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DISSERTATION

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Mimotopes for therapy of grass pollen induced asthma: proof of concept studies in a pathophysiologically relevant memory mouse model

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CONTENT

INTRODUCTION

1. Grasses and allergy

Grass pollen allergens are the most important causes of hay fever and allergic asthma during summer in cool temperate climates. About 25% of the population is affected by grass pollen mediated type I allergy. Symptoms can range from rhinitis, conjunctivitis, hay fever, asthma, urticaria, chronic eczema up to severe, life-threatening anaphylactic reactions [1]. The underlying key pathomechanism of the allergic disease is the production of IgE antibodies, which bind with high affinity to the IgE-specific receptor FcεRI on effector cells like mast cells and basophils. Upon exposure to the respective allergen, cross-linking of the IgE and FcεRI occurs which leads to effector cell degranulation resulting in the release of preformed mediators such as histamines, prostaglandins, tryptase and heparin. These mediators collectively cause increased vascular permeability, vasodilation, contraction of bronchial and visceral smooth muscle and local inflammation. The reaction starts within minutes after allergen exposure and is referred to as immediate hypersensitivity. In addition, the release of cytokines and chemotactic factors is essential for the recruitment and activation of further inflammatory cells involved in the development of a late asthmatic response, which will be described in chapter 2.

Among the most abundant airborne grass pollen allergen sources are meadow grasses such as timothy grass (*Phleum pratense*), orchard grass (*Dactylus glomerata*) and meadow foxtail (*Alopecurus pratensis*), each of them belonging to the *Poaceae* family [2]. With very few exceptions, all grass pollen types show a very high degree of crossreactivity [3]. In this aspect, crossreactive grass pollen antigens of different species have been classified in groups, e.g. group 1, 2, 3 … allergens [4].

The most complete set of allergens has so far been isolated and cloned from timothy grass pollen (*Phleum pratense*). Members of group 1 and 5 allergens appear to be the clinically

most relevant with an IgE reactivity of 65-85% of grass-pollen sensitized patients tested [5]. Group 5 allergens from at least seven species of the *Pooideae* subfamily have been identified so far, whereby Phl p 5 is one of the most allergenic members. Phl p 5 has been localized in the cytoplasm of the pollen grain and the surface of the exine [6]. It exists in two isoforms, i.e. Phl p 5a and Phl p 5b with molecular weights of 33 and 29 kD [7].

The availability of grass pollen allergens depends on the allergen release from the pollen grain which primarily occurs under humid conditions. When the pollen grain comes into contact with the oral, nasal or conjunctival mucosa, it bursts under osmotic shock and releases allergens by expulsion of cytoplasmic fragments or parts of the pollen wall.

However, as pollen grains are too large (approximately 10 μm in diameter) to penetrate the lower airways, they release allergen-containing starch granules (SG) [8]. Once expulsed from pollen, these microparticles (< 5 μm) can create inhalable allergenic aerosols capable of triggering an early asthmatic response and are implicated in thunderstorm-associated asthma [9]. In thunderstorms, strong downdrafts and outflows cause the pollens to entrain into the cloud base where pollen rupture is enhanced. Respirablesized pollen debris are subsequently transported to the ground level and are distributed by outflows ahead of the rain [10-12].

In addition, the pollen grain itself releases proinflammatory mediators such as eicosanoids, which act as chemoattractants and can activate inflammatory cells present in the upper part of mucous membranes. Pollen associated lipid mediators (PALMs) are known to resemble leukotrienes and prostaglandins in function and thereby activate innate immune cells like neutrophils and eosinophils, shifting the immune response into a Th2-biased direction [13].

The prevalence of allergic airway disease is furthermore linked to the increasing air pollution, which implicates the emission of volatile organic compounds. With the emission of ozone and particles in suspension, including the so called diesel-exhaust particles (DEPs), irritative problems with the airways occur. Via physical contact, DEPs can cause pollen rupture which leads to the release of paucimicronic particles. By their absorptive capacity and small size (<

0.1 µm-10 µm), DEPs can easily transport the allergenic particles and facilitate their penetration of the human airways [14].

In sum, many factors influence the likelihood of developing clinically significant sensitization to allergens. This might in atopic patients only be explained by the inherited predisposition for allergies. There is, however, agreement today that also the type of allergen, the allergen concentration and whether exposure occurs together with environmental conditions and/or adjuvant agents which enhance the sensitization process.

2. Allergic asthma

Allergic asthma is a chronic inflammatory airway disease, characterized by airway inflammation, persistent airway hyperresponsiveness (AHR) and intermittent, reversible airways obstruction [15]. An estimated 300 million people worldwide suffer from asthma, whereas 70% of these patients have allergies. The prevalence of allergic airway disease is rapidly increasing world-wide in all age groups; in many countries it reaches over 30% in children and more than 10% in adults [16, 17]. In many cases asthma is associated with the genetic predisposition to generate type I hypersensitivity against otherwise harmless common environmental antigens.

However, the predominant role of allergens in the course of asthma is questioned by the fact that suppression of symptoms, which occur most frequently already in childhood, have no influence on the outcoming of asthma later in life [18].

Dendritic cells are known to play a crucial role in the initiation of asthma by regulating early inflammatory events at epithelial surfaces. Particularly children in the first years of life do not exhibit yet airway dendritic cells (ADC) in the absence of inflammation. However, the chance of a respiratory virus infection which induces inflammation and subsequent ADC maturation with a preferential bias towards a Th2 immune response is high. In consequence, respiratory

viruses or other components of the inhaled environment predispose to allergic sensitization [19, 20].

