equipped with a flame-ionization detector, using a HP5 fused silica column with He as the carrier gas. Identification was performed on the basis of the coincidence of the retention time of the sample components with those previously measured for known monosaccharide reference standards analyzed under identical conditions and using inositol as internal standard. The protein content was measured by the Bradford assay.

For control purposes, one part of the same batch of the allergen extract remained untreated (native allergen, N) or subjected to the above protocol but without mannan to obtain a conventional mannan-free polymerized (P) allergoid. In some experiments, PM was mildly oxidized with 5 mM *m*-periodate (PM-Ox) as described (Sirvent et al., 2016).

2.4. Nuclear magnetic resonance (NMR) studies

NMR spectra were obtained for samples (4 mg/mL in D₂O) at 298 K as described (Manzano et al., 2016). Briefly, Standard 1H NMR and 2D-NMR experiments were employed using Bruker Avance 500 or 600 MHz spectrometers (Bruker Ltd., Germany). Standard conditions were employed for proton one dimension experiments and were acquired with 32 scans. The two dimension diffusion ordered spectroscopy (2D-DOSY) experiments were carried out by recording 64–128 scans for each gradient step, a linear gradient of 16 steps between 2% and 95%, a diffusion time (big delta) between 0.2 and 0.4 s, and the length of the diffusion encoding gradient pulses (little delta) between 2 and 4 ms. All spectra were processed with the protocols implemented in Topspin software (Bruker Ltd).

2.5. Gel electrophoresis (SDS-PAGE) and immunoblotting

Non reduced *D. farinae* allergen samples (N, P and PM) were subjected to protein separation in 12.5% polyacrylamide gels under denaturing conditions with sodium dodecyl sulfate (SDS-PAGE) and Coomassie blue protein staining as described (Manzano et al., 2016).

