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Quantitative assessment of immediate cutaneous hypersensitivity in a mouse model exhibiting an IgE response to Timothy grass allergens

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Background:	A variety of allergic reactions can be induced in mice, as measured by the induction of specific IgE. Functional read-out parameters include skin reactions and airway constriction. The aim of this study was to establish an improved quantitative assessment of the immediate cutaneous hypersensitivity reaction in a mouse model.
Material/Methods:	Timothy grass pollen was used to sensitize BALB/c mice, which were monitored for hypersensitivity reactions by Western blot analysis of the IgE response, determination of specific immunoglobulin subclasses by ELISA, and a skin test. The immediate cutaneous hypersensitivity reaction (ICHR) was assessed by pretreating the animals with an intravenous injection of Evans blue before anesthesia and provoking a reaction by intradermal application of the allergen. Ensuing blue coloring at the injection site could be seen as an indication of mast cell degranulation and the areas of 'wheal and flare' reaction were documented for digital imaging and quantitative analysis.
Results:	Analysis using an antigen-specific ELISA showed that sensitization with pollen extract induced the production of specific IgE, IgG1, IgG2a and IgG2b antibodies. Western blot analyses demonstrated the production of specific IgE antibodies analogous to the IgE reactivity pattern found in allergic patients. The production of IgE was concurrent with a positive ICHR to these allergens. Quantitative analysis demonstrated that the area of ICHR increased in size in correlation to the dose of allergen applied.
Conclusions:	This model and the mode of skin reactivity analysis may serve as a tool to evaluate new hyposensitization and therapeutic strategies for pollen allergy.
key words:	BALB/c mice • immediate cutaneous hypersensitivity reaction • Phleum pratense • pollen extract • quantitative analysis
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BACKGROUND

More than 20% of the population in the Western hemisphere suffers from symptoms of type I allergy [1]. Substances inducing this type of allergy are often inhalant allergens from house dust mites, animal dander, or grass and tree pollen. Conventional immunotherapy is performed by subcutaneous injection of high doses of complete natural allergen extracts; however, these large amounts of complete allergens can lead to anaphylactic side reactions, posing a risk for the patients thus treated. Furthermore, the mechanism of this empirically discovered immunotherapy is only partly understood, and, especially for type I allergy to house dust mites, animal dander, and pollen, it is not always successful. Consequently, new concepts are needed for successful immunotherapy of type I allergy.

A recent study has shown that the highest IgE (immunoglobulin E) responses among 209 patients demonstrating grass pollen allergy were observed against *Poa pratensis*, followed by *Festuca rubra*, *Phleum pratense*, and *Dactylis glomerata*, with 57% of the patients showing IgE reactivity to the two *P. pratense* major allergens Phl p 1 and Phl p 5 [2]. We have established a mouse model, which demonstrates hyperreactivity reactions to the clinically relevant allergens of *Phleum pratense* pollen extract [3], in order to create a foundation for testing new hyposensitization and therapeutic strategies.

The antigens used to sensitize mice are rarely chosen from the array of clinically relevant allergens, and one of the most widely used animal models uses ovalbumin for sensitization [4]. In the ovalbumin model, parameters for assessing functional disease-relevant symptoms are the development of ICHR [5] and increased responsiveness of airway [6] and airway smooth muscle [7]. In the assessment of ICHR and smooth muscle responsiveness, a method is used which requires the sacrifice of the animals [5, 7], and measurement of increased airway responsiveness requires a complicated procedure and expensive equipment [6]. Thus a further quantifiable functional assay for hypersensitivity reactions, which is easy to perform and does not require the sacrifice of the animals but rather leaves them available for further investigations, would be a valuable complementary tool.

In this report, a method was devised for the *P. pratense* pollen allergy mouse model, which allows for an easy quantitative assessment of immediate cutaneous hypersensitivity reactions and can be implemented for the evaluation of desensitization and therapeutic strategies.