Antigen inhalation of sensitized atopic patients with asthma results in an acute early asthmatic response (EAR) which develops immediately after challenge and reaches a maximal bronchoconstriction between 15 and 30 min resolving within 1-3 hrs [21]. EAR or type I immediate hypersensitivity (as described above), reflects the secretion of mediators at the affected site. Among them are histamines, prostaglandins and leukotrienes, which act via activation of different G-protein coupled receptors and thereby augment many features of asthma, like airway hyperresponsiveness, eosinophilia and mucus hypersecretion [22]. Th2 associated cytokines IL-4 and IL-13 act together with leukotrienes to worsen the features of asthma. IL-4 is on the one hand essential for the activation and function of B cells by augmenting the expression of MHC class II molecules and thereby facilitating the allergen-presentation to Th_2 cells. On the other hand, it induces isotype switching which leads to further IgE production by B cells [23]. IL-13 stimulates in overlap with IL-4 multiple features of asthma such as IgE production, airway hyperreactivity, goblet cell hyperplasia, smooth muscle hyperplasia and subepithelial fibrosis. The most important Th2 associated cytokine associated with eosinophils is IL-5, which regulates eosinophilic growth, maturation, differentiation, survival, activation and recruitment to the airways [23-25]. Several other, newly described cytokines are known to actively contribute to the development of allergic airway diseases, like IL-9 which exerts synergistic actions with IL-4 and 13, or IL-25 which stimulates release of IL-4 and 5, thereby, in turn, promotes IgE synthesis and eosinophilia [26].

Depending on the intensity and duration of the stimuli, approximately 60% of asthmatic patients are dual responders, i.e. the early asthmatic response is followed by a late asthmatic response (LAR). LAR starts between 3-4 hrs after the allergen provocation, peaks between 6 and 12 hrs and resolves within 24 hrs. Via cytokine-induced endothelial expression of leukocyte adhesion molecules such as E-selectin or ICAM-1, the recruitment of leukocytes

into tissues can be easily promoted. Inflammatory cells such as eosinophils, neutrophils, T-cells, macrophages, dendritic cells, endothelial cells, airway smooth muscle cells and bronchial epithelial cells produce a variety of cytokines and chemokines which elicit airway wall thickening, subepithelial fibrosis, goblet cell hyperplasia, myofibroblast hyperplasia, airway smooth muscle cell hyperplasia and hypertrophy.

Thus, the late phase of the asthmatic response is characterized by excessive inflammation of the airways resulting in structural changes. In chronic allergic asthma this re-structuring is referred to as airway remodeling [27]; inflammation affects all layers of the airway walls and is associated with changes in the epithelium including an increased number of mucus producing goblet cells, increased secretion of chemokines and cytokines, substantial inflammation of the submucosa, enhanced development of myofibroblasts, augmented vascularity and increased thickness of the muscular layer of the airways with greater size, number and function of smooth muscle cells [28].

3. Mouse models of allergic asthma

Although insights from mouse asthma models can only approximate human clinical disease, they are widely used to investigate the immunological mechanisms and inflammatory responses in allergic asthma.

The induction protocols for acute or chronic asthma in mice are principally all based on systemic intraperitoneal priming followed by respiratory boosts with the respective allergen. Airway provocation is usually performed via aerosolization, intranasal or intracheal administration and can take one to nine days in the acute and four to twelve weeks in chronic mouse models [29]. Systemic sensitization is most often described to be achieved by administration of the allergen either dissolved in PBS or administered with alum as an adjuvant [30]. Mice which are exposed to aerosolized antigen in the absence of systemic immunity do not develop lung disease [31, 32]. However, primary systemic sensitization can also

trigger systemic immune and non-specific airway responses on consecutive airway challenges with an unrelated, not cross-reactive, secondary allergen [33].

The most commonly used mouse strain for allergen challenges is BALB/c as it is known to develop a solid, Th2-biased immunological response [34]. However, also other strains like C57BL/6 and A/J mice have been successfully used for allergen challenge studies [35]. Ovalbumin (OVA) derived from chicken egg is frequently used as a model allergen because it induces a robust pulmonary allergic inflammation in mice and is, moreover, relatively inexpensive. OVA, however, is seldomly implicated in human asthma, therefore sensitization with alternative allergens with higher clinical relevance, such as house dust mite [36] or grass pollen [37], are envisaged.

Upon sensitization and airway challenges with the respective allergen, eosinophilic infiltration of the lung, airway hyperresponsiveness (AHR), increased IgE, mucus hypersecretion and, eventually, airway remodeling in mice are observed [31].

Due to the relative short-term nature of many acute models, the key feature of asthma, namely airway remodelling is minimal and other parameters like eosinophilic infiltration and AHR are transient. Airway inflammation and AHR have been shown to resolve within a few weeks after the final allergen challenge. However, acute models have provided important information about inflammatory processes like the role of Th2 cells in asthma and the implication of eosinophils in AHR. Furthermore, many cellular and molecular targets for the treatment of asthma could be evaluated in the acute models, e.g. IL-4 and IL-5 antagonists [38-40].

Chronic allergen challenge models which more closely mimic the chronic aspects of human asthma with respect to epithelial hypertrophy, subepithelial fibrosis and limited smooth muscle hyperplasia have been established by several groups [41-43]. The problem with chronic models is that long-term challenges of mice often induce tolerance and rather downregulate

inflammation and AHR [44]. Thus, the strain of mice, the allergen concentration and the route of administration are critical factors that determine whether tolerance is induced or not.

Major differences between mouse and human physiology have to be considered when working with mouse asthma models: whereas patients suffer from methacholine induced AHR even in symptom free periods, mice exhibit only transient methacholine-induced AHR after airway provocation. Furthermore, an apparent lack of eosinophil degranulation could be observed in the majority of mouse asthma models and there is no or little recruitment of mast cells into the airway wall or epithelium [45-47].

Although mouse models of allergic asthma have limitations that need to be considered when extrapolating findings from mouse to the human disease, many questions on susceptibility, pathogenesis, airway remodeling, immunological memory, and treatment are currently and successfully answered in mouse models [31].

4. Treatment strategies for allergy and asthma

As exposure with allergens is the prerequisite to develop any allergic disease, allergen avoidance of allergen exposure before or after sensitization should be beneficial as primary or secondary prophylaxis. This is difficult for ubiquitary allergens such as grass pollens.

