

Immunological mechanisms involved in the human response to a dog dander allergoid

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ABSTRACT

Background: Dog allergens are a common cause of allergic sensitisation and trigger respiratory symptoms worldwide. However, clinical evidence regarding dog immunotherapy is limited. Therefore, the aim of this study was to analyse the immunomodulatory properties of a new allergoid from dog dander, thereby deepening the understanding of the molecular mechanisms involved in the reestablishment of the tolerogenic response.

Methods: Three independent batches of dog dander native and allergoid allergen extracts were manufactured and characterised. Allergenic profiles were analysed by the identification of all dog allergens and quantification of the major allergens Can f 1 and Can f 5. The allergenicity profile of the allergoid was studied using biological potency and basophil activation tests. *In vitro* immunomodulatory parameters were evaluated as the capacity of the allergoid to induce IgG antibodies that block IgE binding to the allergen and cytokine promotion (IFN- γ , IL-4, IL-6, IL-10, IL-13, and TNF- α) in PBMCs from allergic donors.

Results: The presence of all dog allergens, including Can f 1 and Can f 5, was confirmed in both types of extracts. The new allergoid showed a low IgE binding capacity, which significantly affected the activation of effector cells, such as basophils. The IgG antibodies induced by the allergoid in rabbits blocked human IgE binding epitopes on the dog native extract and induced Th1 and Treg responses by increasing IFN- γ and IL-10 levels in PBMCs from allergic donors.

Conclusion: This new dog dander allergoid containing Can f 1 and Can f 5 showed a low capacity to bind IgE and to activate basophils in dog allergic patients. Furthermore, it showed potent activation of Th1 mediators and induction of tolerance through Treg activation. This allergoid could offer a safer profile than the native extract and could be an effective immunotherapy treatment for dog allergic patients.

1. Introduction

The greater presence of dogs in homes in urbanised countries has increased the frequency of allergic reaction to these animals (Schoos et al., 2021). Pet owners and professionals involved in animal care, are most affected by this respiratory allergy, the main symptoms of which include rhino-conjunctivitis and asthma (Dávila et al., 2018).

To date, eight dog allergens from different anatomical sites, including the mouth cavity (saliva), skin (dander/hair), and prostate

(urine), have been identified (www.allergen.org). In terms of biochemical function, Can f 1, Can f 2, Can f 4, and Can f 6 are members of the lipocalin family, whereas Can f 3 is a dog serum albumin that displays a high homology to other mammalian albumins, and Can f 7 is a member of the MD2-like lipidic recognition family (Wintersand et al., 2019). Recent studies have shown increasing importance of Can f 5 (Mattsson et al., 2009; Suzuki et al., 2019; Kailaanmäki et al., 2016), which is a 28 kDa prostatic kallikrein (Canine Prostatic Specific Esterase, CPSE) secreted mainly in male dog urine (González-de-Olano et al., 2018; Suzuki et al., 2019; Kailaanmäki et al., 2016; Liccardi et al., 2018;

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Nomenclature

AIT	Allergen immunotherapy
BAT	Basophil activation test
DE	Depigmented extract
DPE	Depigmented and polymerised extract
MS	Mass spectrometry
NE	Native extract
PBMCs	Peripheral blood mononuclear cells

Schoos et al., 2020). Today, Can f 1 and Can f 5 are considered to be the major dog allergens responsible for more than 70% of allergic sensitisation. Recently, Can f 8 has been observed in dog dandruff and described as a member of the cystatin family (www.allergen.org).

Currently, allergen immunotherapy is the only therapeutic treatment that can change the course of allergic diseases providing clinical efficacy with a long-term benefit. However, although the molecular mechanisms involved in the AIT response have been studied, they have not been elucidated in their entirety. Commonly, successful therapeutic intervention against type 1 allergic diseases has been associated with production of high-titred blocking IgG antibodies. Furthermore, AIT is believed to promote the absence of proinflammatory signals towards a peripheral tolerance response (Calzada et al., 2018). Thus, it induces major changes in allergen-specific T cell subsets, modulating the Th2 to a Th1 response and promoting regulatory T and B cells. Their main roles are to maintain integrity of the body through avoiding excessive immune responses that may result in harmful immune pathology by the secretion of cytokines as IL-10 (Kucuksezer et al., 2020). However, evaluation of the clinical efficacy of dog allergen immunotherapy (AIT) yields modest results. Until date, the number of available studies on subcutaneous immunotherapy with dog are limited. The scientific literature offered very modest results with respect to the clinical efficacy of AIT with dog extracts possibly due to the use of extracts without significant and detailed characterisation of the allergens, particularly Can f 5, present in it. This can be explained by variability of allergenicity, as well as the complexity of the allergenic profile of dogs (Dávila et al., 2018). Furthermore, the molecular mechanisms involved in dog AIT response have not been adequately studied. Thus, better characterisation of dog allergen extracts and better understanding of the effectiveness of dog AIT are needs that are, as yet, unmet.

Moreover, the development of chemically modified allergen extracts by polymerisation with glutaraldehyde has been postulated as an alternative in immunotherapy, maintaining/increasing the efficacy while reducing the risk of adverse reactions (Ibarrola et al., 2004). The resulting products are high molecular weight allergen chains which contain all the allergens present in native extracts (NE). An intermediate patented process known as “depigmentation” (Carnés Sánchez, J., n.d.) consists on the purification of the NE for the removal of irrelevant allergenic substances with the objective to increase the concentration of the individual allergens. To date, this method has yielded good results in other aeroallergens, including mites and pollens (Gallego et al., 2010; Pfaar et al., 2015).

Based on these concepts, the objective of this study was to investigate the immunological response of a new allergoid for treating dog allergies.