MATERIAL AND METHODS

Eight-week-old female BALB/cAnNCrIBR mice were obtained from Charles River Inc, Extertal, Germany, and were certified free of viral, bacterial and parasitic infections by the supplier. The animals were used for the experiments after an acclimatization period of at least one week. The mice were housed in the Research Center Borstel animal care unit with a 12:12 h light:dark cycle (lights on 6:00 h), temperature $22 \pm 1^\circ\text{C}$ and a relative

humidity of 50% in polycarbonate cages (dimensions, 40×25×15 cm, l×b×h) with softwood fiber bedding (ssniff Spezialdiäten GmbH, Soest, Germany) in groups of five mice/cage. The mice were given ozone-treated germ-free water and ssniff R/M-H complete maintenance diet (ssniff Spezialdiäten GmbH, Soest, Germany) *ad libitum*.

The experiments were carried out with the approval of governmental authorities (Animal Protection Authority of the Ministry of Environment, Kiel, Germany, #1252-72241.123-3(4-1/01)) strictly according to the animal welfare regulations contained in German national legislation and controlled by the Länder ministries.

Two groups of BALB/c mice (treated and control; five or ten mice per group as indicated) were sensitized systemically at intervals of one week by five intraperitoneal (i.p.) injections of 100 μg *Phleum pratense* pollen extract (PE) (ARTU Biologicals, Lelystad, The Netherlands), prepared as previously described [8] and absorbed to 50 μg hydrated aluminum hydroxide gel (WDT, Garbsen, Germany) in a total volume of 200 μl phosphate buffered saline (PBS: 137 mM NaCl, 3.9 mM KH_2PO_4 , 16 mM $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$). Control animals were treated according to the same regimen, excluding the pollen extract. Before the first and seven days following each inoculation, tail vein blood – two filled 75 mm long, 1.3 mm diameter hematocrit capillaries (Hirschmann Laborgeräte, Eberstadt, Germany), corresponding to 120 μl /mouse – was collected for preparation of sera, which were frozen at -20°C until further analysis.

Pollen extract specific immunoglobulin levels were determined by ELISA, using pollen extract-coated plates (NUNC Maxisorp F 96, #44 2404, Nunc, Wiesbaden, Germany). Murine serum immunoglobulins were detected after binding with the respective biotinylated or alkaline-phosphate coupled specific anti-mouse immunoglobulin reagent (rat anti-mouse IgG1, clone A85-1; rat anti-mouse IgG2a, clone R19-15; rat anti-mouse IgG2b, clone R12-13, rat anti-mouse IgE, clone R35-92; all from BD-Biosciences Pharmingen, Heidelberg, Germany). Bound detection antibody was visualized using streptavidin-coupled alkaline phosphatase (#016-050-084, Dianova, Hamburg, Germany) and p-nitrophenyl-phosphate (Sigma, Deisenhofen, Germany) as the substrate. Extinction at 405 nm was measured in an ELISA reader. Statistical analysis was performed using the Student's t-test for independent samples. A value of $p < 0.05$ was considered to be significant.

The IgE-specific response against PE allergens was analysed by Western blot. PE (4 μg) was separated by 12.5% SDS-PAGE and transferred onto nitrocellulose (NC) membranes (pore size 0.2 μm , Roth, Germany) by semi-dry blotting. NC membranes were incubated with the sera and binding of mouse IgE was detected using a rat-anti-mouse IgE monoclonal antibody (clone R35-72, BD Biosciences-Pharmingen, Heidelberg, Germany) followed by incubation with an alkaline phosphatase (AP) conjugated secondary goat anti rat antibody (Dianova, Hamburg, Germany) and NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) as the

chromogenic substrate for visualization. As a direct comparison, blots were also incubated with sera from patients allergic to Timothy grass, which were collected by informed consent and approved by the local ethics committee from grass pollen-allergic patients with a clinical history of hay fever and EAST classes ≥ 3 (EASTRV; Allergopharma, Reinbek, Germany) using *P. pratense* extract. IgE reactivity was detected with an AP conjugated anti-human IgE antibody (Allergopharma, Reinbek, Germany).

