



Immunoprophylaxis with DNA vaccination inhibits Th2-mediated responses and airway inflammation in mice sensitised with the major timothy grass pollen allergen Phl p 5b

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Abstract

DNA vaccination is discussed as having the potential of improving therapy of type I allergies. A mouse model was used to investigate the effect of DNA vaccination against sensitisation with the clinically relevant Phl p 5b major allergen from timothy grass pollen in an immunoprophylactic experimental setup. Pre-treating the mice with Phl p 5b-DNA-construct significantly inhibited the production of specific IgE and IgG₁ antibodies and prevented the development of a lung pathology associated with inflammation. Thus, immunoprophylaxis with an expression vector containing the Phl p 5b sequence totally prevented the emergence of characteristics associated with type I allergy and chronic bronchitis.

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1. Introduction

Grass pollen is one of the most frequent and potent elicitors of type I allergy worldwide and it has been reported that at least 40% of type I allergic patients are sensitised against grass pollen allergens [1]. Since hypersensitivity against these aeroallergens is an accepted major risk factor for asthma the development of therapeutic strategies against allergic diseases resulting from exposure to the major allergens of grass at an early stage is a goal to aim for. During specific immunotherapy (SIT) small but increasing doses of allergen extracts are subcutaneously injected in order to achieve a state of tolerance to subsequent allergen exposure. Although SIT is the only known curative treatment for pa-

tients suffering from type I allergy to airborne allergens [2] the application of allergen extracts bear the risk of inducing allergic reactions and anaphylactic shock [3,4]. Thus, research is focused on new approaches to provide a safer and more efficient form of specific immunotherapy. DNA vaccination, first described in 1990 by Wolff et al. [5], induces a strong Th1-mediated immune response against the encoded genes and is thus discussed as a means to protect against atopic disorders [6–13]. Immunostimulatory DNA sequences (e.g. CpG) in the vectors used are involved in evoking a predominant Th1-mediated immune response and thus in inhibiting a subsequent sensitisation with an allergen by weakening a Th2-mediated immune response [7,8]. These motifs can be administered in different ways, encoded with either an allergen complementary DNA sequence for production of the allergen protein in the host [6], given alone [14,15] or together with the allergen [16].

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The major allergenic components of grass pollen belong to the grass groups I and V allergens. The timothy grass (*Phleum pratense*) pollen major allergen Phl p 5b is responsible for 90% of the allergenic activity found in grass pollen sensitised patients [17] and is an aeroallergen which has no posttranslational modifications [18,19]. In this study a mouse model was used to investigate whether allergen-encoding DNA applied in a prophylactic approach could influence the immune response to subsequent sensitisation using Phl p 5b as a clinically relevant allergen. Previous investigations had demonstrated that mice develop hypersensitivity reactions highly analogous to the situation in human patients with respect to IgE reactivity against the major allergens of *P. pratense* pollen extract [20], whereby Phl p 5b proved to be the most dominant allergen as assessed by kinetics of specific IgE production [21]. To mimic the human corollary sensitisation process more closely, treatment of the animals with allergen was performed without aluminium hydroxide (AlOH₃; alum) as adjuvant for a Th2-mediated immune response. The study documents that immunoprophylaxis could inhibit the production of Th2-associated immunoglobulins and the appearance of pathologic alterations in the lung resembling chronic bronchitis.

2. Materials and methods

2.1. Construction of DNA immunisation vectors

An 891 bp fragment was amplified from *P. pratense* cDNA by PCR. The sequences of the 5' and the 3' primers were 5'-GCGCGCACGCGTATGGCCGATGCCGGCTACGCC-3' (*Mlu*I restriction site underlined) and 5'-GCGCGCTC-TAGAACACATACCGTACAGTAGTATG-3' (*Xba*I restriction site underlined) respectively, encompassing the entire phl p 5b sequence. The PCR product was digested with *Mlu*I and *Xba*I and cloned into the mammalian expression vector pCI (Promega, Mannheim, Germany) at the corresponding sites. The sequences of the construct were verified using an AbiPrism™ Genetic Analyser (Perkin-Elmer, Norwalk, USA). Plasmid propagation was performed in *Escherichia coli* XL1-Blue strain (Stratagene, La Jolla, USA).

Endotoxin-free plasmid purification was done using the EndoFree Plasmid Mega Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of DNA were analysed by absorption at 260 and 280 nm and by agarose gel electrophoresis. The LPS/endotoxin content of every batch of pCI and pCI/phl

p 5b plasmid DNA was determined with the Chromogenix Coatest Endotoxin Limulus Assay Kit (Haemochrom Diagnostika GmbH, Essen, Germany) and was found to be below 22.4 pg/ml resulting in less than 2.7 pg in the volume injected per mouse. This is below the level that affects cytokine activation in cell culture [22].

2.2. Expression control of pCI/phl p 5b in 3T3 mouse fibroblast cells

Isolated plasmid DNA was added to 100 µl Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Eggenstein, Germany), mixed with 10 µl Metafectene (Biontex, Munich, Germany) in 90 µl DMEM and incubated at room temperature for 20 min. The mixture was then added to 5×10^5 3T3 fibroblast cells growing on microscope slides and incubated at 37 °C/5% CO₂ for 24 h before immunohistologic analysis (see below). Treatment of animals was performed only after the expression of Phl p 5b by pCI/phl p 5b transfected 3T3 fibroblast cells could be confirmed (data not shown).