2. Materials and methods

2.1. Allergen extract preparation

One hundred grams of dog dander (Greer, Lenoir, NC, USA) were extracted in phosphate-buffered saline 0.01 M-NaCl 0.15 M over two stages with continuous magnetic stirring for 12 h at 4 °C. The resulting product was termed the native extract (NE) that was depigmented by a

mild acid treatment with HCl (pH 4) and extensively dialysed against bi-distilled water in 3.5 kDa cut-off dialysis membranes (Millipore, Bedford, MA, USA) (Carnés, 2011). Then, the pH was adjusted to 7.4. The resulting depigmented extract (DE) was then polymerised with glutaraldehyde and dialysed against bi-distilled water in 100 kDa cut-off dialysis membranes (Millipore). Finally, the polymerised product was freeze-dried, yielding a dog depigmented-polymerised extract (DPE) (Morales et al., 2017, 2018). Three batches were thus manufactured to confirm the consistency of the process. More details are indicated in the [Supplementary Material](#).

2.1.1. Protein content characterisation

The protein contents of the NE, DE and DPE were measured using the Bradford method (Thermo Fisher Scientific, Waltham, MA, USA) and total nitrogen content with the TN analyzer multi N/C 2100 S (Analytikjena, Jena, Germany) according to the manufacturer's instructions. To determine the protein profile, an SDS-PAGE (2.67%C-15%T acrylamide) was performed under reducing and non-reducing conditions and stained with Coomassie-Blue R-250 (Bio-Rad, Hercules, CA, USA). Additionally, the molecular weight distribution of each type of extract was determined by high-performance size exclusion chromatography using a Bio SEC-3 column (Agilent Technologies, Santa Clara, CA, USA). To analyse the proteolytic activity of the extracts, a gelatine zymography SDS-PAGE was used. It indicates if the sample contains proteases that utilise gelatin as a substrate. After Coomassie-Blue R-250 (Bio-Rad) staining, areas of degradation are visible as clear bands against a darkly stained background.

2.1.2. Allergen characterisation

2.1.2.1. Allergens identification. Dog allergens were identified in whole NE, DE, and DPE by MS. Additionally, in the NE, bands from the SDS-PAGE corresponding around 23 and 28 kDa were cut and sequenced by MS to detect Can f 1 and Can f 5, respectively. Briefly, the samples were digested with trypsin and the peptide mixtures were analysed in a nanoAcquity liquid chromatography system coupled to an Orbitrap Velos (Thermo Fisher Scientific) mass spectrometer. A database search was performed by Thermo Proteome Discover against the UniProt database.

Additionally, to confirm the presence of major allergens, western blotting with anti-Can f 1 (EL-CF1, Indoor Biotechnologies, Charlottesville, VA, USA) and anti-CPSE (BVT, La Seyne sur Mer Cedex, France) were used to detect Can f 1 and Can f 5, respectively.

2.1.2.2. Major allergen quantification. Can f 1 and Can f 5 were quantified by ELISA. Can f 1 was measured using EL-CF1 (Indoor Biotechnologies) and the goat anti-rabbit IgG-HRP (1/300.000) (Bethyl, Montgomery, TX, USA) antibody. Can f 5 was measured using the Odelis CPSE kit (BVT), according to the manufacturer's instructions.

2.1.2.3. Allergenic properties.

- **Western blotting:** The allergenic properties of NE, DE, and DPE proteins were studied using a western blot assay. The SDS-PAGE gels were transferred to a Trans-Blot® Turbo™ Transfer system (Bio-Rad). Then, the membrane was incubated overnight with different sera from patients allergic to dog dander and incubated with mouse anti- α -human-IgE-HRP (Southern Biotech, Birmingham, AL, USA). Finally, the reaction was developed using a Clarity™ Western ECL substrate (Bio-Rad).

- **Biological potency:** The biological potency of each extract was calculated using ELISA competition assays, as previously described (Casanovas et al., 1994). Each extract was compared with the in-house reference preparation, previously standardised *in vivo* (data

not shown). The loss of potency percentage was calculated as the difference in biological potency between the NE and the DPE divided by NE potency. The results were expressed in histamine equivalent units (HEP)/mg, given that 10 HEP of extract elicits a similar skin reaction as a histamine prick of 10 mg/ml.

- **Basophil activation test (BAT):** BAT was performed to study the functional capacity of the NE and the DPE. Twelve dog allergic patients and six healthy controls were clinically classified by respiratory symptoms, skin prick test results and the specific IgE to dog dander tested by Immunocap (Thermo Fisher Scientific). Ethical approval was obtained from the Ethical and Research Committee of the Clinic Hospital Ref. HCB/2020/0139; Barcelona, Spain. Inclusion criteria are summarised in the [Supplementary Material](#). Basophils from the peripheral blood of each volunteer subject were stimulated by both the NE and the DPE at three different concentrations (0.1, 1, and 10 µg/ml), and the expressions of the specific markers CD203c and CD63 were evaluated by flow cytometry. The number of activated cells was reported as a percentage (cells expressing CD63 among all basophils gated; a minimum of 500 basophils were analysed in each case). Data were analysed using the Diva Software (Becton Dickinson).

2.2. Immunological studies

2.2.1. Humoral response

Polyclonal antibodies were used for IgG-binding assays to allergen extracts. Specific antibodies were induced into two New Zealand white rabbits after the administration of DPE and after three immunisations with 200 µg of DPE, with complete Freund's adjuvant being used for the first immunisation and incomplete Freund's adjuvant being used for the second and third immunisations every three weeks. All procedures were approved by the Biolab Institutional Review Board (Biolab, Colmenar Viejo, Spain) and followed the local ethics rules for animal experimentation.