IgE reactivity to the major allergens of Timothy grass was shown to be present after 5 i.p. inoculations in all mice [3] and this was verified and confirmed before performing a skin test. One week before skin-testing, the animals were anaesthetized by intramuscular injection of 25 μ l of a mixture of Ketamine® (WDT, Garbsen, Germany) (45 mg/kg body weight) and Rompun® (Bayer AG, Leverkusen, Germany) (8 mg Xylazine Hydrochloride/kg body weight) and challenged intranasally with 5 μ g Timothy pollen extract in PBS using a 10 μ l micropipette. In order to visualize the possible effects of mast cell degranulation and consecutive 'wheal and flare' reactions in the skin, the fur of the abdomen was gently removed the day before the test using a depilatory cream (WDT, Garbsen, Germany). Fifteen minutes before the test, the animals were injected intravenously (i.v.) with 100 μ l of a 0.5% Evans Blue/PBS solution and were anaesthetized as described above. The allergens to be tested were dissolved in 0.9% NaCl and injected intradermally (i.d.) in a total volume of 20 μ l at different doses with ample space between the injection sites to avoid confluency of the reactions. The histamine releasing factor compound 48/80 (20 μ l of a 33 μ g/ml solution in 0.9% NaCl; Sigma, Deisenhofen, Germany) served as a positive control and 0.9% NaCl as a negative control. In a preliminary assessment of the appropriate allergen concentration using 5 mice per group, a range of 100, 250, 500 and 750 ng allergen was applied i.d. The reactions as seen by bluing of the skin around the injection sites were assessed 15 minutes after the injection of the allergen and were documented by photography. The results of this first skin test demonstrated that less than 100 ng would be more suitable for the evaluation of ICHR, since the bluing reactions spilled into each other and the areas were not amenable to quantitative evaluation. Thus a second experiment using 5, 25, 50 and 75 ng of PE and ten mice per group was performed.

To quantify the results, digitalized images of the photographs were generated, making sure that the images were of the same magnification and resolution (dpi). The blue colored skin reaction areas were selected using Adobe Photoshop (version 6.0) and rendered into grayscale TIFF-images for area analysis using Scion Image freeware (Scion Corporation, Maryland, USA, www.scioncorp.com).

Statistical analysis

Statistical analysis was performed using the Student's t-test for dependent samples, and a value of $p < 0.05$ was considered to be significant.

RESULTS

The IgE-response of PE-sensitized mice was evaluated by immunoblotting of pollen extract and was compared to the IgE-reactivity found in patients suffering from Timothy grass allergy. None of the control PBS-treated mice exhibited an IgE response to PE, whereas all sensitized animals showed an allergen-specific IgE response to different components of grass pollen after the fifth sensitization, as demonstrated by the binding pattern on the blot (Figure 1A) and as shown before [3]. This pattern was similar in the mice and the three patients except for one prominent band, which was not present in the mice and in one of the patients.

The analysis of anti-pollen extract specific antibodies of different isotypes using an antigen-specific ELISA showed that sensitization with PE induced significant production of specific IgE, IgG1, IgG2a and IgG2b antibodies ($p < 0.05$, Figure 1B).

Skin testing was performed in order to visualize effects of mast cell degranulation, and the analysis demonstrated that control mice sensitized with buffer showed an ICHR response only to the positive control, as represented by the intradermal injection of compound 48/80. The PE-sensitized mice treated i.d. with 5, 25, 50 and 75 ng allergen showed the ICHR pattern demonstrated in Figure 2A: a strong reaction with the positive control, little or no reaction with the negative control, and apparently a reaction correlating in area to the varying amounts of allergen applied in the test reactions. In order to consolidate this observation a quantitative analysis of the bluing reactions was performed as described in the methods section. Figure 2B exemplifies the quantitative assessment of the area of individual ICHR spots from one experiment for 6 out of 10 different sensitized mice. The Evans Blue i.v. injection is a critical step in achieving successful ICHR, and since this failed in four mice these could not be included in the analysis. The applied concentrations of 5, 25, 50 and 75 ng correlated with an increase in area size of the ICHR in all six mice, lying between the ICHR areas obtained with the positive and negative controls and increasing steadily from 5 ng to 75 ng. Statistical analysis revealed that all applied antigen concentrations resulted in a positive skin reaction area significantly larger than the negative control ($p < 0.01$). The differences in area between increasing antigen concentrations were only significant between the 0 and 5 ng concentrations ($p < 0.01$) and the 25 and 50 ng concentrations ($p < 0.001$). There was a strong increase in skin reaction area size from 5 ng to 25 ng ($p = 0.14$) and from 50 ng to 75 ng PE, though this fell just short of statistical significance ($p = 0.054$).