At present, the only curative treatment with long-term effects in patients is allergen-specific immunotherapy (SIT), which involves the administration of allergen extracts aiming to induce tolerance to those allergens which cause symptoms in the individual. The clinically most effective form of SIT is subcutaneous immunotherapy (SCIT), which comprises repeated subcutaneous injections of the sensitizing allergens. Typically, patients receive a course of injections, starting with a very low allergen dose being built up gradually until a plateau or maintainance dose is reached. The "build-up phase" consists of several weekly injections and is followed by a "maintainance phase", in which the allergen is given in a 4 to 6-week interval over a period of 3-5 years [48]. The beneficial effects of SIT comprise the induction of allergen-specific T regulatory cells which produce anti-inflammatory cytokines IL-10 and TGF-β and thereby downregulate the activity of effector Th2 cells, suppress IgE production and induce isotype class switching to IgA and IgG4 [49, 50]. IgG4 antibodies, also known as allergen-specific blocking IgG, compete with effector cell bound IgE for allergen binding and therefore may prevent the activation of mast cells and basophils. In the same way, IgE facilitated allergen presentation via binding to low affinity IgE receptor CD23 on antigen-presenting B cells is impeded [51]. When complexed with an allergen, blocking antibodies can moreover mediate inhibitory signals via co-aggregation of the IgG receptor FcγRIIb and IgE receptor FcεRI and, thus, again avoid activation/degranulation of effector cells and, more importantly, inhibit IgE synthesis from B cells [52-54].

However, the benefit of SIT becomes less favorable when considering the increased risk of allergen-induced, IgE-mediated systemic side effects and *de novo* sensitization against other

components of the allergen extracts administered. To improve safety, sublingual immunotherapy (SLIT), i.e. application of allergens via the oral mucosa has only recently gain acceptance. Although higher doses of allergen are used, side effects are relatively mild [55]. However, the treatment benefit was found to be approximately half of that achieved with SIT [56].

In the past few years, intensive research has been done to improve efficacy and safety of immunotherapy in allergy. Among the current developments of SIT is the generation of recombinant allergens which allow consistency in quality and quantity of the active ingredient [57]. However, as IgE reactivity is still preserved, modified versions like hypoallergenic recombinant molecules, deletion mutants, fragments or peptides have been developed [58-62]. Many of these approaches include T cell epitopes aiming to stimulate peripheral T cell tolerance while lacking IgE antibody binding sites to exclude the risk of IgE mediated crosslinking. Nevertheless, several studies using T cell peptides for treatment of allergic airways disease have instead reported on the recruitment of inflammatory T cells and manifestation of chronic allergic inflammation [63, 64].

Another possibility to bypass the activation of allergen-specific T cells in this aspect is the use of mimotopes, which exclusively mimic B cell epitopes of allergens. As B cell epitopes most often appear conformational and discontinuous in nature, phage display offers an elegant method for defining structures that mimic binding sites for antibodies [65]. In a peptide phage library, random oligonucleotides are inserted into the genome of a filamentous bacteriophage for subsequent presentation in context with either minor phage coat protein pIII or major coat protein pVIII. In a process called “biopanning”, repetitive screening of phage libraries with allergen-specific antibodies of interest is performed.

Besides peptide libraries, antibody-derived phage display systems are also suitable for selection of potential B-cell epitope-specific candidates in biopanning. For the construction of an e.g. combinatorial Fab antibody phage library, cDNAs of heavy and light chains from the

B cell repertoire of an allergic individual are combined and presented by phagemid systems [66]. When screening with anti-allergen Ig, the selected Fab represents an anti-idiotypic specificity. It represents a complete antigen and is suitable for immunizations. In contrast, a mimotope peptide is a hapten, being much smaller in size and therefore requires T-cell help to gain sufficient immunogenicity. Bystander T cell help must be provided by e.g. immunogenic carriers or immunostimulatory agents, such as KLH or tetanus toxoid (TT) [67]. Moreover, due to the monovalent nature of anti-idiotypic Fab fragments, the risk of IgE crosslinking on effectors cells is limited [68]. In the past we and other groups could identify mimotopes and anti-idiotypic Fabs from a number of different allergens [68-72]. It could be shown that the selected clones were able to interfere with the high affinity binding of IgE and to trigger allergen-specific immune responses without the activation of allergen-specific T cells [73, 74] .

In previous studies, our group identified a peptide mimotope of grass pollen major allergen Phl p 5 which was later on applied for therapeutical treatment in a mouse model of acute allergic asthma. For the first time it was shown that a mimotope could successfully downregulate allergic airway inflammation and moreover protect from disease upon allergen re-challenge by airway provocation [37].

Besides protein-based immunization strategies, application of allergen-encoding DNA in rodents has yielded promising results in the field of allergy. Side effects which are connected to protein immunization like IgE cross-linking or *de novo* induction of anaphylactic antibodies could be effectively avoided upon the administration of pure DNA. DNA vaccines consist of the foreign gene, e.g. the allergen of interest, which is cloned into a bacterial plasmid engineered for optimal expression in eukaryotic cells [75].

Experiments in animal models have demonstrated that injection of plasmid DNA induces a Th1 cytokine milieu being efficient either in a preventive or therapeutic approach [76-79]. Plasmid DNA is taken up by either APCs or somatic cells like keratinocytes, which transcribe and translate the information into the respective allergen. As a result, peptides can be

presented via MHC I and thereby active Th1 cells which secrete IFNγ and drive B cells towards IgG production or via MHC II which leads in conjunction with Th1 cells to activation of cytotoxic T cells.

Immunization of DNA vaccines can be performed via several administration routes including epidermal, mucosal, intramuscular, intravenous or biolistic gene transfer. Injection of plasmid DNA into skin or muscle requires high amounts of DNA whereas the biolistic device (gene gun) renders immune responses with at least 100-fold lower quantities of DNA. However, gene gun bombardment was in the past predominately reported to be associated with a Th2 biased immune response [74, 80].

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AIMS

We previously generated peptide mimotopes and anti-idiotypic Fab fragments being B cell epitope mimics and completely devoid of allergen-specific T cell epitopes. As a model allergen, we chose grass pollen major allergen Phl p 5. The overall intention of this thesis work was to achieve for the first time the proof of concept for a therapeutic mimotope vaccine in a mouse model of allergic disease.

Therefore, our first aim was to adapt a mouse model of acute allergic asthma to sensitization with grass pollen allergen Phl p 5 and to evaluate its pathophysiological significance. For this we used a novel, open-circuit indirect calorimetric system which enabled us to measure respiratory gas exchange and motor activity of mice within the acute asthma attack. To dissect allergen-specific asthma from non-specific airway provocation, we introduced an irrelevant antigen for airway challenge.