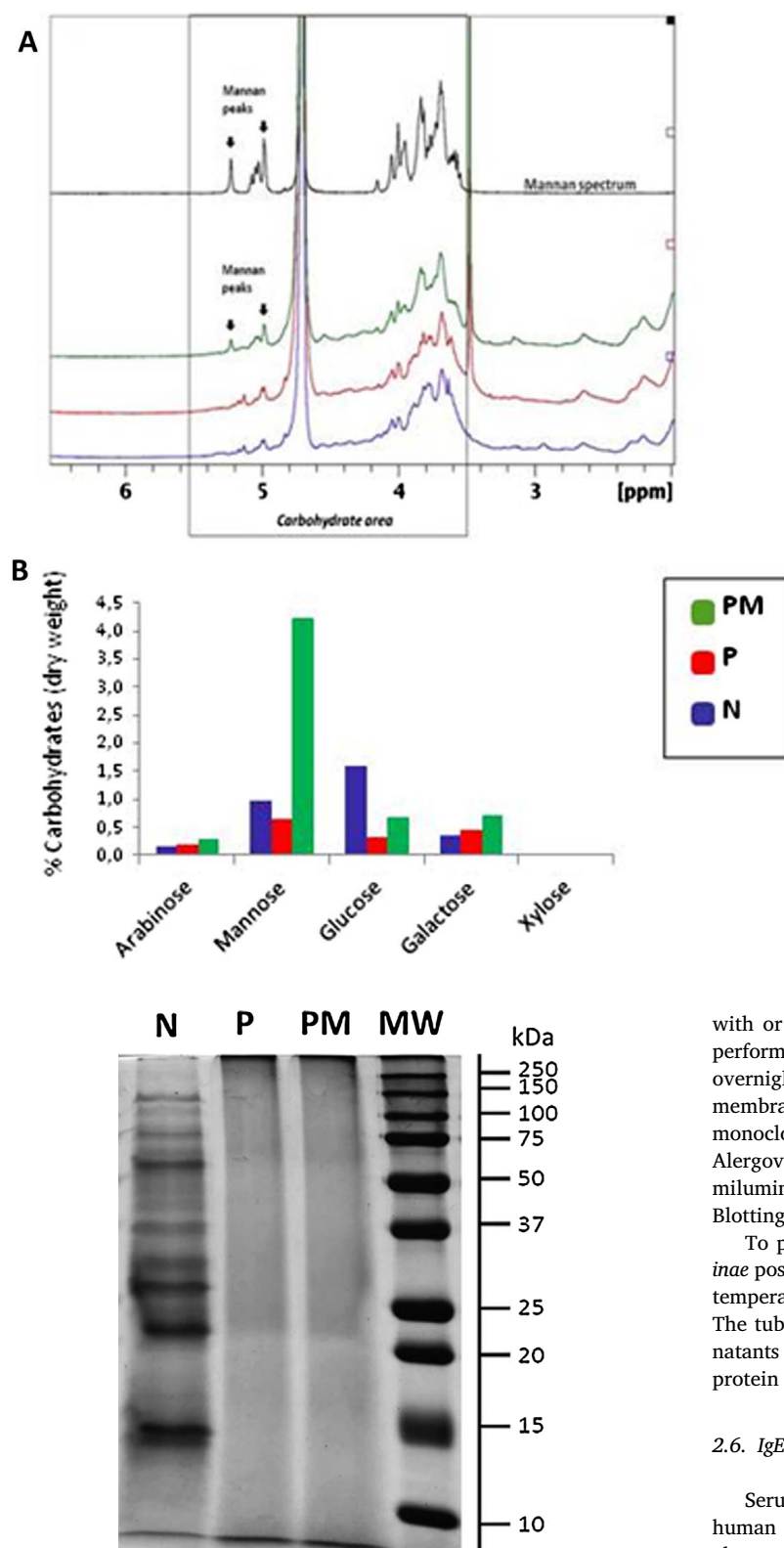


Fig. 1. Structural analysis and carbohydrate content of *D. farinae* allergen preparations. PM (green): Polymerized allergoid-mannan conjugate; P (red): Polymerized allergoid; N (blue): Native (unmodified) allergen. A) Representation of the different ^1H NMR spectra for the purified mannan and allergen preparations. Arrows indicate mannan-specific sugars. Carbohydrate (5–3 ppm) region is displayed. B) Percentage of neutral monosaccharides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. *D. farinae* allergen preparations subjected to SDS-PAGE. N: Native (unmodified) allergen; P: Polymerized allergoid; PM: Polymerized allergoid-mannan conjugate; MW: Molecular weight standards. Protein bands detected by Coomassie blue staining.

Immunoblots were performed by electro-transferring the proteins of *D. farinae* allergen preparation (N) separated by SDS-PAGE to PVDF membranes (Bio Rad, Germany). The membranes were blocked with 5% bovine serum albumin in PBS 0.1 M-0.5% Tween 20 and incubated with a pool of *D. farinae* positive dog sera (Alergovet, Madrid, Spain)

with or without a prior absorption with the allergen preparations to perform inhibition assays (see below). The incubation was performed overnight at $+4^\circ\text{C}$ with orbital shaking. After a washing step, the PVDF membranes were incubated with a mixture of three specific anti-dog IgE monoclonal antibodies conjugated with peroxidase (Olygo.3mAb, Alergovet, Madrid, Spain) diluted 1:20,000 in the same buffer. Chemiluminescence was used for reaction development (ECL Western Blotting System; Buckinghamshire, UK).

To perform IgE immunoblotting inhibition assays a pool of *D. farinae* positive dog sera was mixed separately in tubes during 3 h at room temperature with the same amount of protein (7.5 μg) from N, P or PM. The tubes were then centrifuged (10,000 rpm; 10 min) and the supernatants incubated with the above PVDF membranes containing the protein bands from native *D. farinae* (N).

2.6. IgE-ELISA-Inhibition studies

Serum IgE reactivity to the native *D. farinae* allergens (N) from both human and dog was assayed by ELISA-Inhibition in the presence or absence of each *D. farinae* allergen preparation (N, P, PM). Briefly, 96 well ELISA plates (Greiner bio-one, Germany) were coated with N (1 $\mu\text{g}/\text{well}$) in bicarbonate buffer, pH = 9.6. Once the wells were washed, a constant serum dilution preincubated with a $\frac{1}{2}$ serial dilutions (100 $\mu\text{g}/\text{mL}$ to 0.01 $\mu\text{g}/\text{mL}$) of each sample, were added. The plates were incubated with the serum-allergen mixture overnight. After a washing step, the wells were incubated with peroxidase conjugated monoclonal anti-canine IgE (Olygo.3mAb; Alergovet, Madrid, Spain) or anti-human IgE (Southern Biotech, USA) at a 1:3,500 or 1:2,000 final dilution, respectively. After a final washing, the substrate solution