2.3. Animals

Female 8–10-week-old BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals were certified as pathogen and virus-free according to the supplier, were kept in a standard environment and fed with a maintenance diet and water ad libitum. Experiments were performed according to the national animal protection guidelines and were approved by the governmental authority (Animal Protection Authority of the Ministry of Environment, Kiel, Germany).

2.4. DNA vaccination and allergen sensitisation

Six groups of five BALB/c mice each were immunised by six intradermal (i.d.) injections of 20 µl each amounting to a total of 5 µg rPhl p 5b (Allergopharma, Reinbek, Germany), 50 µg pCI, 100 µg pCI, 50 µg pCI/phl p 5b, or 100 µg pCI/phl p 5b. These injections were performed at three different time points (see Fig. 1), and the control group was treated with 6 × 20 µl of saline each time. The groups of mice were termed according to these stated types of pre-treatment they had received. A subsequent intraperitoneal (i.p.) sensitisation with 200 µl of saline or 5 µg rPhl p 5b was performed at the time points indicated. All mice were challenged intranasally with 5 µg rPhl p 5b in 20 µl saline. Mice were sacrificed at day 139 to collect the lung for histological analysis.

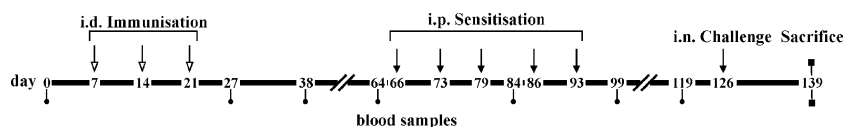


Fig. 1. Experimental set up of the immunisation and sensitisation strategy. Arrow marks indicate the days on which mice were treated with DNA or protein. Sera for the analysis of specific antibodies were taken at days marked with points.

Tail vein blood samples were taken at seven different time points according to Fig. 1 and aliquots of sera were frozen at -20°C until use.

2.5. Determination of antigen-specific IgE production by Western blot

Recombinant Phl p 5b was separated by SDS-PAGE ($T = 15\%$, $C = 4\%$) and blotted onto nitrocellulose (NC) membranes with pore sizes of $0.2\ \mu\text{m}$ (Schleicher & Schuell, Dassel, Germany). NC membranes were blocked with TBS-Tween (TBS-T: 1 M Tris, 1 M NaCl, 25 mM MgCl_2 , pH 7.4, 0.05% Tween 20), cut into strips and incubated overnight at room temperature with the respective 1:1000 diluted murine sera. After washing with TBS-T antigen bound serum IgE was detected with a horseradish peroxidase labelled rat anti-mouse IgE monoclonal antibody (LO-ME-2, 1:1000, Serotec, Düsseldorf, Germany). Before adding the Lumi-Light^{Plus} Western Blotting Substrate (Roche, Mannheim, Germany) blots were washed twice with TBS-T and TBS, respectively. To determine the luminescence blots were exposed for 120 s in a Chemi DocTM Documentation System (BioRad, Munich, Germany). Densitometric analysis was performed using Quantity One Software (BioRad, Munich Germany).

2.6. Determination of antigen-specific IgG₁, IgG_{2a} and IgG_{2b} production by ELISA

Mouse sera were analysed for IgG₁, IgG_{2a} and IgG_{2b} anti-Phl p 5b antibodies by using an ELISA. The values of the test sera were calculated from two different standard sera pools that were previously titrated. The standard serum for the IgG₁ ELISA was taken from mice sensitised with rPhl p 5b. The standard serum for the IgG_{2a} and IgG_{2b} ELISA was taken from mice immunised with the pCI/phl p 5b-DNA-construct. Both standard serum pools were assigned a value of 1×10^6 U.

For ELISA, 96-well microtitre plates (MaxiSorp, Nunc, Wiesbaden, Germany) were coated with rPhl p 5b at $0.5\ \mu\text{g}/\text{ml}$ in Tris buffered saline (TBS: 1 M Tris, 1 M NaCl, 20 mM MgCl_2) overnight at 4°C and then washed with TBS-T using the Elx405 washer (Bio-Tek Instruments Inc., Wirooski, USA). Non-specific binding sites were blocked with 1% bovine serum albumin (BSA, fraction V, ICN Biomedicals, Eschwege, Germany) in TBS-T at room temperature for 7 h. Test sera were serially diluted in TBS-T containing 0.5% BSA to determine the inflexion point. The respective standard serum was titrated on each plate. After overnight incubation at 4°C the plates were washed and incubated with alkaline phosphatase labelled goat anti-mouse IgG₁, IgG_{2a} or IgG_{2b} (Pharmingen, San Diego, USA) at 1:1000 dilution in TBS-T containing 0.5% BSA at room temperature for 1.5 h. After washing $1\ \text{mg}/\text{ml}$ *p*-nitrophenyl phosphate (pNPP, Sigma, Deisenhofen, Germany) in diethanolamine buffer (1 M, 0.5 mM MgCl_2 , pH 9.8) was

added. After 30 min absorbance at 405 nm was read with Expert 96 (Asys Hitech GmbH, Eugendorf, Austria).