- **IgE Blocking antibodies:** The capacity of DPE to induce allergen-specific polyclonal IgG antibodies that block IgE-binding to the allergen was evaluated by ELISA inhibition, as previously described ([Lopez-Matas et al., 2013](#)). The percentage of inhibition was calculated by comparing IgE binding after incubation with preimmune or final bleeding polyclonal antibodies. We evaluated the capacity of DPE to induce specific IgG that can inhibit the IgE binding of a pool of sera from dog allergic patients (Plasmalab) to dog dander NE. The percentage of inhibition was calculated as follows: percentage of IgE binding = $100 - (\text{ODf}/\text{ODP}) \times 100$. Odf and ODP are the optical densities of serum after preincubation with the rabbit's final sera and the corresponding preimmune sera, respectively.

2.2.2. Cell response

- **Cytokine profile:** The capacity of the NE and the DPE to modulate the levels of cytokines in donors allergic to dog dander ($n = 8$) and in healthy controls ($n = 4$) was evaluated by ELISA-based Milliplex®. The study was approved by the Ethics Committee of the Regional Research of Malaga, Spain (26thSept-2019). Inclusion criteria are summarised in the [Supplementary Material](#). Briefly, peripheral blood mononuclear cells (PBMCs) (1×10^5 cells/50 µL) were isolated from donors using Ficoll density gradient and stimulated in duplicates with the NE or the DPE, cells were resuspended in culture media RPMI supplemented with foetal calf serum at 20%. The cytotoxicity of the products was determined using Live/Dead NearIR (Life Technologies-Invitrogen) after incubating the cells with different concentrations of NE or DPE. Higher concentrations of both products, showing more than 70% cell viability, were selected for

cytokine stimulation assays. The production of IFN-γ, IL-4, IL-6, IL-10, IL-13, and TNF-α by stimulated PBMCs was measured after 72 and 120 h of incubation at 37 °C and 5% CO₂, with the optimal concentration of the products (25 µg/ml). PBMCs incubated with only culture media and those stimulated with concanavalin A (5 µg/ml) were used as negative and positive controls, respectively. The cytokine levels in the supernatants were quantified using HCYTO-MAX-60 K-06 Human Cytokine Magnetic kits by a MAGPIX® 4.2 (Luminex®) (EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions.

2.3. Statistical analysis

Statistical analyses were carried out using the GraphPad Prism 9.0 software. The Wilcoxon test and The Mann-Whitney were used to analyse and compare specific IgE levels, basophil activation test was to compare data from the same or different clinical groups, respectively. Different paired tests were performed for the comparisons of more than two (Friedman) or two (Wilcoxon) cytokine release experimental conditions from the same clinical group.

3. Results

3.1. Allergen extract characterisation

Three independent batches of dog dander DPE were manufactured from their corresponding NEs. According to Bradford method, mean protein content of the three NE batches was 91.6 ± 4.6 µg protein/mg NE. In the case of DE and DPE, the protein contents were 130.7 ± 7.9 and 162.0 ± 27.2 µg protein/mg lyophilised respectively. Regarding the total nitrogen analyses the values were 506 ± 28 µg for NE; 728 ± 23 µg for DE and 724 ± 60 µg in DPE ([Fig. 1 A.4](#)).

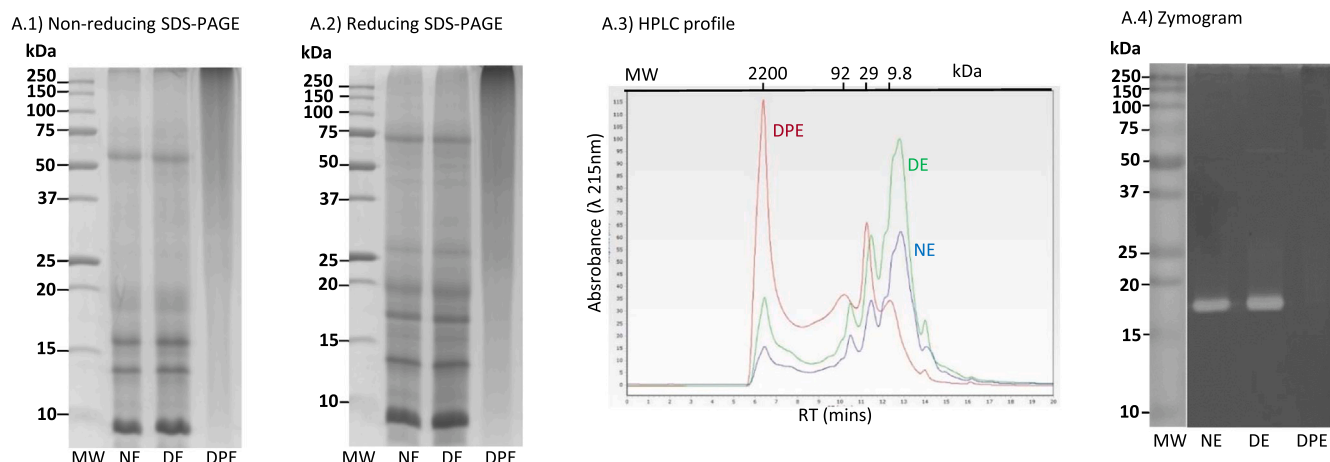
[Fig. 1 A](#) shows the main results according to the protein profiles. NE and DE had similar profiles. In the case of DPE, only one band with a high molecular weight (>100 kDa) was observed in the SDS-PAGE, under both reducing and non-reducing conditions. Furthermore, molecular weight distribution showed a significant modification of the chromatographic profile of the DPE compared with that of the NE and the DE. DPE had a low number of peaks with a prominent peak around 2200 kDa, which is consistent with efficient protein polymerisation. The chromatographic profile showed high consistency between the different batches. With respect to proteolytic activity, there was a qualitatively important decrease in the DPE compared with that of the DE and the NE in the zymogram gel.