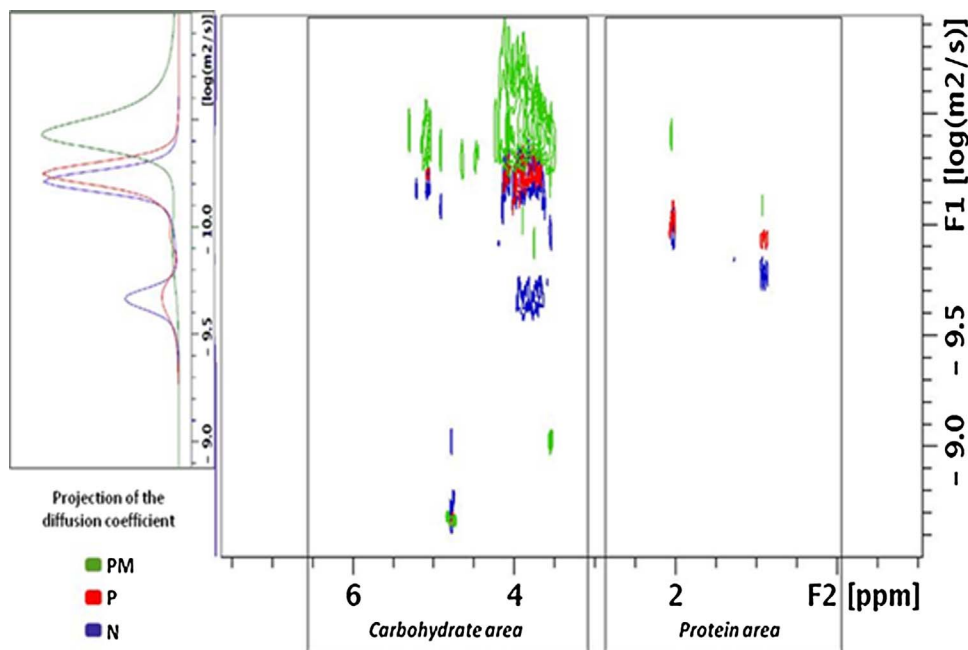


Fig. 3. Two dimension diffusion ordered spectroscopy (2D-DOSY). Superimposition of the different DOSY experiments recorded for *D. farinae* allergen preparations. PM (green): Polymerized allergoid-mannan conjugate; P (red): Polymerized allergoid; N (blue): Native (unmodified) allergen. Both the carbohydrate and the protein areas are displayed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(TMB) was added, the reaction stopped after 30 min adding HCl 2N and the color developed read at 450 nm.

2.7. Monocyte-derived canine DCs

Canine peripheral blood mononuclear cells were obtained from the buffy-coats by means of Ficoll density gradients following standard method. Monocytes were isolated with anti-CD14 coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the supplier's protocol. Blood monocytes were differentiated *in vitro* to DCs as described (Wang et al., 2007) with slight modifications. Cultures were performed with RPMI-10% FBS containing canine rIL-4 (35 ng/mL), human rGM-CSF (50 ng/mL), and human rFlt-3L (10 ng/mL), all cytokines from R & D System, USA. Cells were cultured during 6 days replacing half of the medium with fresh one on day 3. Before using in the experiments, monocyte-derived DCs were characterized phenotypically by flow cytometry. Briefly, 10^5 cells were stained (30 min at 4 °C) with anti-HLA class-II DR (mAb clone L243 reactive with dog; Antibodies On-line, Atlanta, USA), anti-CD80 (mAb clone 16-10A1 reactive with dog; Antibodies On-line), anti-CD86 (polyclonal antibody, cat. #ABIN736708 reactive with dog; Antibodies On-line), or with appropriated isotopic controls (BD Biosciences/Pharmingen). Samples were analyzed on a FC-500 Beckman Coulter flow cytometer using FLOWJO V.10 software.

2.8. Allergen uptake by canine DCs

N, P and PM preparations from *D. farinae* were labeled with Alexa 488-maleimide (Thermo Fisher Scientific) using thiol reactive groups to achieve a homogeneous labeling among samples as described (Sirvent et al., 2016). Labeling was controlled by measuring the fluorescence in each sample preparation (Fluostar Optima reader, BMG Labtech). In some experiments, the labeled-PM preparation was further mildly oxidized with 5 mM *m*-periodate as previously described (Sirvent et al., 2016).