2.7. Lung histology

Lungs were fixed overnight in 4% formalin at room temperature. After embedding in paraffin, $2\ \mu\text{m}$ tissue sections were stained with periodic acid-Schiff (PAS) using standard protocol [23,24] or Giemsa using Accustain (Giemsa Stain, modified from Sigma, Deisenhofen, Germany) following instructions provided by the manufacturer.

The stained sections of each mouse were analysed by light microscopy by two independent observers and pathologic alterations were graded semi-quantitatively on a scale of 0–3.

To determine the severity of goblet cell hyperplasia, bronchioles in each PAS stained section were counted. The appearance of no goblet cell per bronchiole scored '0', less than 25% '1', between 25 and 50% '2' and more than 50% '3'. The number of bronchioles exhibiting peribronchial infiltration were rated '0' for less than 15%, '1' for 16–30%, '2' for 31–45% and '3' for more than 45%. Based on severity and extent of mucus production and of eosinophils in infiltrates stained sections were scored '0' representing none, '1' mild, '2' moderate and '3' severe, respectively.

2.8. Immunohistology

Immunohistologic detection of the proliferation associated Ki-67 protein and the expression of Phl p 5b in transfected 3T3 cells were performed using the indirect peroxidase method.

For detection of the Ki-67 protein in paraffin sections of lungs with the MIB-5 antibody (Dako, Glostrup, Denmark) [25], antigen retrieval is necessary. Therefore, after dewaxing, sections were immersed in 10 mM citric acid, pH 6.0, and boiled at 95°C for 60 min in a water bath. Subsequently, slides were rinsed with TBS (10 mM Tris, 150 mM NaCl, pH 7.2–7.4), and endogenous peroxidase was blocked by incubating the slides in 3% H_2O_2 TBS for 20 min. Sections were then incubated with the primary MIB-5 antibody ($40\ \mu\text{g}/\text{ml}$) in 2% BSA/0.05% NaN_3 /PBS for 30 min.

For the detection of Phl p 5b in 3T3 cells slides were washed twice with phosphate buffered saline (PBS: 154 mM NaCl, 8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.5) and once with 10% foetal calf serum (FCS) in PBS. Afterwards the slides were dried at room temperature for 4 h before fixation in acetone and chloroform 15 min each. Slides were placed into wet chambers and incubated with anti-Phl p 5b monoclonal antibody Bo9 [17] in 10% FCS/TBS for 30 min.

In both cases, the secondary reagent (horseradish peroxidase-conjugated goat anti-mouse IgG (H + L, Dianova, Hamburg, Germany)), diluted 1:75 in 10% FCS/TBS, was applied for 30 min after rinsing with TBS. After a washing step samples were incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG (H + L, Dianova, Hamburg,

Germany), diluted 1:75 10% FCS/TBS for 30 min. Slides were developed with diaminobenzidine peroxidase substrate (D-4168, Sigma, Deisenhofen, Germany) in TBS for 10 min, counterstained with haematoxylin and mounted in Kaiser's glycerol gelatine.

The stained sections of each mouse were analysed by light microscopy. Based on the extent of proliferation observed in the bronchiolar epithelium stained sections were semi-quantitatively scored as '0' representing none, '1' mild, '2' moderate and '3' severe.

2.9. Statistical analysis

All data are expressed as means $\pm$ S.E. using SSPS[®] 11.0 software. One-way analysis of variance (ANOVA) was used to determine statistically significant differences between groups. Comparisons for all pairs were performed by Tukey-Kramer HSD and Bonferroni test. p -values <0.05 were considered statistically significant.

3. Results

3.1. Specific antibody titres after intradermal immunisation

To investigate the immune response after three intradermal injections with saline, rPhl p 5b, pCI or pCI/phl p 5b the quantity of allergen specific IgE, IgG₁, IgG_{2a} and IgG_{2b} was determined and is shown in Fig. 2. The control group injected with saline showed no production of any immunoglobulin subclass.

The mice treated intradermally with rPhl p 5b showed a marked increase of specific IgE 6 weeks after the third immunisation in comparison to all other groups, which did not notably produce specific IgE above background levels (Fig. 2A). Furthermore, these mice produced significant amounts of specific IgG₁ from day 6 after the third injection onward ($*p < 0.05$, Fig. 2B). In contrast, no specific IgG_{2a} (Fig. 2C) was detectable and the levels of specific IgG_{2b} increased