DISCUSSION

A method was devised for a Timothy grass pollen extract hyperreactivity mouse model that allows the quantitative assessment of the size of the allergen-induced skin reactivity, which may serve as a tool to be implemented in the evaluation of studies investigating hyposensitization and therapeutic strategies of type I allergies. Allergy to

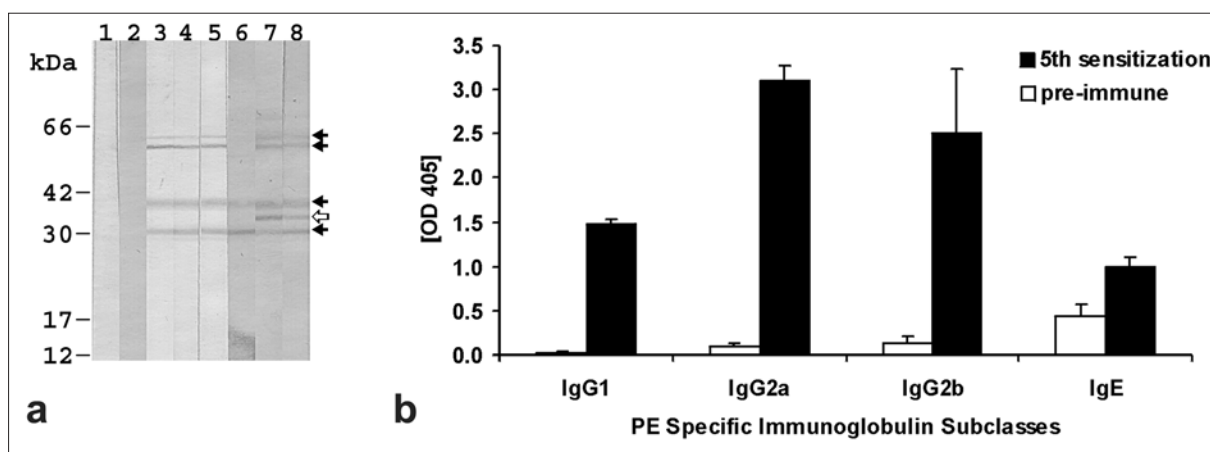


Figure 1A. Western blot analysis of IgE-specific antibodies against *Phleum pratense* antigens: Mice (3 out of a group of 5 are shown) sensitized with pollen extract (PE) exhibited an IgE-specific response to four different prominent antigens (lanes 3-5; black arrows), whereas non-sensitized PBS-injected control mice (n=5) showed no IgE-reactivity (a representative blot shown in lane 1). The sera from three different patients allergic to Timothy grass pollen showed a similar pattern (lanes 6-8; negative control lane 2) in IgE-reactivity as the sensitized animals except for one prominent band (lanes 7 and 8, open arrow) corresponding to Phl p 1 (see discussion). This reactivity was missing in the patient sera tested in lane 6, which additionally did not show reactivity with the upper two higher molecular weight antigens.

Figure 1B. Pollen extract specific immunoglobulin levels of the IgG1, IgG2a, IgG2b and IgE subclasses, shown as averages with standard deviation (n=5). Results of statistical analysis are given in the Results section.