Uptake experiments with DCs were performed as previously described (Sirvent et al., 2016). Briefly, canine DCs (3×10^5) were incubated (30 min at 37 °C) with 100 µg/mL of each labeled preparation in RPMI. Incubation was stopped by adding cold PBS. Next, cells were recovered by centrifugation and their fluorescence analyzed by flow cytometry (FC-500 Beckman Coulter) using FLOWJO V.10 software.

Data were normalized against the mean fluorescence intensity (MIF) obtained with N.

2.9. IL-4 and IL-10 cytokine production by canine DCs

Canine monocyte-derived DCs (10^6 /mL) were cultured in RPMI with or without the different allergen preparations (100 µg/mL) for 72 h. Levels of IL-4 and IL-10 were measured in culture supernatants using commercially available dog ELISA kits (R & D Systems, USA).

2.10. Statistics

Data are expressed as mean $\pm$ s.e.m. of the indicated parameters. Statistical differences were determined with the Student's *t*-test. Significance was defined as **p* < .05, ***p* < .01 and ****p* < .001.

3. Results

3.1. Conjugation of *D. farinae* allergens with nonoxidized mannan produces polymerized allergoid-mannan complexes of high molecular weight

The result of coupling nonoxidized mannan with *D. farinae* allergens by means of glutaraldehyde is shown in Fig. 1. After the conjugation process, specific mannan peaks were present as detected by NMR (Fig. 1A). The total content of mannose increased more than 8-fold (Fig. 1B), as compared with native (unmodified) *D. farinae* (N) or glutaraldehyde-treated *D. farinae* allergens without mannan (P). Fig. 2 shows the protein pattern of N, P and PM as detected by SDS-PAGE and protein staining. The protein bands detectable in the native *D. farinae* allergen (N) almost disappeared in lanes loaded with glutaraldehyde-treated preparations (P and PM). This reflects the protein polymerization process and the further dialysis step. Fig. 3 shows the 2D-DOSY NMR spectra of N, P and PM. A reduced translational diffusion was seen in PM in comparison with N and P, indicating an increase of the average molecular weight size. A bigger molecular entity after allergen conjugation with mannan is seen in PM as compared with N and P, indicating that high molecular weight complexes were formed after the coupling process.