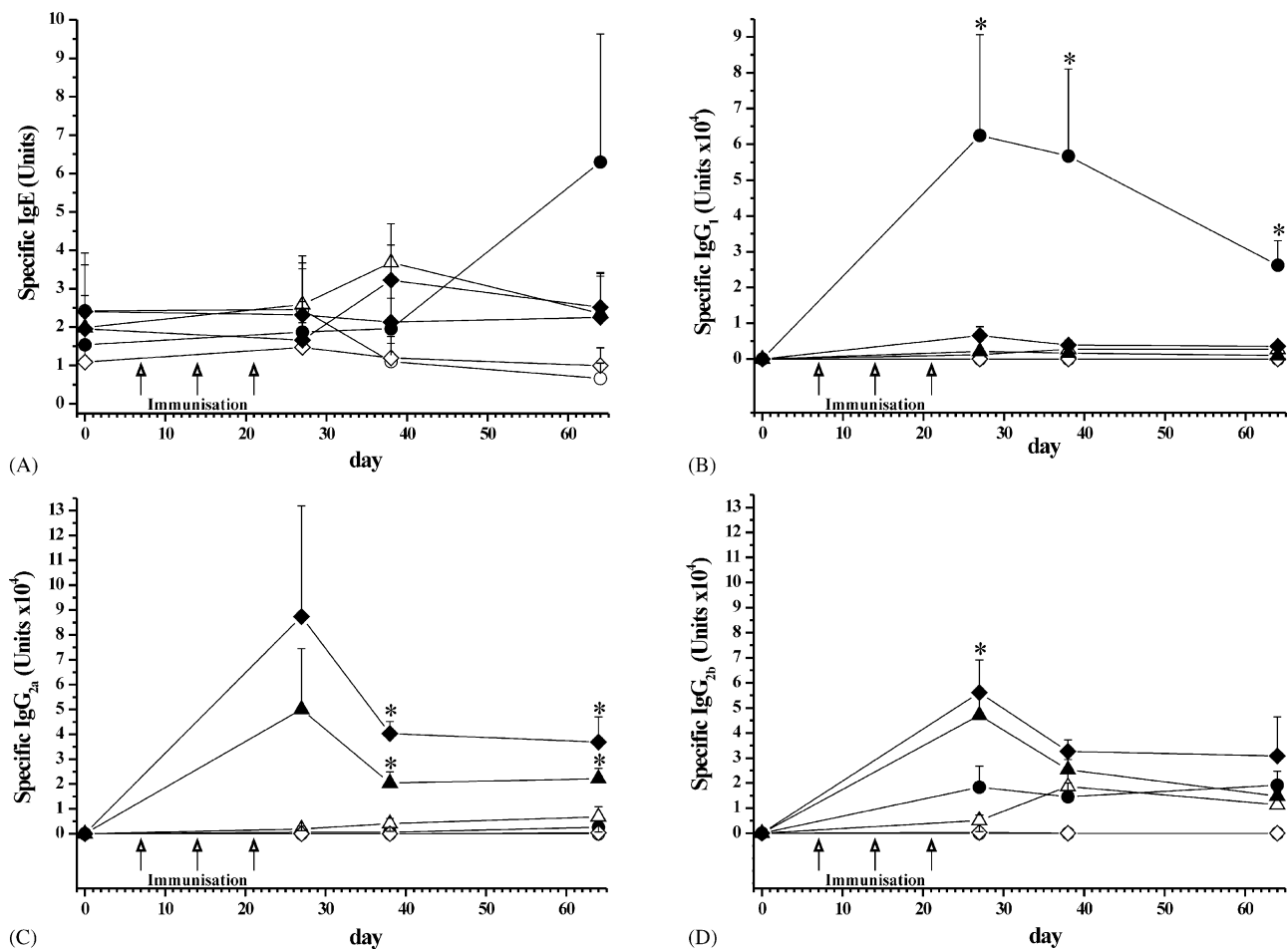


Fig. 2. Specific IgE, IgG₁, IgG_{2a} and IgG_{2b} responses of BALB/c mice to intradermal injection of saline (○), 5 µg rPhl p 5b (●), 50 µg pCI (△), 100 µg pCI (◇), 50 µg pCI/phl p 5b (▲) or 100 µg pCI/phl p 5b (◆). Each data point represents the mean $\pm$ S.E. of five mice per group. Significant differences in IgG₁ levels of mice immunised with rPhl p 5b (●) in comparison to all other groups were observed ($*p < 0.05$). IgG_{2a} levels were significantly higher in both pCI/phl p 5b treated mouse groups (▲; ◆) than in the other groups ($*p < 0.05$). Only the 100 µg pCI/phl p 5b immunised mice (◆) showed significantly higher IgG_{2b} levels than rPhl p 5b treated mice (●) at one time point ($*p < 0.05$).

marginally. These data infer that treatment with the recombinant allergen elicited a Th2-mediated response in this group of mice.

The mice treated with pCI vector control showed no specific immune response as detected by the production of Phl p 5b-specific IgE, IgG₁ or IgG_{2a} (Fig. 2A–C). For unexplained reasons a minor amount of specific IgG_{2b} production was detected in two out of five mice in the 50 µg pCI vector control group (Fig. 2D).

The two groups of mice treated intradermally with the pCI/phl p 5b showed a significant production of specific IgG_{2a} compared to the group injected with rPhl p 5b ($*p < 0.05$). The amounts of Phl p 5b-specific IgG_{2a} correlated with the dose of DNA-construct applied, since the 100 µg pCI/phl p 5b treated mice showed markedly higher antibody titres than the 50 µg pCI/phl p 5b group (Fig. 2C). An analogous kinetic was observed for specific IgG_{2b} levels in these two groups, although significant differences to the group injected with rPhl p 5b could be observed only in the mice treated with 100 µg pCI/phl p 5b at day 27 ($*p < 0.05$, Fig. 2D). The 100 µg pCI/phl p 5b but not the 50 µg pCI/phl p 5b treated mice showed a very slight production of specific IgG₁ antibodies (Fig. 2B). As measured by these different subclasses of antibody titres the intradermal application of the DNA-construct resulted in a pre-

dominant Th1-mediated response in these two groups of animals.