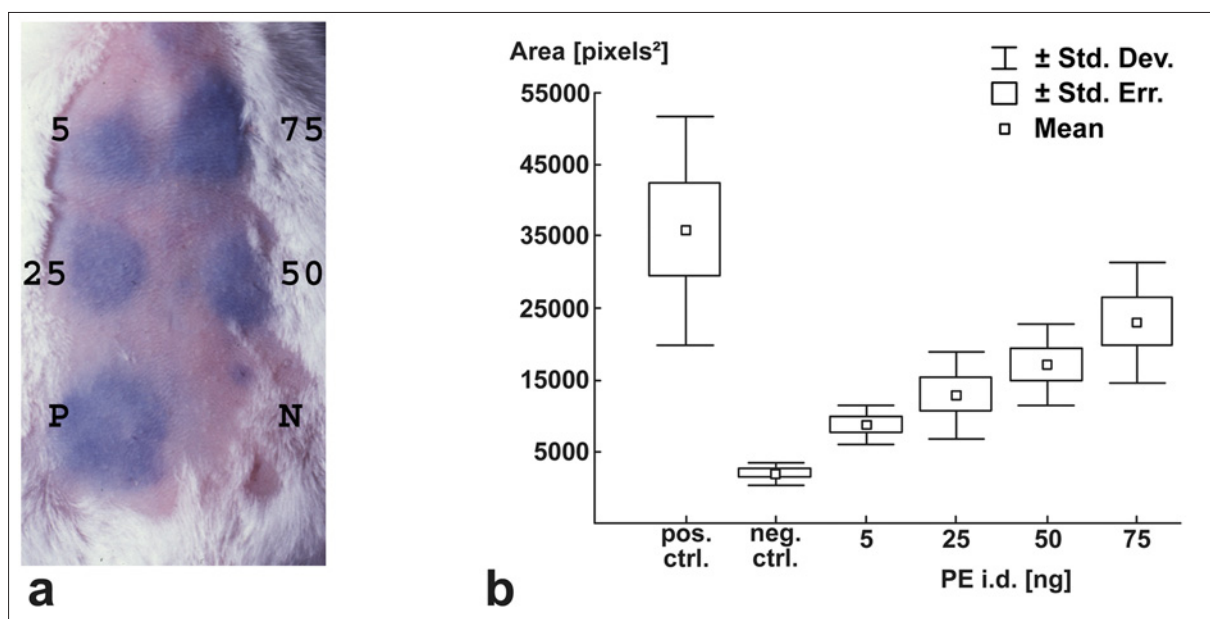


Figure 2A. Immediate cutaneous hypersensitivity reaction (ICHR) in mice sensitized with pollen extract: After the injection of Evans blue i.v., ICHR was induced by intradermal injections of PE (5, 25, 50 and 75 ng), the positive control 48/80 (P), and the negative control (N) as indicated. The bluing reaction was photographed 15 min after injection. Six out of ten mice included in the study responded in this fashion. Four of the ten mice did not react, since the Evans blue i.v. injection was not successful.

Figure 2B. Quantitative assessment of the area of ICHR in six mice: The area of ICHR in six out of ten sensitized mice was determined as described in detail in the Methods section. A clear increase in ICHR area is seen in correlation with an increased dose of PE injected i.d. Results of statistical analysis are given in the Results section.

airborne antigens such as grass pollens has become increasingly common in many countries during recent years, thus representing a significant cause of immediate type hypersensitivity, which may affect more than 20% of the population [1]. Apart from the irritating effects of hypersensitivity reactions for affected patients, allergy is

also acknowledged as a major risk factor for developing asthma [9], and the successful treatment of type I allergy is thus of interest. The development of therapeutic strategies for allergic disease requires appropriate models, and intensive research has been done to establish animal models that mimic the development of allergic

sensitization and the subsequent disease process. Specific IgE-responses to pollen extract from Timothy grass and to the recombinant Timothy grass allergens Phl p 1, Phl p 2 and Phl p 5 in mice has been demonstrated before [10, 11], and recently we could show that the components in PE to which an IgE-response occurs correspond to major allergens [3]. It may be concluded that PE induces a similar immune response with respect to the production of specific IgE-antibodies in BALB/c mice as in human patients, as demonstrated also by the analogous IgE reactivity pattern shown in this study, making this model very interesting for the investigation of the disease mechanisms of allergic type I reactions. The only difference seen in the IgE reactivity pattern concerns a band of approximately 37 kDa, which is present in most human patients (96% [12]) but not in the BALB/c mice and which corresponds to Phl p 1 [3]. The reasons for the missing induction of a specific IgE response against this protein is unclear and currently under investigation.

The analysis of the functional consequences of the production of specific IgE-antibodies to PE showed that the mice also develop an ICHR comparable to the skin prick test described for the human system [13,14]. Since none of the control animals produced specific IgE antibodies or exhibited a similar skin reaction as the sensitized animals, it is quite reasonable to suggest that the development of the skin reaction coincides with the production of allergen-specific IgE antibodies. This type of hyperreactivity in correlation to an IgE-response has also been demonstrated for other allergens (house dust mite extract [4], ovalbumin [5], recombinant mosquito salivary allergen rAed [15], birch pollen extract [16]), and is thus also a valid deduction in the system described here.