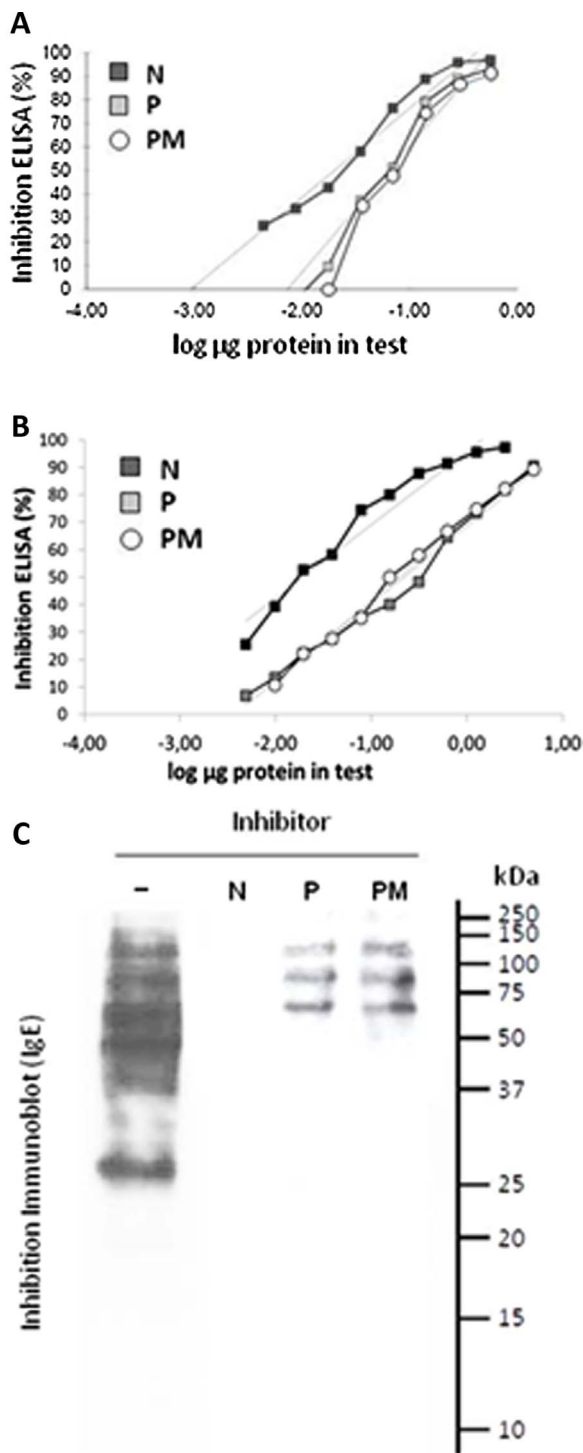


Fig. 4. Dog and human IgE inhibition studies assayed onto native (unmodified) *D. farinae* allergen preparation. Inhibitions were performed using *D. farinae* allergen preparations as inhibitors. N: Native (unmodified) allergen; P: Polymerized allergoid; PM: Polymerized allergoid-mannan conjugate. A) ELISA-Inhibition (dog IgE) using increasing amounts of each inhibitor; B) ELISA-Inhibition (human IgE) using increasing amounts of each inhibitor; C) Immunoblot (dog IgE) inhibition using a single amount of each inhibitor.

3.2. *D. farinae* allergens coupled to nonoxidized mannan show a reduced IgE reactivity with the sera from atopic dogs

IgE-ELISA inhibition assays using pooled sera of atopic dogs were performed with the three allergen preparations. A loss of IgE reactivity was detected with P and PM, as compared with the inhibition achieved with the native (N) allergen preparation (Fig. 4A). The decrease was

similar for P and PM (65–70% of loss of the initial activity defined by N). When testing pooled sera from *D. farinae* sensitized human patients, the reduction of IgE reactivity was even higher (above 90%) for both P and PM (Fig. 4B). Fig. 4C displays the IgE pattern of reactivity of the atopic dog pooled serum with the protein allergen bands from the native *D. farinae* allergens by immunoblotting. This assay was performed with or without prior serum absorption with each allergen preparation. While N preparation was able to completely inhibit IgE reactivity, P and PM were not at the same allergen protein concentration. Allergens in the range of 65–150 kDa seem to be more sensitive to glutaraldehyde treatment, since these bands were less inhibited by both P and PM.

3.3. *D. farinae* allergens coupled to nonoxidized mannan improve their uptake by canine DCs and induce a higher IL-10/IL-4 ratio production

Canine DCs were derived from blood monocytes. Before using in the experiments, the expression of the DC cell surface markers (MHC-II, CD80 and CD86) was confirmed by flow cytometry (Fig. 5). Allergen uptake by DCs was performed with all three preparations N, P and PM equally labeled with a fluorochrome (Alexa Fluor 488). As shown in a representative experiment (Fig. 6A), the percentage of Alexa positive cells was greater when incubated with PM (62%) than with N (19%) or P (39%). When PM was oxidized (PM-Ox), without affecting the intensity of fluorescence emission (data not shown), the percentage of positive cells dropped to 23% (Fig. 6A). Fig. 6B shows the cumulative results from independent experiments considering the fluorescence intensity of each preparation after allergen uptake. These results indicated that the incorporation of nonoxidized mannan to PM significantly enhanced allergen uptake by canine DCs and that this feature was highly dependent on the integrity of mannoses.

Cytokines (IL-10 and IL-4) were measured in the supernatants of canine DCs cultures after stimulation with N, P and PM allergen preparations (Fig. 7). The production of IL-10 was significantly increased in PM-stimulated cultures compared to N or P. IL-10 induction by PM was significantly impaired after mild oxidation (PM-Ox). However, IL-4 production by canine DCs was decreased after PM stimulation. Thus, the ratio IL-10/IL-4 was significantly increased in PM cultures in comparison to N, P or PM-Ox (Fig. 7). These results indicate that PM is able to enhance a tolerogenic profile in canine DCs, which is dependent of the use of nonoxidized mannan.