With respect to allergen characterisation, the presence of all described dog allergens was confirmed in the NE and the DE by MS ([Table 1](#) and [Supplementary material](#): Table). Can f 8 was not identified in the DPE. Bands from the non-reduced SDS-PAGE corresponding to 28 and 23 kDa were sequenced and identified as Can f 5 and Can f 1, respectively, by MS. Both major allergens were quantified using ELISA sandwich analyses. Mean levels of Can f 1 were 6.8 ± 0.6 µg/mg lyophilised in NE and 9.9 ± 0.8 µg/mg in DE, while mean Can f 5 levels were 0.93 ± 0.01 µg/mg in NE and 1.37 ± 0.30 µg/mg lyophilised in DE. Both allergens were unquantifiable in the DPE. Additionally, the presence of the major allergens Can f 1 and Can f 5 in NE and DE was demonstrated by Western blotting using specific antibodies ([Fig. 1B](#)).

The allergenicity of each extract was demonstrated by immunoblotting using varying sera from different dog dander allergic patients and ELISA competition assays ([Fig. 2](#)). The sera recognised several bands in the NE and the DE across the 10–100 kDa range with pool 1. on the other hand, serum 2 mainly recognised a band of 25 kDa. Furthermore, mean biological potencies of the NE and DE were 64.67 ± 4.93 and 93.33 ± 8.08 HEP/mg, and residual potency in the DPE was 1.43 ± 0.51 HEP/mg lyophilised. This shows that there was a 97.80% reduction in IgE binding capacity in the DPE compared with that of the NE.

BAT assays ([Fig. 3](#)) showed a significant reduction in the amount of

A) Protein profile



B) Allergen identification

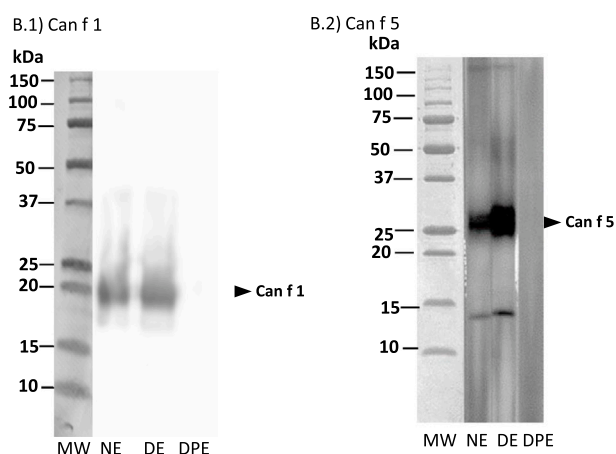


Fig. 1. Extract characterisation. MW: molecular Weight; NE: native extract; DE: depigmented extract; DPE: depigmented and polymerised extract. An example of each experiment is represented, the consistency was demonstrated analysing three different batches of each type of extract. A) Protein profile A.1) A.2) by SDS-PAGE (15%T-2.67%C): 100 µg of each type of extract in each lane A.3) Size exclusion high-performance liquid chromatography profile. A.4) Zymogram assay: 100 µg of each type of extract in each lane; white bands refer to proteins with proteolytic activity. B) Major allergens identification: detection with B.1) an anti-Can f 1 and B.2) an anti-Canine Prostatic Specific Esterase specific monoclonal antibodies under non-reducing conditions.

Table 1

Allergen identification. Mean values obtained in the three independent batches of each type of extract.

Allergen (Sequence)	Biochemical name	MW (kDa) ^a	Sequence coverage (%)		
			NE	DE	DPE
Can f 1 (O18873)	Lipocalin	23–25	59.96 ± 2.32	57.85 ± 6.93	48.85 ± 10.97
Can f 2 (O18874)	Lipocalin	19 or 27	45.37 ± 14.21	42.60 ± 13.79	32.96 ± 2.10
Can f 3 (P49822)	Serum albumin	69	56.63 ± 5.40	54.22 ± 6.25	40.68 ± 1.90
Can f 4 (D7PBH4)	Lipocalin	16 (non-red.) 18 (red.)	70.31 ± 2.66	67.43 ± 4.31	59.19 ± 3.20
Can f 5 (P09582)	Arginine esterase, prostatic kallikrein	28	16.28 ± 10.29	18.21 ± 10.62	14.36 ± 3.11
Can f 6 (H2B3G5)	Lipocalin	27 and 29	32.46 ± 7.01	29.47 ± 3.65	26.14 ± 1.21
Can f 7 (Q28895)	Epididymal Secretory Protein E1, or Niemann Pick type C2 protein	16	40.72 ± 10.53	39.15 ± 14.46	20.58 ± 0.39
Can f 8 (F1PHB6)	Cystatin A	11	52.04 ± 0.00	52.04 ± 0.00	–

^a By SDS-PAGE.

activated basophil cells after DPE stimulation compared with that after NE stimulation ($p < 0.05$) in blood from allergic patients. No extracts showed basophil activation in healthy controls. The clinical characteristics of the study population are shown in Table 2a.