The aim of the second study was to examine for the first time the therapeutic efficacy of a decameric peptide mimotope termed Phl mim 5 by use of the previously established memory mouse asthma model. We evaluated whether mimotope vaccination would a) downregulate airway inflammation and moreover b) protect from acute asthma upon re-exposure to the allergen. Re-challenge with the aerosolized allergen was performed after vaccination therapy. Effects on the humoral and cellular immune response were analyzed in sera, bronchial lavage fluid (BAL) and lungs.

The third and last aim of the thesis was to investigate whether an immunization strategy with minigenes would be advantageous over peptide administration. As genetic vaccination has shown to efficiently downregulate ongoing Th2 immune responses in rodent models in the past few years, we decided to construct a DNA vaccine encoding the mimotope sequence and tested whether it would elicit allergen-specific immune responses in naive BALB/c mice.

As the allergen route might critically affect the outcome of the immune response, we intended to compare head to head two different routes of gene vaccine administration, i.e. intradermal injection and gene gun immunization.

Anti-Ids in Allergy: Timeliness of a Classic Concept

Julia Wallmann, MSc, Isabella Pali-Schöll, PhD, and Erika Jensen-Jarolim, MD

Abstract: Anti-idiotypic antibodies (anti-ids) are part of natural immune responses with regulatory capacity. Their effect on an antigen-specific, so-called Ab1 antibody response, is dependent on 1) the original antigen, which they mirror, being Ab2 antibodies, and 2) their isotype. In the case of IgE-mediated allergy, natural anti-ids against allergen-specific IgE represent internal images of allergen molecules. A key biologic feature of allergens is that they can crosslink IgE, expressed by B-lymphocytes or passively bound via high affinity receptors to effector cells, which renders cellular activation. Therefore, the IgE cross linking capability of anti-ids determines whether they dampen or enhance immediate-type hypersensitivity. Correspondingly to classic antiallergen blocking IgG antibodies, anti-ids may also interact with inhibitory FcγRIIb receptors and, thereby, down-regulate T_H2-type inflammation. Anti-ids and other B-cell epitope mimetics, like mimotopes and DARPins, represent antigen surrogates, which can be used for vaccination. Intriguingly, they may induce antibody responses without activating potentially proinflammatory, antiallergen T-lymphocytes. Taken together, collective evidence suggests that anti-ids, although representing immunologic classics, are a timeless concept in allergology.

Key Words: anti-idiotypes, allergy, vaccination, FcεRI, FcγRIIb, blocking

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INTRODUCTION

Besides a specific antigen binding site, antibody molecules possess antigenic determinants themselves. When these epitopes are located within the variable region of an antibody, they are designated idiotopes. Hence, each Fab arm of an antibody displays a set of idiotopes, representing epitopes for complementary antibody molecules (Fig. 1), so called anti-idiotypic antibodies (anti-ids). Anti-ids are part of regular immune responses and are thought to result in a web of interacting idiotypes. Classically, Jerne defined the order of an antibody response as follows: Antibody Ab1 is produced in response to an antigen and induces the production of anti-idiotypic Ab2, which can in turn stimulate the synthesis of an anti-(anti-idiotypic) antibody Ab3, and so forth (Fig. 1). Anti-idiotypic antibodies (Ab2) can be classified into several categories according to their fine specificity (Fig. 2): 1) conventional Ab2α antibodies recognize idiotopes of Ab1 outside of its antigen binding site, but still within the variable region; 2) internal image Ab2β antibodies recognize idiotopes directly within the antigen binding site of Ab1 and, therefore, mimic its original antigen epitope like "internal images"; 3) in case the idiotope recognized by Ab2 is not completely overlapping but close to the antigen binding site of Ab1, it may still be able to interfere with the antigen binding and is called Ab2γ. Consequently, binding of antigen by Ab1 is not affected by Ab2α, may be blocked by Ab2γ and is completely blocked by Ab2β.

ANTI-IDS IN EXPERIMENTAL STUDIES: THE BEGINNING

The first experiments regarding anti-ids were animal studies, which were performed to evaluate the effect of anti-ids either on an upcoming or ongoing immune response.

For instance, Cosenza and coworkers designed a mouse study where they used a monoclonal IgA antibody derived from the myeloma cell line TEPC 15, specific for the hapten phosphorylcholine.[1] To generate an anti-idiotypic immune response, mice were immunized with the myeloma antibody Ab1 and sera were harvested. Parallel, they immunized another group of BALB/c mice with heat-killed pneumococci to induce a phosphorylcholine-specific Ab1 response. As a proof for the induction of specific antibodies, erythrocytes attached to either pneumococcal C polysaccharide or phosphorylcholine were incubated with the splenocytes from the latter group of mice, resulting in hemolytic plaques because of immune complex-mediated complement activation.[2] Importantly, when spleen cells were preincubated with the anti-idiotypic serum generated upon immunization with myeloma IgA, plaque formation was specifically inhibited because of blockage of Ab1. This principle was later also shown for anti-ids directed against Ab1 specific for group A streptococcal antigen.[3]

However, anti-ids could not only inhibit effector responses, but also prevent de novo induction of specific immune responses in vivo. Naive BALB/c mice were administered the anti-idiotypic serum derived from immunization with TEPC myeloma IgA and subsequently intravenously immunized pneumococci. Antibody responses to phosphoryl-