4. Discussion

In a previous study we have demonstrated that polymerized grass pollen allergens can be coupled to nonoxidized mannan (Manzano et al., 2016), and that it results in a hypoallergenic mannan-allergoid conjugate with improved properties for ASIT (Manzano et al., 2016; Sirvent et al., 2016). Herein we show that this can also be achieved in dogs using mannan conjugates with allergens derived from the mite *D. farinae*, an important allergen in the pathogenesis of canine atopic dermatitis (Nuttall et al., 2006; Nuttall et al., 2001a; Youn et al., 2002).

Conjugation of nonoxidized mannan with *D. farinae* allergens was achieved successfully by using glutaraldehyde as detected by NMR. Conjugation is likely made through the reaction of glutaraldehyde with the free amino groups from both the allergen proteins and the manno-protein tail linked to the mannan carbohydrate backbone (Manzano et al., 2016). The coupling efficiency of nonoxidized mannan to *D. farinae* allergens was slightly lower than to pollen (*Phleum pratense*) (approx. 8-fold increase in total mannoses vs. approx. 20-fold-increase, respectively) (Manzano et al., 2016); this may be due to the lower initial content of mannoses in this latter (0.5% vs 0.1%).

The importance of coupling to mannan without a prior oxidation step (Mislovicová et al., 2002) was stressed previously (Manzano et al., 2016; Sirvent et al., 2016) and supported in our experiments. All functional features ascribed to PM in the present study were lost by breaking the manno-pyranose rings of mannan following a mild

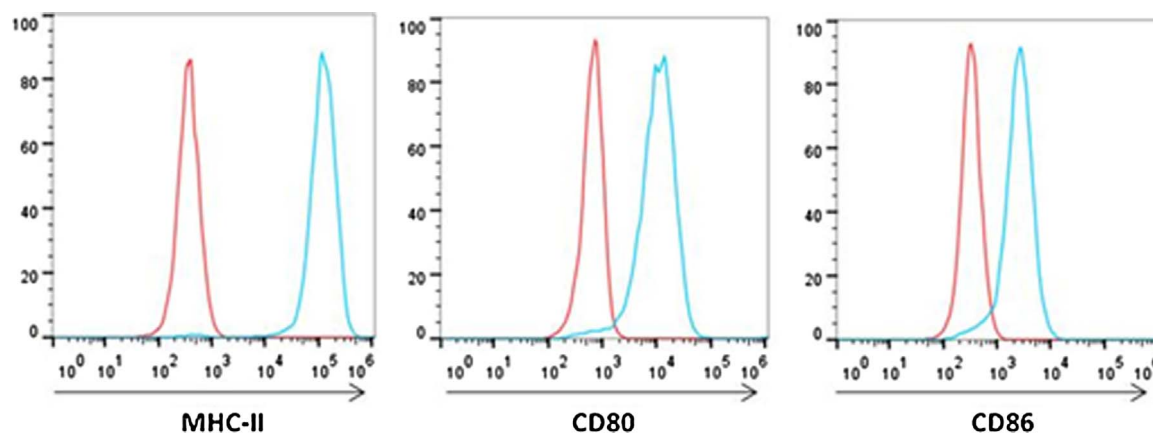


Fig. 5. Expression of cell surface markers on canine DCs derived from peripheral blood monocytes as detected by flow cytometry. The phenotypic characterization was assayed after 5 days cell culture with recombinant canine IL-4, recombinant human GM-CSF and recombinant human Flt3L. Isotype controls (red) and specific antibodies (blue) are displayed for each cell marker (a representative experiment out of three). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