3.2. Specific antibody titres after i.d. immunisation and subsequent i.p. sensitisation

To analyse the protective effect of DNA vaccination all groups of the pre-treated mice except the saline control group were sensitised by five intraperitoneal injections with rPhl p 5b. Serum samples were collected at the time points indicated and levels of specific IgE, IgG₁, IgG_{2a} and IgG_{2b} were analysed (Fig. 3). There was no production of specific antibody of any immunoglobulin subclass in the control group injected with saline.

The rPhl p 5b pre-treated mice which exhibited a Th2-mediated response after the immunisation phase showed a highly significant increased production of rPhl p 5b specific IgE and IgG₁ ($**p < 0.0001$, Fig. 3A and B) during and after the sensitisation phase. These animals also showed no detectable amounts of specific IgG_{2a} (Fig. 3C) and only low levels of specific IgG_{2b} (Fig. 3D).

Compared to the group treated with allergen throughout the experiment the mice pre-treated with vector alone showed an 87% suppressed production of specific IgE and a 79% suppressed production of IgG₁ after sensitisation (Fig. 3A

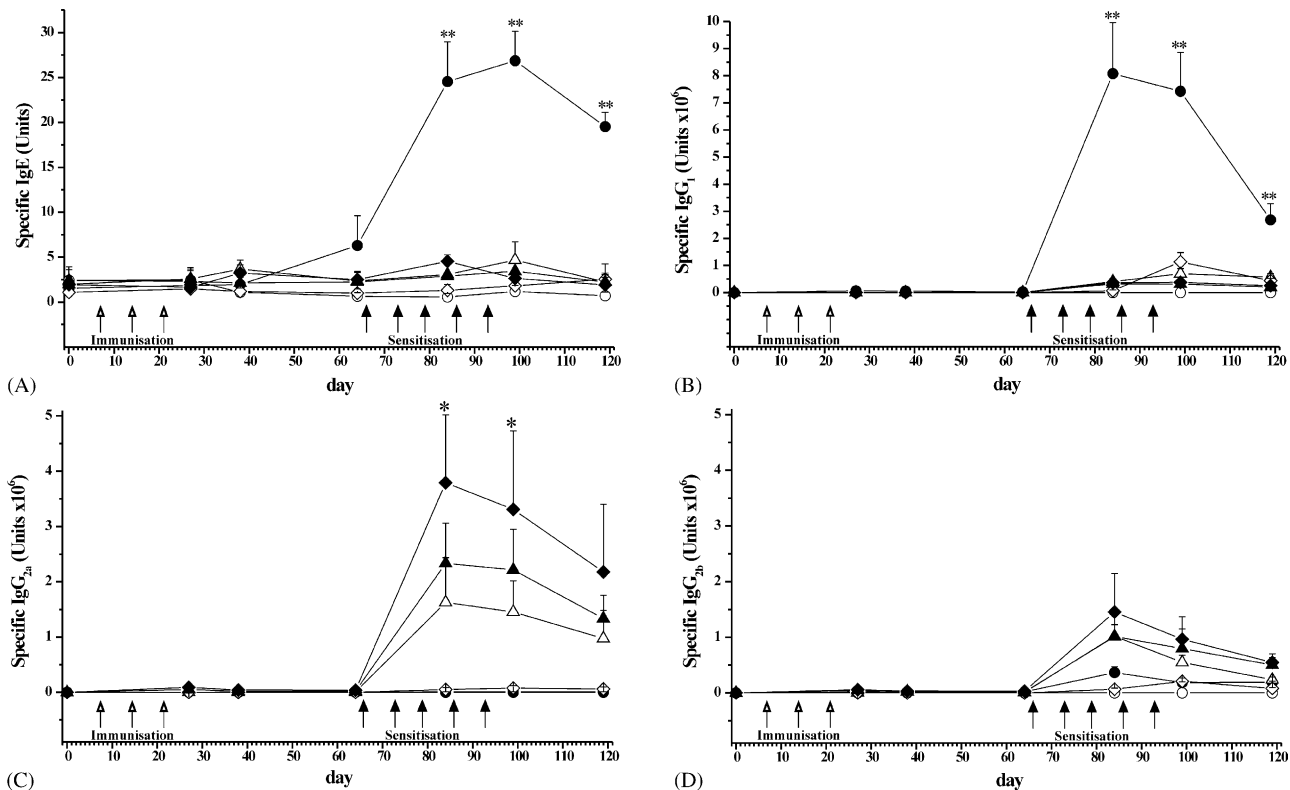


Fig. 3. Specific IgE, IgG₁, IgG_{2a} and IgG_{2b} responses of BALB/c mice pre-treated with saline (○), 5 µg rPhl p 5b (●), 50 µg pCI (△), 100 µg pCI (◇), 50 µg pCI/phl p 5b (▲) or 100 µg pCI/phl p 5b (◆) and then sensitised with five intraperitoneal injections of 5 µg rPhl p 5b each. Each data point represents the mean $\pm$ S.E. of five mice per group. Mice treated with rPhl p 5b throughout the experiment (●) showed significantly higher levels of IgE and IgG₁ compared to all groups ($**p < 0.0001$). Mice pre-treated with 100 µg pCI/phl p 5b (◆) before sensitisation developed significantly higher IgG_{2a} titres than the rPhl p 5b group (●) ($*p < 0.05$).