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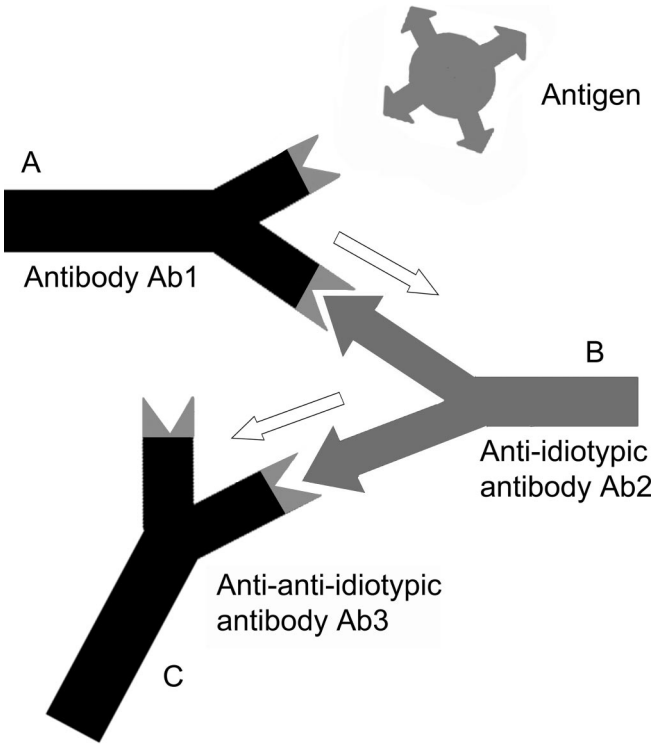

FIGURE 1. The anti-idiotypic network amplifies antigenic signals. (A) An antibody Ab1 is produced in response to a specific antigen. (B) With a defined idiotype, Ab1 induces the production of an anti-idiotypic antibody Ab2. This Ab2 may resemble the original antigen as an internal image. (C) Ab2 can stimulate the synthesis of an anti(anti-idiotypic) antibody Ab3 which principally is of the same specificity as Ab1.

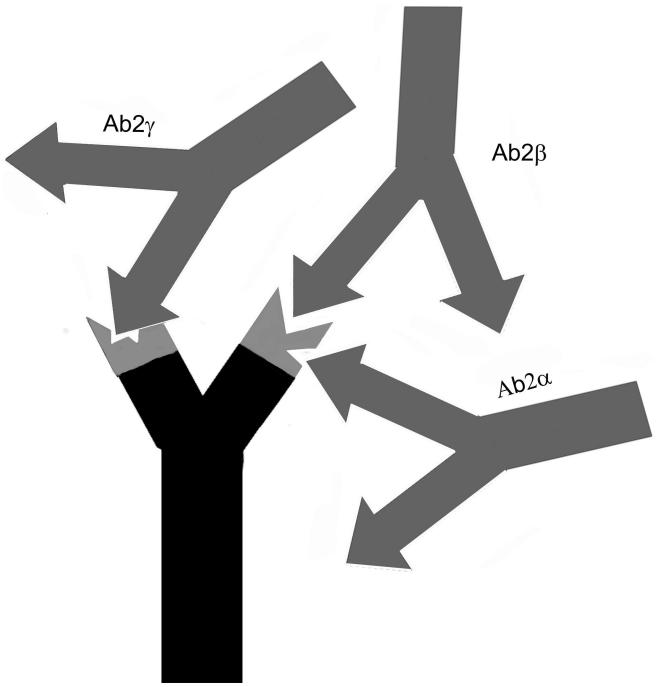

FIGURE 2. Categories of anti-idiotypic antibodies. Ab2α antibodies recognize idiotopes outside the antigen binding site, but still within the variable region of Ab1. Ab2γ recognizes idiotopes close to the antigen binding site of Ab1 and thus may interfere with the antigen binding. Internal image Ab2β binds directly within the antigen binding site.

choline but not to an irrelevant control allergen were completely blocked. Thus, anti-ids were discussed to be directed also against membrane immunoglobulins of naive antigen-specific B cells. Accordingly, it was hypothesized that membrane and secreted immunoglobulins share similar variable domains even when belonging to different isotypes.[1,4] The key result of these studies was, however, that anti-ids act at the cellular level to specifically suppress antibody formation.

Hart and his group generated anti-ids by immunization of rabbits with *p*-azophenylarsonate-specific mouse antibodies. When naive mice were then injected with the anti-idiotypic rabbit serum and afterward challenged with KLH (keyhole limpet hemocyanin)-linked azophenylarsonate, de novo antibody formation was suppressed up to 97%.[5] However, the suppression was not complete. This might have been because of the appearance of antibody responses directed against either the carrier protein KLH or the hapten (*p*-azophenylarsonate) as such. It seemed probable that the induced antiphenylarsonate antibodies (and their anti-ids), induced by carrier-hapten immunization, possessed idiotypes other than those elicited by either KLH or the hapten. Again, the conclusion of this study was that anti-ids acted suppressive at the cellular level via binding to immunoglobulins expressed by B-lymphocytes.

Furthermore, it became apparent that T cell receptors carry idiotypes similar to those of immunoglobulins, meaning that anti-ids could potentially regulate both, B-lymphocyte and T-lymphocyte function.[6] The idiotypic relationship between hypervariable domains of T and B lymphocyte receptors has indeed been repeatedly documented.[7–10] Furthermore, it has been shown that anti-ids can mediate cooperation between T and B lymphocytes (Fig. 3C). Furthermore, when B-lymphocytes expressing membrane Ab1 (mAb1) are targeted by Ab2, they internalize and process the anti-id (Fig. 3B) like the original antigen (Fig. 3A). Consequently, an anti-idiotypic peptide of Ab2 is presented in an MHC II context to T cells, again resulting in T cell-help. This would be an additional explanation for how B-lymphocytes recruit T cell help. In the early 1980s, also Blaser et al identified idiotypes on specific T-helper cells similar to the antigen-specific IgG. However, they dissected an antihapten from an anticarrier response and proposed that, in contrast to the above, especially an anticarrier anti-id might block T-helper cell function, rendering reduced antibody production.[11]

Taken together, already at that timepoint, functional similarity between antigen and anti-id could be shown. There was mostly evidence that administration of anti-ids suppressed specific idiotype production.[4,12] However, other studies[13] suggested that under certain conditions anti-ids could enhance immune responses. For instance, administration of anti-ids of the IgG1 isotype raised in A/J mice against an antistreptococcal antigen A antibody (Ab1) could prime animals for a secondary boost to the antigen. Interestingly, anti-ids of the IgG2 isotype were rather