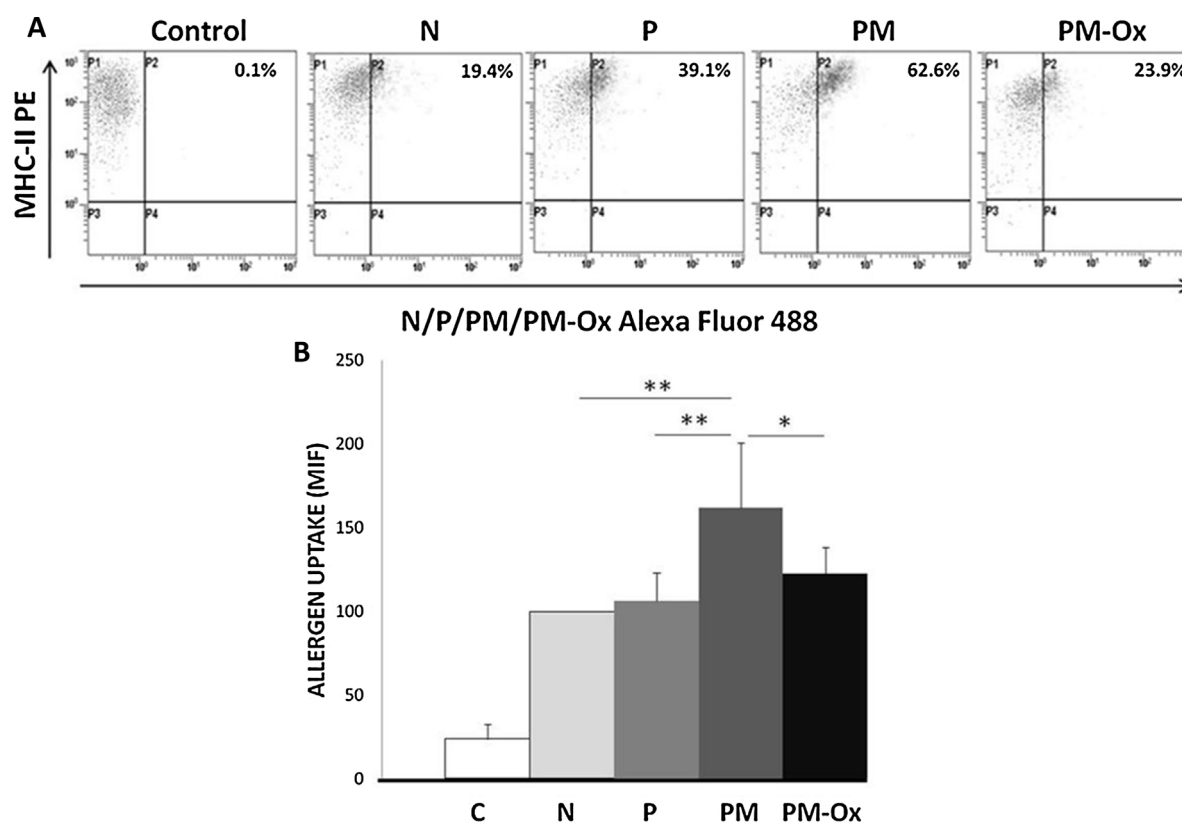


Fig. 6. Uptake of fluorescence-labeled *D. farinae* allergen preparations by flow cytometry after 30 min of incubation with canine DCs. N: Native (unmodified) allergen; P: Polymerized allergoid; PM: Polymerized allergoid-mannan conjugate; PM-Ox: PM further oxidized with sodium *m*-periodate. A) Dot plots images of a representative experiment; B) Mean fluorescence intensity $\pm$ s.e.m. of 6 independent experiments. Statistical differences were analyzed with Student's *t*-test. **p* < 0.05, ***p* < 0.01.

oxidation. The *D. farinae*-mannan conjugate results in a high molecular weight entity as detected by DOSY NMR spectra and SDS-PAGE. The higher molecular weight is due, in part, to the allergen polymerization process associated with glutaraldehyde treatment which explains the reduction of IgE reactivity in P and PM allergen preparations (Lee et al., 1986; Sirvent et al., 2016). By using pooled sera from allergic dogs, this reduction was of approximately 65–70%, which is a lower reduction in IgE reactivity as compared with human IgE (above 90% loss).

Major allergens of *D. farinae* recognized by allergic dogs are in the range of 80–140 kDa and are different molecules than major allergens for humans (in the range from 15 to 30 kDa) (Nuttall et al., 2001a, 2001b). It seems that polymerization reduces preferentially the reactivity of dog IgE with the high molecular weight major allergens, as

detected in the present study by immunoblot inhibition assays. Glutaraldehyde treatment, with or without mannan, produces hypoallergenic *D. farinae* allergoids for allergic dogs and therefore, the mannan-conjugates keep the hypoallergenic features of conventional polymerized allergoids. This represents an important advantage when using rush protocols for the buildup phase in veterinary and human medicine (Mueller et al., 2004). Moreover, hypoallergenic preparations have the additional advantage of avoiding Th2 promoting events such as IgE-facilitated antigen presentation or basophil activation (Akdis and Blaser, 2000; Suurmond et al., 2014).