3.2. Immunological studies

3.2.1. Humoral response

According to the humoral response, the immunisation of rabbits with DPE induced the production of specific IgG against dog dander extracts, as demonstrated by direct ELISA (Fig. 4A). The efficacy of these

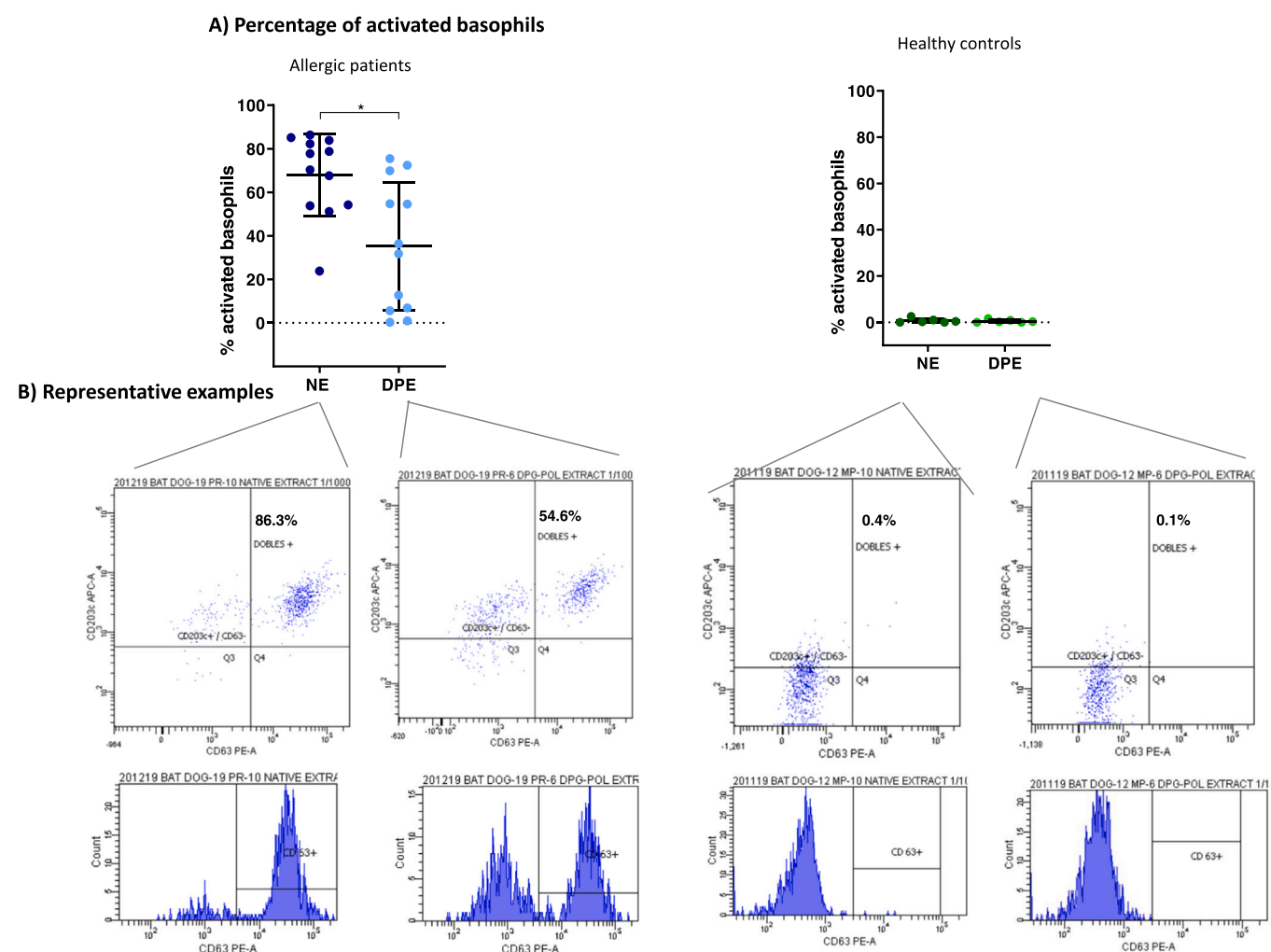
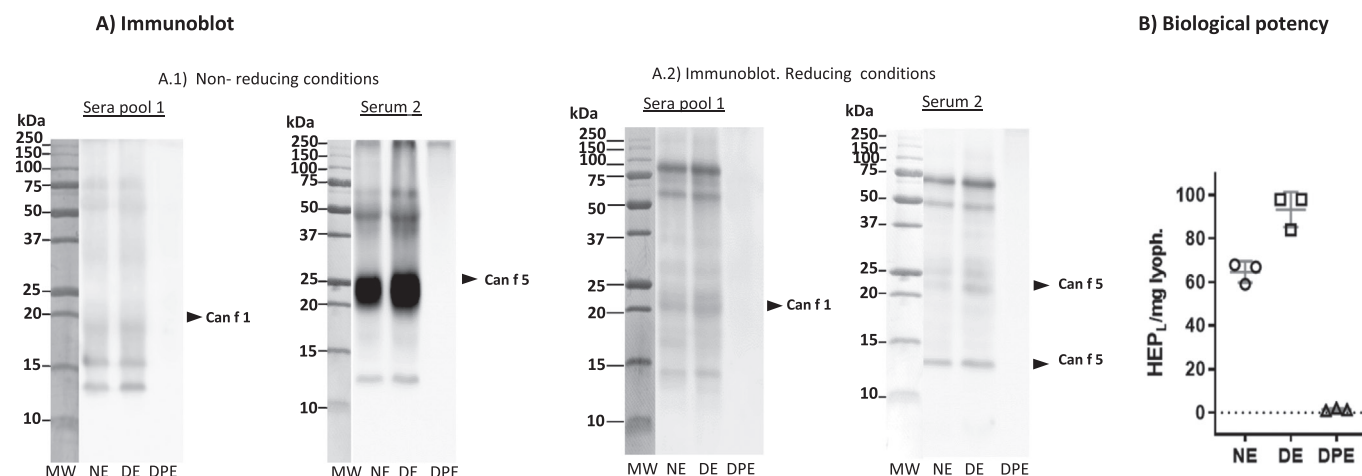


Table 2

Study populations.