and B). In contrast to the group pre-treated with 100 μ g pCI, which exhibited no production of IgG_{2a} or IgG_{2b} antibodies, the group which had received 50 μ g pCI showed a marked increase of specific IgG_{2a} and IgG_{2b} production levels (Fig. 3C and D).

Mice, which had been pre-treated with the allergen DNA-construct pCI/pHl p 5b before sensitisation produced no detectable specific IgE and only minor amounts of IgG₁ antibodies (Fig. 3A and B). Both the 50 and 100 μ g pCI/pHl p 5b groups showed a very high increase of specific IgG_{2a} (Fig. 3C). Only the 100 μ g pCI/pHl p 5b group had significantly elevated levels compared to the rPhl p 5b and 100 μ g pCI pre-treated mice (* p < 0.05) after three and five intraperitoneal inoculations with rPhl p 5b. A comparable immune response with albeit lower levels was observed for the production of specific IgG_{2b} (Fig. 3D). For both the IgG_{2a} and IgG_{2b} subclasses the levels of antibody production correlated

with the concentration of DNA-construct used. Animals pre-treated with 100 μ g of the DNA-construct consistently produced more specific IgG_{2a} and IgG_{2b} than animals pre-treated with 50 μ g.

3.3. Pathologic alterations of the lung

Lung sections of each mouse were analysed after intranasal challenge with the allergen with regard to pathologic alterations resembling the histopathology of chronic bronchitis such as goblet cell hyperplasia, mucus production, peribronchial inflammation, eosinophilia and proliferating cells in bronchiolar epithelium. Representative results obtained from these analyses are depicted in Fig. 4.

In comparison to the control mice treated only with saline, where no obstruction of the alveolar system was present and the ciliated epithelium was normal (Fig. 4A–C), the group