One of the main purposes of the present study was to assess the effect of the allergoid-mannan conjugates on canine DCs. The fact that the functional studies with these conjugates were performed previously

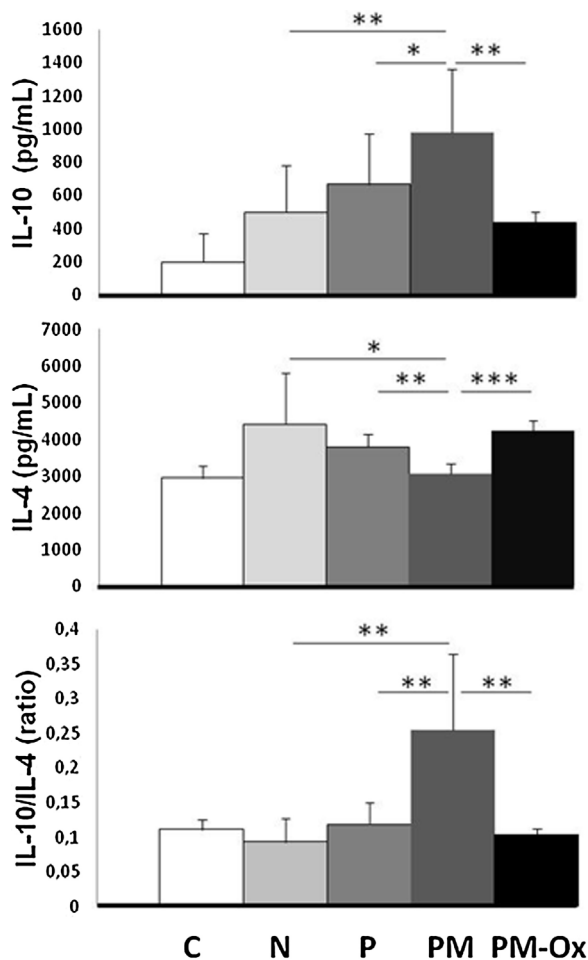


Fig. 7. Cytokine production *in vitro* by canine DCs stimulated with *D. farinae* allergen preparations. N: Native (unmodified) allergen; P: Polymerized allergoid; PM: Polymerized allergoid-mannan conjugate; PM-Ox: PM further oxidized with sodium *m*-periodate. Cell culture supernatants were collected after 72 h culture. Results are the mean $\pm$ s.e.m. of 6 independent experiments. Statistical differences were analyzed with Student's *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

with human DCs (Sirvent et al., 2016), together with some doubts in relation to the expression of mannose receptor (CD206) on canine DCs (Ricklin Gutzwiller et al., 2010), prompted us to clarify this issue. A variety of C-type lectin receptors have been shown to be involved in the mannan-DC interaction, and not only CD206 (Palomares and Subiza, 2017; Sirvent et al., 2016). However, suitable reagents are not currently commercially available for these receptors in dogs. We have shown that DCs derived from canine blood monocytes show a similar functional behavior as in humans. Thus, *D. farinae* allergoid coupled to non-oxidized mannan is better taken up by canine DCs while favoring IL-10 production by these cells. This, along with a lower production of IL-4, makes such allergoid-mannan conjugates more convenient for ASIT (Schülke and Vieths, 2016).

The *in vivo* implications of the above allergoid-mannan conjugates have been emphasized in mice and rabbits (Sirvent et al., 2016), but not in experimental allergy models. A pilot study with the same *D. farinae* allergoid-mannan preparation has been successfully completed in dogs suffering from atopic dermatitis (González et al., in preparation). Thus, these polymerized allergoids coupled to nonoxidized mannan represent a good alternative to conventional allergens for ASIT in veterinary medicine. Since they target DCs increasing allergen uptake, it is conceivable that, at the same allergen dose, efficacy could be improved. Furthermore, as they also promote a tolerogenic phenotype, these novel vaccines targeting DCs may well represent a new generation of allergen

vaccines for improved ASIT.

Conflict of interest

J.L.S. is the founder of Immunotek and shareholder together with M.C. and E.F.C. Immunotek is a manufacturer of allergy vaccines and has submitted a patent related to the vaccine described herein (WO/2014/162036).

F.J.C., J.J.B. and O.P. have received grants from Immunotek.

I.S., A.I.M., B.C. and J.L.R. are employees of Immunotek.

J.A. and A.M.F. are employees of Alergovet, a company that markets immunotherapy treatments for veterinary.

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