The quantitative assessment of ICHR demonstrated that an increase in area size of ICHR correlated with the allergen concentrations tested, and can serve as a parameter for ICHR evaluation. Other reports in the literature also describe ICHR reactions in mice. Saloga et al. [5] and Wang et al. [15] describe a similar approach to the one described here regarding the use of living animals. In contrast to the method presented here, however, they do not quantify the results, but rather set criteria for distinguishing a positive from a negative reaction. Daser et al. [16] describe a quantitative method, which however requires the sacrifice and skinning of the animal. The test described in the present paper therefore has several advantages: i) it is conducted on living animals allowing further experiments to be performed, ii) it is inexpensive and easy to perform, and iii) it produces an additional informative quantitative parameter for assessing hyperreactivity response. This parameter may also be seen as a complementary one to the assessment of lung function in mouse models. The small size of the animal requires a number of technical challenges to be overcome to measure lung mechanics, resulting in compromises being made between precision and noninvasiveness [17]. This in turn has an uncertain bearing on the response of the lung, thus also influencing the correlation of the data with changed lung function [17]. Accordingly, assessing the ICHR as described here may be considered as a tool to consolidate lung function data.

It should be emphasized that for ICHR measurement an optimal dose response must be determined for each sensitization protocol and allergen used, since high concentrations eventually lead to unquantifiable results due to confluency of the bluing reaction around the different injection sites (data not shown). The use of Evans blue for determining vascular leakage and estimating plasma volume is based on the fact that this non-toxic dye binds to serum albumin and thus is slowly cleared from the bloodstream [18]. In our experience the use of Evans blue has no pathological side effects on treated mice. Furthermore, in subsequent experiments we did not notice an influence of the Evans blue treatment in comparison to untreated mice.

CONCLUSIONS

The mode of sensitization with the clinically relevant allergens from *P. pratense* pollen extract, resulting in an IgE response analogous to that in the human system, along with the analytical approach chosen for the assessment of subsequent ICHR in BALB/c mice, should provide an interesting basis for devising and testing therapeutic strategies against Timothy grass allergy. As mentioned in the introduction, conventional immunotherapy is performed by subcutaneous injection of high doses of complete natural allergen extracts as introduced in 1911 by Noon [19], with the attendant risk of leading to anaphylactic side reactions. One approach discussed to improve desensitization side effects is the use of defined, recombinantly expressed allergens. In the case of Timothy grass allergens, a number of these have been cloned and are able to be recombinantly expressed [20–24]. Thus, these recombinant allergens could be assessed in an experimental setup as described in this investigation with respect to their potential usefulness in the hyposensitization of Timothy grass pollen allergy.

New concepts for successful immunotherapy of type I allergy are also under discussion, such as the destruction of conformational IgE-binding epitopes leading to reduced IgE-binding epitopes. Thus these allergens would not be able to crosslink FcεRI receptor-bound IgE on basophiles or mast cells, and, therefore, should not be able to induce negative side effects, such as histamine release or even release of IL-4 from basophiles [24–27]. Such mutants have been generated for the major allergen Phl p 5b of Timothy grass and have been shown to exhibit significantly reduced IgE reactivity and histamine releasing capacity, compared with the wild-type Phl p 5b. Furthermore, skin prick tests on allergic patients showed that the deletion mutants had a significantly lower potency to induce cutaneous reactions, while on the other hand retaining comparable proliferation of T cell clones after stimulation with allergen variants and wild-type Phl p 5b [28]. The mouse model and the method introduced in this report may be used to evaluate the hyposensitization potential or even prophylactic implementation of this IgE-reactivity depleted mutant.

A further promising approach for immunotherapy of allergy is DNA vaccination [29]. In fact, intramuscular injection of rats with a plasmid DNA encoding a house

dust mite allergen resulted not only in the induction of a specific immune response but also prevented the induction of IgE synthesis, histamine release and airway hyperresponsiveness in rats challenged with allergen [30]. Similar promising results were obtained in experiments with DNA-constructs containing birch pollen allergen, in which a humoral specific response but no specific IgE response was produced against this allergen [31]. An analogous approach for DNA constructs containing *Phleum pratense* allergens could be investigated in our model with respect to the therapeutic and prophylactic effects of such a DNA vaccine, and besides looking at the humoral response, also using the additional read-out parameter for functional effects using the skin test described.

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