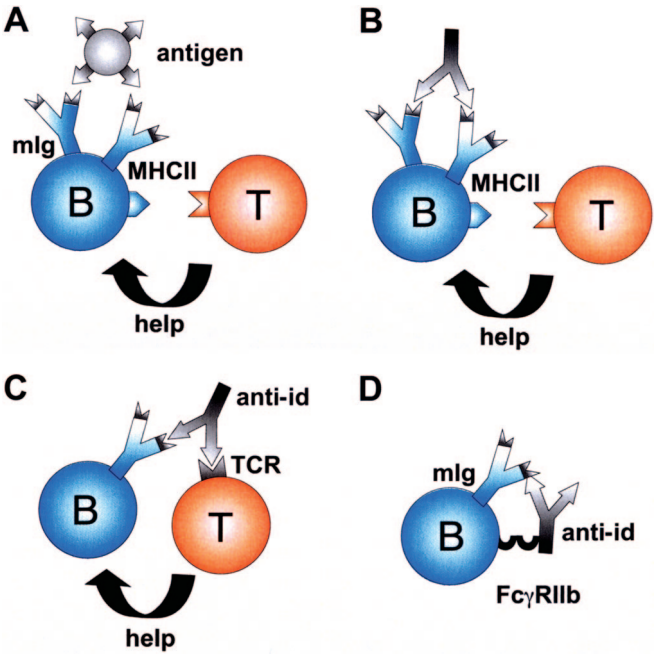

FIGURE 3. Mechanisms of anti-idiotypic antibodies on B (B) and T cell (T) interaction. (A): B cells recognize antigen by membrane-expressed immunoglobulins (mIg) and receive bystander help from T-helper cells. (B) Alternatively, an anti-id may bind to mIg of a B-cell. This leads to processing and MHCII display of an anti-idiotypic peptide rendering T cell activation and help. (C) Because of idiotypic similarities between B and T cell receptors, an anti-idiotypic antibody may also force cooperation between B and T cells by binding to both. (D) Inhibition of B cell proliferation is achieved by simultaneous binding of the anti-id to its corresponding idiotype on mIg and via its Fcγ domain to FcγRIIb on the B-cell.

associated with suppressive effects on naive, antigen-sensitive B cells.[12] Therefore, besides antigen-specificity of the anti-idiotypic system, also the induced isotype seemed to contribute to the regulatory capacity of anti-ids.

NATURAL ANTI-IDs TO ALLERGEN-SPECIFIC IGE: QUALITATIVE AND QUANTITATIVE STUDIES

In allergy research, Malley et al reported the influence of anti-ids on primary and secondary IgE antibody responses to timothy grass (*Phleum pratense*) pollen extract.[14] An anti-idiotypic antibody was produced to the grass pollen-specific IgE and was administered intraperitoneally followed by immunization of mice with timothy grass pollen extract. This anti-id profoundly prevented IgE production toward the allergen extract, and suppressed even up to 75% of the IgE response to a secondary boost. This suppression persisted for at least 35 days.[14]

Despite optimistic results such as these, the role of anti-idiotypic immune responses in humans induced during long-time immunotherapy could at that time only be postulated.

Bose and colleagues did the first quantifications of anti-ids in rye grass-allergic patients' sera.[15] Rye I-specific IgG anti-ids were purified from a ryegrass allergic patient who had been previously hyposensitized. In vitro, these IgG specifically blocked the binding of IgE and IgG to group I allergens of rye grass in sera of 20 individual allergics. This result pointed toward the recurrence of idiotypes and a constricted repertoire of human antibodies specific for an allergen. The authors discussed similar idiotopes of IgG and IgE within their antigen binding site. However, in this study it remained unclear whether the same anti-ids were able to inhibit antigen binding to both, IgE and IgG.

A functional distinction between paratope-associated idiotopes versus bystander idiotopes (ie, idiotopes located outside the antigen-binding site) of IgE and IgG was made by the group of Saint-Remy.[16] They observed cross-reactivity of house dust mite-specific IgE and IgG, but only with respect to bystander idiotopes.[17] They inhibited Ab1-Ab2 interactions with house dust mite allergens and thereby found that the majority of natural anti-ids in mite-allergic patients was of the Ab2β type, thus resembling internal images of the allergen. Still, the authors suggested the epitope-specificity of IgE and IgG to be different.

Interestingly, anti-ids specific for ragweed were also found in nonatopic individuals although to a much lesser extent than in allergic and immunotherapy-treated patients.[18–20] The authors proposed that anti-ids detected in nonatopics probably represent a basal level of Ab1/Ab2 responsiveness because of the presence of allergen-specific IgG, but not IgE antibodies, in health.

Anti-id levels in untreated patients sensitive to grass pollen allergen Lol p I and in hyposensitized patients were compared with levels in allergic donors not sensitive to Lol p I, and nonallergic donors by Bose et al using sepharose purification. Via binding to radiolabeled, affinity-purified $F(ab')_2$ fragments of Lol p I monoclonal antibodies, the amounts of anti-ids and the ratio to allergen-specific antibodies were calculated.[21] Again, anti-ids were observed to be present not only in allergic individuals but also in nonallergics, although the latter did not show any detectable IgG or IgE (Ab1) anti-Lol p I. Anti-id levels in allergics were shown to directly correlate with the level of pollen exposure or the hyposensitization status. In the majority of patients treated by immunotherapy an inverse relationship between serum levels of anti-Lol p I IgE and IgG antibodies (Ab1) and of anti-ids (Ab2) could be observed. An initial increase of specific idiotype (Ab1) levels was associated with a drop of the anti-id (Ab2) level. However, like during hyposensitization, the anti-id level later on increased toward a plateau. An increase of Ab2 was also reported by Castracane et al in a ragweed allergic human patient undergoing immunotherapy. They used an antiragweed specific $F(ab')_2$ fragment for coating in a solid phase assay.[19,20] In line with earlier observations by Oudin and Cazenave, it had to be considered that a proportion of the raised anti-ids was most probably not specific to Lol p I, but could have also encountered similar idiotypes derived from different allergens.[21,22] Hebert and colleagues measured elevated anti-id levels in sera of hyposensitized patients by the use of 3 different murine monoclonal anti-Lol p I antibodies.[23] These murine antibodies were shown to share cross-reactive idiotypes with human anti-Lol p I IgE and IgG antibodies and therefore suitable in this study.[24]