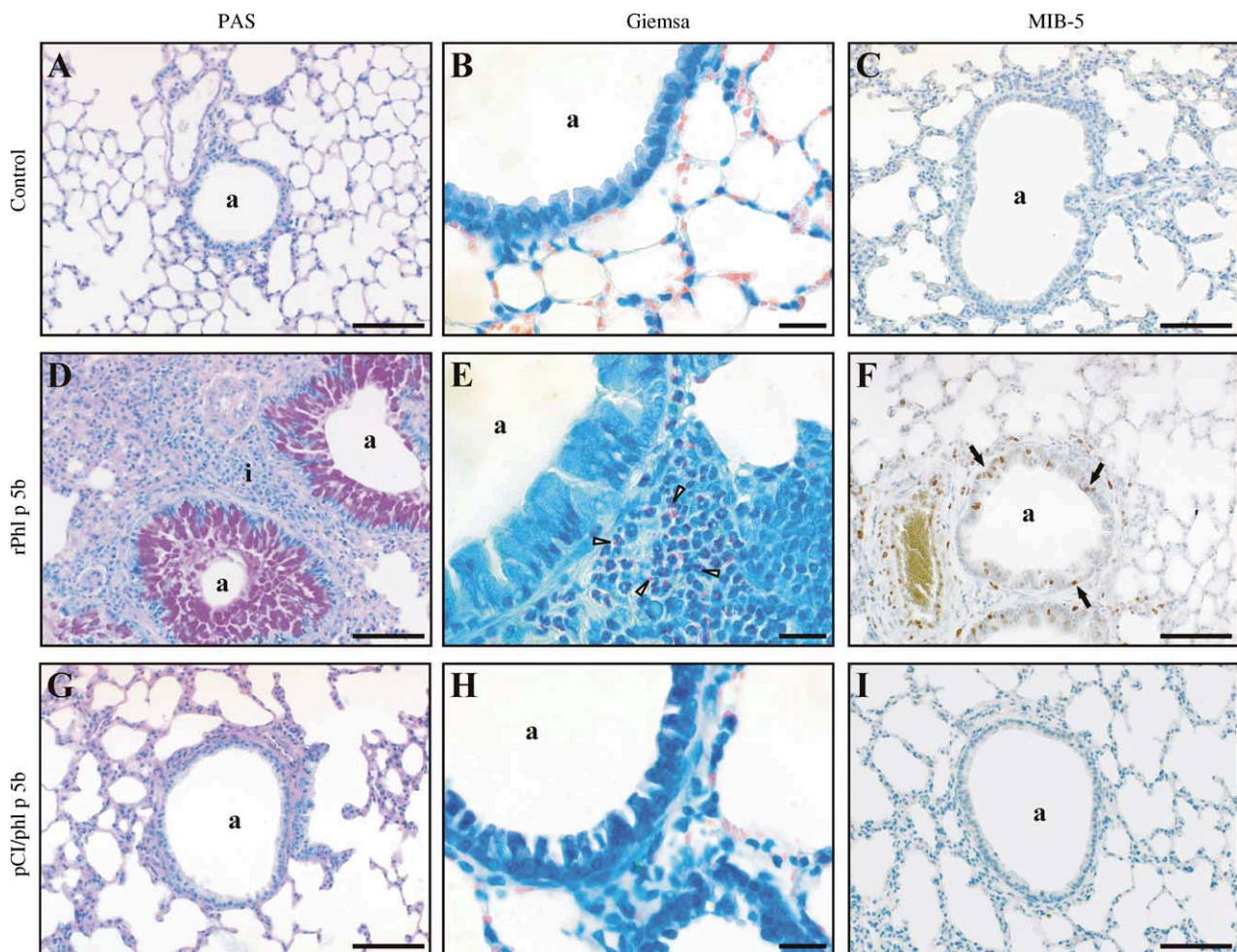


Fig. 4. Histology and immunohistochemistry of murine lung sections after intranasal challenge with the recombinant Phl p 5b allergen. Representative photomicrographs of alveoli (a) and surrounding tissue are shown for PAS (A, D and G), Giemsa (B, E and H) and MIB-5 staining (C, F and I) for control mice treated with saline (A–C), for mice treated with rPhl p 5b throughout the experiment (D–F) and for the 100 μ g pCI/pHl p 5b pre-treated and rPhl p 5b sensitised mice (G–I). In comparison to the control mice, rPhl p 5b-treated mice show massive mucus production as assessed by PAS staining (D), massive infiltration (i) and eosinophilia (E, open arrowheads), and activated bronchoalveolar epithelial cells as determined by staining for the proliferation-associated Ki-67 antigen (F, arrows). Mice pre-treated with 100 μ g pCI/pHl p 5b before sensitisation with rPhl p 5b (G–I) demonstrated none of these pathologic alterations and displayed a similar histology and morphology to the control mice. Size bars: A, D, G, C, F and I: 100 μ m; B, E and H: 10 μ m.

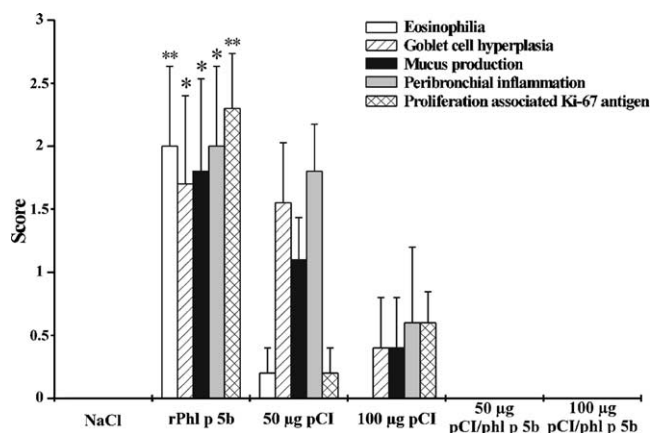


Fig. 5. Scores obtained for the histological analysis of pathologic alterations in the lung. Each data point represents the mean $\pm$ S.E. of five mice per group. Mice which were treated solely with rPhl p 5b showed significant increases of goblet cell hyperplasia, mucus production and peribronchial inflammation compared to control mice and mice pre-treated with the DNA-construct pCI/phl p 5b (* $p < 0.05$). Eosinophilia and proliferating bronchial epithelial cells were significantly increased compared to all other groups (** $p < 0.01$). Mice pre-treated with the vector alone also showed considerable pathological alterations correlating in severity with the amount of vector applied, however less pronounced than in the rPhl p 5b group.

treated with rPhl p 5b throughout the experiment exhibited a large number of goblet cells, peribronchial infiltrates and substantial mucus production (Fig. 4D). The lungs of these mice also had significant eosinophilia (Fig. 4E), and proliferative activity in the bronchiolar epithelium as assessed through detection of the proliferation associated Ki-67 protein was markedly present in these mice (Fig. 4F).

In contrary to the allergen-sensitised mice the 100 μ g pCI/phl p 5b pre-treated mice demonstrated none of the mentioned alterations (Fig. 4G–I) and the lung histology was comparable to the control group (Fig. 4A–C).

The evaluation of the scores obtained for the different histological criteria used to assess pathologic alterations in the lung clearly demonstrated the differences found in the various pre-treated groups (Fig. 5). Mice that were treated with rPhl p 5b throughout the experiment showed a significant increase of goblet cell hyperplasia, mucus production and peribronchial inflammation compared to mice pre-treated with both doses of the DNA-construct pCI/phl p 5b and to the controls (* $p < 0.05$). Eosinophilia and proliferation of bronchial epithelium were significantly increased compared to all other groups (** $p < 0.01$). Mice pre-treated with 50 μ g pCI vector still showed considerable goblet cell hyperplasia, peribronchial infiltrates, moderate mucus production and detectable infiltration of eosinophils. The 100 μ g pCI vector pre-treated group exhibited noticeably reduced pathologic alterations in the lung, most conspicuously the infiltrates did not contain eosinophils. The mice pre-treated with the different doses of pCI/phl p 5b DNA-construct showed no alterations in the lung with respect to the parameters investigated being comparable to the lung morphology found in control mice.