A) Basophil activation test						
Group	N	Mean age (years)	Gender (female/male)	Clinical diagnosis	Skin Prick Test (mm)	Dog dander Specific IgE (kUA/l)
Healthy control	6	42 ± 13	67% / 33%	Non-allergic	-	< 0,35
Dog dander allergic patients ^a	12	39 ± 11	75% / 25%	25% rhinitis 75% rhinoconjunctivitis 58% asthma	6.00 ± 1.22	13.17 ± 16.82
B) Cellular response studies						
Group	N	Mean age (years)	Gender (female/male)	Clinical diagnosis	Dog dander Specific IgE (kUA/l) / % of sensitised patients	
Healthy control	4	39,5 ± 11	100% / 0%	Non-allergic	< 0,35	
Dog dander allergic patients ^a	8	39,5 ± 13	88% / 12%	100% rhinitis 50% conjunctivitis 50% asthma	18.80 ± 19.43	Can f 1: 4.39 ± 2,75 / (87.5%) Can f 5: 6.22 ± 8.48 / (75%)

^a Allergic patients were recruited according to the following inclusion criteria: a) being older than 18 years and b) having voluntarily signed the Study Informed Consent. Allergic donors were further selected based on: a) being diagnosed with rhinitis, conjunctivitis or rhinoconjunctivitis related to dog allergy, with or without allergic asthma, b) having a positive prick test for dogs, c) having dog-specific IgE titre > 0.35 kU/L and d) not having previously received specific immunotherapy with extracts of aeroallergens.