THE FUNCTIONAL ROLE OF ANTI-IDS IN ALLERGY

Levels of anti-ids were generally found to be higher in healthy individuals than in untreated atopic patients. In the latter, levels can be elevated by immunotherapeutical treatment to the levels found in healthy volunteers.[20,24]

Valacer and colleagues inhibited IgE binding from allergic patients to ragweed antigen by administration of anti-ids purified from sera of nonallergic individuals. Strikingly, this effect could be achieved although no allergen-specific IgG or IgE could be detected in the sera of the healthy donors. The authors discussed that anti-ids in healthy persons could regulate the IgE response by suppressor mechanisms.[25] Indeed, a dramatic suppressive effect of anti-ids on the IgE response was observed in experimental studies. A single anti-id directed against an idiotope of a murine antibody specific for a major epitope of grass pollen allergen Lol p IV down regulated the allergen-specific IgE response profoundly, whereas levels of other isotypes, for example, IgG1 and IgG2 were less affected.[26]

The enthusiasm about potential beneficial effects of anti-ids in the course of immunotherapy was dampened by the sudden awareness that anti-ids against allergen-specific IgE antibodies potentially could also target IgE bound to effector cells. This could lead to either mediator release by IgE-cross-linking (Fig. 4B) like it is the case with the original antigen (Fig. 4A), or to the inhibition of allergen-induced release (Fig. 4D). In fact, Geha et al demonstrated that passive cutaneous administration of an antitetanus toxin anti-id in a human patient elicited positive immediate-type skin reactions.[27] In another study, passive cutaneous anaphylaxis experiments in rats were chosen to analyze the in vivo effects of anti-ids on mast cell bound IgE. Anti-ids were raised in syngeneic mice by immunization with dinitrophenol (DNP)-specific mouse monoclonal IgE. The anti-idiotypic serum induced a passive cutaneous anaphylactic reaction in skins of rats sensitized with the DNP-specific mouse IgE. In contrast, the same anti-id did not elicit a reaction in DNP-IgE sensitized rats when they simultaneously were passively administered grass pollen-specific IgE. Thus, a specific anti-id seemed to be more effective in histamine release when less IgE specificities were present in the sensitized individual. From this it was concluded that anti-idiotypic stimulation within a polyclonal response, like in human atopy, would rather inhibit mediator release (Fig. 4C). Furthermore, successful immunotherapy was considered to rely on continuous anti-id targeting of the mast cells rendering internalization and, therefore, depletion of cell bound specific IgE.[28]

The capability of human anti-idiotypic IgG purified from mite allergic patients to recognize cell bound IgE idiotypes of other donors, and to induce mediator release of basophils in the absence of allergen was demonstrated.[29] Consequently, the possibility that degranulation in response to anti-ids could be a general event in allergy was proposed. However, the degranulating activity of anti-id preparations varied from one patient to the other. Several mechanisms were discussed: First, the precise specificity of anti-ids could play a role, because framework idiotopes could be private and, in contrast, paratope-associated idiotopes of antibodies

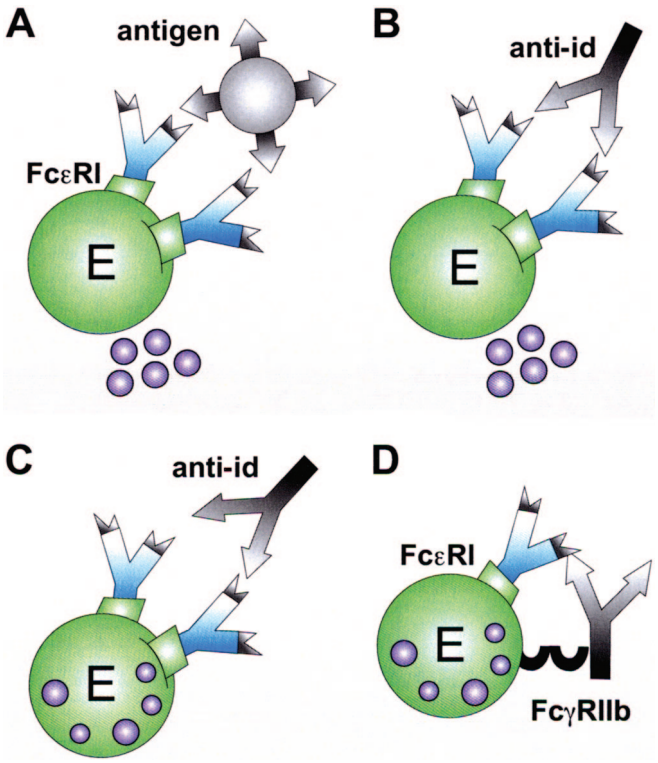

FIGURE 4. Mechanisms of anti-idiotypic antibodies on effector cells. Mediator release of effector cells (E) is triggered by cross-linkage of 2 receptor-bound IgE either via (A) recognition of the antigen or (B) the anti-id. (C) Effector cell inhibition is achieved by binding of the anti-id to its specific idiotype within a polyclonal response. (D) Cross-linkage of FcεRI and the inhibitory FcγRIIb receptor via an anti-idiotypic IgG antibody also inhibits degranulation.

were found to be more common among different individuals. Hence, the relative proportion of these 2 types of anti-ids could explain the varying degranulating activity of single anti-id preparations on basophils of different donors. Secondly, the authors discussed the repertoire of anti-ids to be dependent on the individually varying antiallergen IgE response, limiting their degranulation capacity when testing basophils of other donors.