4. Discussion

This study evaluated the immunoprophylactic effect of plasmid DNA encoding the clinically relevant major timothy grass pollen allergen Phl p 5b to investigate whether DNA vaccination can inhibit a subsequent sensitisation with the allergen and protect against the development of pathologic alterations in the lung.

During the pre-treatment phase, the strategy applied resulted in the initiation of a Th2-biased immune response in the allergen treated animals versus a Th1-biased immune response in the DNA-construct treated animals. In contrast vector treated animals as expected did not mount a specific immune response. Interestingly, these respective polarised immune responses were significantly augmented during the course of the sensitisation phase with the allergen. This is a strong indication that on the one hand the application of the Phl p 5b allergen per se (without the Th2-adjuvant alum) initiates a Th2-mediated immune response which is significantly amplified upon repeated exposure to the allergen in the presented experimental setup. On the other hand, the specific Th1-polarised immune response elicited by the application of the allergen DNA-construct remained and was also significantly boosted by application of the allergen, pointing out that the initiation of a Th2-mediated response during sensitisation was repressed in these animals. In addition, the vector pre-treated group also mounted a Th1-polarised immune response during the treatment phase with the allergen, strongly suggesting that the CpG motifs contained in the vector acted as an adjuvant to promote the production of a Th1-immune response upon allergen exposure and thus suppressed a Th2-mediated immune response [26,27]. In comparison, however, the experiments with the DNA-construct dose dependently elicited a much higher Th1-immune response than the vector alone, which only induced a notable Th1-response at the higher dose applied. Nonetheless, both pre-treatment regimens, regardless of the doses applied, led to a significant and in magnitude comparable repression of the Th2-mediated response as assessed by quantitative immunoglobulin production.

The findings on the specific immunoglobulin levels correlate well with findings by other groups investigating DNA vaccination in allergy [6–13], however they show an interesting divergence namely concerning the effects observed in the vector control animals. In most reports the vector treated animals displayed no significant differences compared to untreated BALB/C mice, thus also no effect on the Th2 immune response after receiving a protein boost [6–9,11,13], regardless of the vector system used. This is an intriguing aspect, as these vectors as well as the vector used in this study contain CpG-motifs and considering that the application of CpG-motifs themselves are discussed as a therapeutic strategy in allergy [28,29].

Besides these striking effects on the production of specific immunoglobulin subclasses the lung histology of the animals was also analysed after provocation with the aller-

gen to determine the functional consequences of the various treatment regimens. The animals treated with allergen throughout the experiment displayed a fulminant pathological alteration in the lungs resembling the histopathology of chronic bronchitis, namely manifest goblet cell hyperplasia, production of large amounts of mucus, peribronchial infiltration and eosinophilia in these infiltrates. Since epithelial cells have been found to undergo proliferation in remodelling airways and since lesions of the epithelial barrier are frequently observed in asthma [30,31] we also investigated the proliferation of the bronchial epithelial barrier. As analysed by immunohistology the expression of the proliferation associated Ki-67 nuclear antigen was significantly increased in the bronchial epithelia of these highly sensitised animals indicating that epithelial damage followed by reparation through proliferation, migration and differentiation may have been taking place.

In strong contrast to the findings observed in allergen sensitised animals, mice pre-treated with Phl p 5b-DNA-construct showed absolutely no pathologic alterations in the lungs, being equivalent to the morphology observed in untreated animals. Thus, the pre-treatment with the allergen DNA-construct completely inhibited the development of these morphological correlates of chronic bronchitis. On the other hand, animals pre-treated with the vector alone showed a partial protection against developing these pathological alterations. Taking into consideration the results of the specific immunoglobulin levels it appears that the induction of a Th1-mediated immune response against the allergen protected the BALB/c mice from developing chronic bronchitis after sensitisation and challenge with the allergen. Since protection was only partial in the vector pre-treated animals it is reasonable to speculate that although CpG motifs and mechanisms of innate immunity appear to be involved in inducing a Th1-response and thus protection, the involvement of a specific immune response as induced by the DNA-construct is even more efficient and perhaps necessary to convey complete protection against hypersensitivity reactions in this model. Other studies have also shown a protective effect of DNA-vaccination on inflammatory airway reactions in the airways [32,33], however, in analogy to the findings on the antibody response, most do not report on effects in the vector control animals. An exception to this is the study by Jilek et al. [34], who could show a protective effect of vector against anaphylactic shock to bee venom phospholipase A₂. In contrast to the findings presented here, however, there was no significant difference observed compared to the DNA-construct treated animals. Future experiments are aimed to elucidate the separate roles of innate and adaptive immunity in conferring protection in the Phl p 5b-model and the mechanisms involved.

In conclusion, this study emphasises the possible potential of DNA vaccination in protection against development of type I allergy, in particular against developing pathological alterations in the lung after allergen exposure using a clinically relevant aeroallergen as represented by Phl p 5b.

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