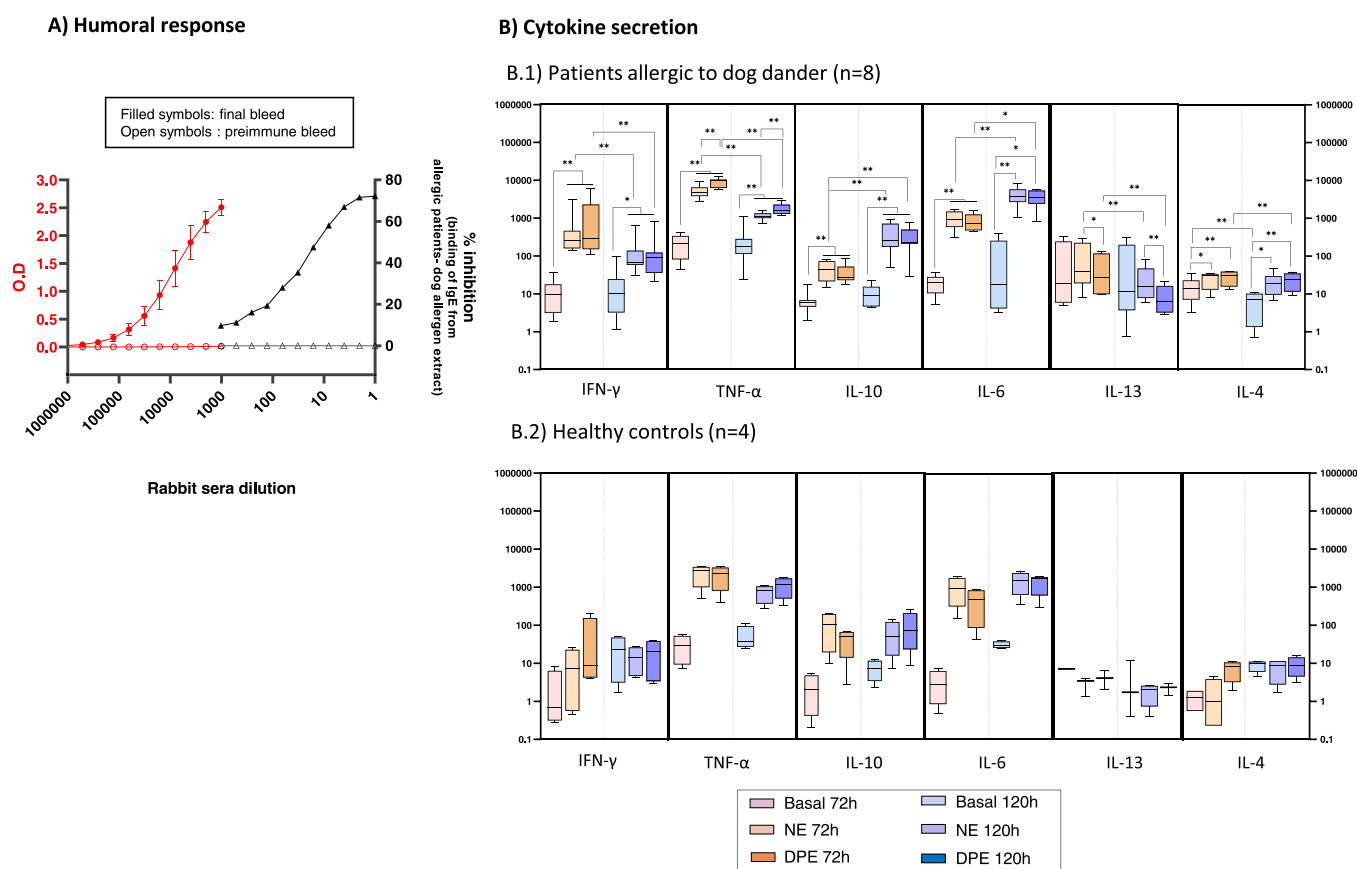


Fig. 4. Efficacy studies. A) Humoral response. Left Y axis (in red): Titration of specific IgG in preimmune or final bleed sera from rabbits immunised with dog dander depigmented-polymerised extract (DPE). Experiments were performed by duplicate (data are shown as mean ± SD). Right Y axis (in black): Block-binding human IgE-allergen capacity by sera from rabbit before and after immunisation with dog DPE. B) Cytokines levels in PBMCs culture supernatant. PBMCs from patients allergic to dog dander; N = 8 (B.1) and healthy controls N = 4 (B.2) were cultured in absence of stimulus (basal), dog dander native extract (NE) or DPE for 72 or 120 h. Data are shown as box (median and 25th to 75th percentile) and whiskers (min to max) plots. Dotted line separates data from 72 and 120 h. * p < 0.05, ** p < 0.01.

antibodies was demonstrated using ELISA competition assay which showed that the IgG antibodies blocked the binding of IgE antibodies (from sera of allergic patients) to the allergen extract by up to 72% (Fig. 4A).

3.2.2. Cell response

The *in vitro* efficacy of the DPE and the NE were further evaluated by analysing their capacity to modulate the Th1, Th2, and Treg responses in PBMCs from dog dander allergic donors. The clinical characteristics of

the study population are shown in Table 2b. Most patients were sensitised to Can f 1 (87.5%) and Can f 5 (75%). From the cytotoxic assays, 25 µg/ml of both the NE and the DPE were selected to perform cytokine release experiments (data not shown).

In the case of allergic donors, there was a significant increase in the release of the Th1 cytokines, IFN-γ and TNF-α, median levels after PBMC stimulation with NE and DPE, compared with that observed in case of unstimulated cells. This increase was significantly higher after 72 h of treatment with both products (Fig. 4 B1) (p < 0.01), being IFN-γ median

levels: 9.43 (range 1.87–36.93) in absence of stimulation; 248.68 (138.46–3035.12) for NE stimulation and 296.46 (112.05–5492.57) for DPE. In the case of TNF- α there were an increase of 22 and 45 fold for NE and DPE, respectively, compared to basal conditions ($p < 0.01$). With respect to the Th2 cytokines, both compounds induced a significant decrease in the IL-13 levels of PBMCs from allergic donors. This effect was more pronounced after incubation with DPE than NE stimulation 1.4 fold change in the case of 72 h of analysis and 2.5 for 120 of stimulation ($p < 0.01$ in each case) and was not observed for IL-4 under the present experimental conditions.

The regulatory response, measured as the increase in cytokine IL-10 release, was significantly higher after incubation with both products at both time points. This increase was more pronounced (more than 30 times) after 120 h of incubation ($p < 0.01$) (Fig. 4B).

The induction of IL-6 after incubation with both products at both time points was statistically significant ($p < 0.01$), although the increase was more evident after incubation with NE for 120 h (3794.32; 1057.46–8011.23) (Fig. 4B).

In allergic patients, the individual Th1/Th2 ratio (based on IFN- γ /IL-13) and Treg/Th2 ratio (based on IL-10/IL-13 and IL-10/IL-4) increased after stimulation with both compounds, being more marked with DPE (Supplementary material: Fig).

Overall, the immunological response profile to both NE and DPE was among the two groups of study, though the response was less intense and not statistically significant in the case of healthy controls.

4. Discussion

Domestic animals, mainly dogs and cats, are one of the most significant causes of perennial allergies. However, current immunotherapy of dog allergy has several drawbacks related to the limited clinical efficacy. This is mainly due to high variability in the allergenicity of the different available allergen extracts, which is, in turn, a result of the non-standardised presence of dog allergens.

The selection of the most appropriate raw materials for the development of allergen extracts has been extensively discussed (Wintersand et al., 2019; Calzada et al., 2020). This selection is mainly influenced by the wide distribution of allergens across different anatomical sites and the high variability in dog fur, urine, and saliva extracts, with significant variations in dog allergens, regardless of breed, having been previously demonstrated (Wintersand et al., 2019; Calzada et al., 2018; Smith et al., 2016; Liccardi et al., 2017). Moreover, a recent report showed individual differences between allergenic profiles in urine extracts of same breed and similarly aged male dogs (Calzada et al., 2020). In this sense, this issue seems to be directly correlated with the efficacy of the vaccines and remains a pressing concern.

In the present study, we show the development and characterisation of a new depigmented allergoid manufactured using dog dander as raw material, which is the most common source in allergies caused by animals (Fernández-Caldas et al., 2017). It has been proved that allergen extracts prepared from dander contain more IgE binding allergens than other starting materials (Chan and Leung, 2018), including major allergens Can f 1 and Can f 5 among others, since allergens are typically localised in the skin, saliva, or urine and tend to stick to fur. The allergens, thus, efficiently disperse into the environment, for example, with the normal hair loss of animals bearing these allergens (Zahradnik, Raulf, 2017; Wintersand et al., 2021), making fur the most complete allergen source. Here, we investigated the consistency of the manufacturing process in terms of protein content, protein profile, the presence of all dog allergens described thus far and the quantification of Can f 1 and Can f 5. Then, three independent batches of intermediate stages (NE, DE) and the final immunotherapy product (DPE) were manufactured from three independent dander raw material batches.

Several studies have demonstrated the safety profile and multiple benefits of using allergoids to treat allergic patients, thereby relieving the main symptoms and decreasing the inflammatory response in the

respiratory airway, which is associated with a better quality of life (Chan and Leung, 2018; Zahradnik and Raulf, 2017; Wintersand et al., 2021; Mösges et al., 2019; Carnés et al., 2018; Pfaar et al., 2013, 2015). In the case of depigmented-polymerised extracts, it is widely known that the polymerisation process is the most common method for the reduction of allergenicity (Pfaar et al., 2015; Morales et al., 2017). In this sense, the allergoid used in this study led to reduced number of exposed IgE-binding sites. The biological-potency loss in the DPE with respect to the NE was confirmed to be higher than 90%. The functional implications of this reduced capacity were demonstrated by the BAT. The activation of basophils in the peripheral blood of allergic patients was lower upon DPE stimulation than upon NE stimulation. These results reinforce the importance of these cells in the immune regulation of allergen immunotherapy (Chirumbolo et al., 2018). The low stimulation of these effector cells implies that the allergoid had low allergenicity but maintained its immunogenic capacity in accordance with its humoral response. This fact was also demonstrated with other depigmented-polymerised extracts, not only in the case of epithelium like cat dander (Morales et al., 2017), but also in mites (Gallergo et al., 2010) and fungi (Morales et al., 2018).

With respect to *in vitro* efficacy, the dog allergoid induced a strong IgG antibody response in the animal model. More importantly, the model showed how these IgG antibodies block human IgE-allergen interactions, resulting in an efficient method for the control of allergenic response (Mitsias et al., 2020). From a clinical point of view, AIT plays an essential role in changing antibody isotypes, where IgG antibodies are considered to be non-inflammatory or protective antibodies. IgG can act as a blocking antibody, competing with the antigen-binding IgE present on the surface of mast cells and basophils, thereby limiting their activation and degranulation (Calzada et al., 2018). Furthermore, several studies have shown that a decrease in allergen-specific IgE in serum is associated with an increase in IgG4 levels; therefore, the IgG4:IgE ratio in serum is generally studied as an alternative biomarker to monitor the efficacy of immunotherapy (Shamji et al., 2017a, 2017b). Another important aspect of efficacy is the capacity of the immunotherapy to induce immune tolerance, marked by the change in different specific T-cell populations (Komlósi et al., 2020; Boonpiyathad et al., 2020). With regards to this, the present study provides extensive information on how this modified allergen extract induces immune deviation, thereby reducing the proliferation of Th2 cells towards a specific tolerance response induced by cytokine secretion. In the present study, major changes, after both 72 and 120 h of stimulation with NE and DPE, were observed in the levels of IFN- γ , TNF- α , IL-10, IL-6, and IL-4 in allergic patients who were sensitised to Can f 1 and Can f 5 (Table 2b). The highest levels of IFN- γ and TNF- α were found after 72 h of stimulation, whereas the highest levels of IL-10 were found after 120 h. Modification of the levels of these cytokines as molecular mechanisms involved in the use of allergoids have been previously described. It is suggested that the antigen-uptake mechanisms mediate the redirection of antigen presenting cells toward an immunoregulatory response. IFN- γ secreted by Th1 cells is considered to be an important modulatory molecule in allergic response control. The role of IL-10 in tolerance and allergic responses has been extensively studied, also in the case of depigmented and polymerised extracts (Morales et al., 2017, 2018), and its protective role is well-established (Boonpiyathad et al., 2020; Drazdauskaitė et al., 2020). IL-10 is considered to be an immunosuppressive cytokine and plays an essential role in protecting against exaggerated inflammatory responses to allergens (Pfaar et al., 2019; Calzada et al., 2019). With respect to Th2 response, the levels of IL-13 were higher after stimulation with NE than with DPE. The median levels of this cytokine, as well as those of IL-4, showed greater reduction at 120 h than at 72 h of cell culture, suggesting a reduction of this inappropriate Th2 response in favour of Th1 and regulatory response, as was confirmed when the Th1/Th2 and Treg/Th2 cytokine ratios were studied (Supplementary material: Fig). The patients in the present study were sensitised to Can f 1 and Can f 5, and the presence of these allergens was demonstrated and

– standardised in our allergoid. PBMC response in patients showed an activated immunoregulatory response, which can lead to the development of an effective treatment strategy that addresses some of the current limited efficacy issues in the treatment of dog allergies. In contrast, in non-allergic subjects, neither the NE nor the DPE induced a statistically significant modification for the secretion of any cytokine, further confirming the safety of the immunotherapy.

In summary, the new dog dander allergoid demonstrated potent immunological response in patients sensitised to the major allergens Can f 1 and Can f 5. It produced IgG blocking antibodies and a change in the Th2/Th1 and Treg cytokines balance in favour of a tolerogenic response. Moreover, the allergoid displayed a significant reduction in IgE binding capacity. Both parameters suggest that the product might be an optimal immunotherapy strategy for treating dog dander allergy and might be able to address the limited clinical efficacy of the current treatments.

CRediT authorship contribution statement

DC, TA, MG, MR, DB and JC contributed to the design of the work, analysis and interpretation of data, drafting the work, final approval and agree to be accountable for all aspects of the work. JA contributed to the acquisition of data, revising the manuscript, final approval and agree to be accountable for all aspects of the work. CM and MS contributed to design and perform PBMCs studies, review the manuscript critically and final approval. MAO and EdO contributed to perform mass spectrometry assays, the subsequent sequence analysis, review the manuscript critically and final approval. MP contributed to design and perform basophil activation test, review the manuscript critically and final approval.

Declaration of Competing Interest

DC, TA, MG, MR, DB, JA and JC are employees of LETI Pharma. CM and MS report support from LETI Pharma during the conduct of the study. EO, MAO and MP have no conflicts of interest regarding the content of this manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.molimm.2022.02.020](https://doi.org/10.1016/j.molimm.2022.02.020).

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