Taken together, the impact of anti-ids in the regulation of IgE antibody responses to allergens has at that time not been revealed to the same extent as for infectious diseases or in malignancies.[30–32] However, genetic engineering of antibodies opened novel methodological possibilities for precise investigation of the effects of anti-ids in the effector phase of type I allergy. As many studies previously were based on mouse monoclonal anti-ids, it was difficult to extrapolate the findings to the human setting. Therefore, the group around Shakib intended to engineer murine/human chimeric anti-ids and the corresponding antiallergen IgE,[33] taking Der p 1 as a model allergen. Testing these chimerized IgE-anti-idiotypic antibodies on FcγRIIb transfected cells and purified basophils, they found an inhibition of the degranulation through the anti-ids. Interestingly, a novel mechanism of action was proposed, involving FcγRIIb-binding by the Fcγ domain of the anti-idiotypic IgG, and,

consequently, cellular inhibition through the associated ITIM-motif of this receptor (Fig. 4D).[34] In this context it is of interest that also allergen-specific IgG may recognize allergen already complexed with mIgE on B-lymphocytes or with IgE bound to the high affinity receptor. Upon simultaneous binding to FcγRIIb, allergen-specific IgG has been shown to down regulate not only effector cells, but also B-lymphocytes.[35,36] Therefore, we postulate here that similarly anti-ids may exploit this mechanism and act on the B cell level, too (Fig. 3D).

Interestingly, anti-ids may not only dampen the allergic response after allergen immunotherapy, but may also have a protective function in offsprings of allergic mothers. In a mouse model, the maternally derived anti-id against allergen-specific IgE was shown to induce IgE suppression in the offspring which was long-lasting and dose-dependent.[37] As molecular modeling ruled out an internal image function of the anti-ids, possibly also here the binding to FcγRIIb could contribute to the protective function.

ANTI-IDS PRIME AND ENHANCE ALLERGEN-SPECIFIC IMMUNITY

It had been suggested that anti-ids function differently in healthy, allergics, or hyposensitized patients. Bose proposed 1986 that anti-ids in allergics might function as "network antigens", mimicking the allergen and therefore being able to accelerate an immune response.[21] In accordance, an anti-idiotypic Ab2β antibody raised by a monoclonal anti-Lol p 1 antibody was able to successfully prime an allergen-specific antibody response, as a subsequent boost injection with the antigen Lol p I resulted in a further increase of specific IgE and IgG antibody levels.[38] The administration of anti-ids augmented both, idiotype specific and total antibody responses against the allergen,[38] a result that had been predicted before by studies outside the field of allergy.[39–41] However, the exact mechanism for the enhancing effect of an anti-id resembling only a single epitope, remained unclear. One may speculate that upon the administration of Ab2 the induced Ab3 facilitates subsequent antigen presentation, leading to epitope spreading. Especially the IgM isotype was considered to potentiate the immune response against low doses of antigen via the idiotypic network. A further explanation could be that immune complexes formed after antigen immunization directly stimulate antigen-specific B cells.[42]

VACCINATION WITH ANTI-IDS TO CONTROL TOTAL IGE LEVELS

The immunotherapeutical potency of anti-ids for allergic patients was not only investigated with respect to the allergen-specific IgE response, but also regarding total IgE levels in allergics. It had been shown previously that natural anti-IgE isotype antibodies exist, which, depending on the epitope specificity of the Fcε domain, may down-regulate or enhance the effector function of IgE.[43] Stadler et al aimed to generate nonanaphylactogenic IgG antibodies through active immunization with an anti-idiotypic antibody as an antigen surrogate. When they used a beneficial, nonanaphylactogenic anti-IgE antibody (termed BSW17) as a template, anti-ids could be generated from a combinatorial phage library displaying the Fab repertoire of a grass pollen allergic patient.[44] According to the principle of molecular mimicry, these anti-ids resembled an epitope of the constant domain of IgE and actively induced BSW17-like anti-IgE specificities in mice.[45] Therefore, as an alternative to passive anti-IgE therapy with, for example, omalizumab, vaccination with anti-idiotypic molecules for active induction of a protective anti-IgE response was envisaged by the authors.

VACCINATION WITH ANTI-IDS TO CONTROL ALLERGEN-SPECIFIC IGE LEVELS

In our own study, we generated anti-idiotypic Fab antibody fragments for IgE, which was specifically directed against timothy grass pollen allergen Phl p 5.[46] The phage library containing the repertoire of a grass pollen sensitized individual, which is described above,[47] was this time used for screening with allergen-specific IgE. Several high-binding Fab clones with mimicry potential to the allergen's IgE epitope could be isolated. Upon immunization with clones in mice, antiallergen IgG could be induced via molecular mimicry. Furthermore, these anti-idiotypic Fabs resembled naturally occurring IgE epitopes of the allergen as observed by sequence analysis and molecular modeling.[46] More recently, we applied one of the selected anti-idiotypic clones in a memory mouse model of acute allergic asthma to test whether it would be suitable for immunotherapy. According to a protocol by Mojtabavi et al[48] acute allergic asthma was induced by injecting recombinant Phl p 5 intraperitoneally, followed by aerosol challenges with this allergen. Subsequently, groups of mice with acute asthma were vaccinated with 1) the anti-idiotypic Fab fragment, or 2) control antigen KLH before reinduction of acute asthma by an additional allergen aerosol challenge.

Whereas the IgE and IgG1 antibody levels of all groups remained unchanged during treatments, the extent of acute eosinophilic inflammation upon rechallenge with aerosolized allergen was much lower in the Fab-treated group compared with the untreated asthmatic and the nonasthmatic groups (data not shown). As the specific IgE levels were not affected, it is tempting to speculate that the reason for this profound anti-inflammatory property might be (similarly as with peptide mimotopes; see below) the absence of allergen-specific T cell epitopes in the anti-ids.[49] Surface plasmon resonance studies indicated that high affinity polyclonal IgE may exhibit a binding behavior similar to monoclonal antibodies, indicating that each Phl p 5 allergen might only harbor a low number of IgE epitopes.[50] Consequently, the anti-idiotypic strategy is expected to down-regulate IgE specificities being relevant and occurring frequently in the grass pollen allergic population.

DISTINCT TYPES OF ANTI-ID LIKE MOLECULES

Although antibodies are widely used for therapeutic applications, they have some potential drawbacks in large scale production including low expression yields and aggregation tendency. In this context, phage display technology does not only enable the selection of anti-ids, but also of peptide molecules, which structurally mimic B cell epitopes and therefore are called mimotopes.[51] Several allergen mimotopes were already defined by other working groups and ourselves, like for the

panallergen profilin,[52] the major fish allergen parvalbumin,[53] birch pollen allergens,[54] grass pollen allergens,[46,55] or house dust mite allergens.[56] Mimotopes lack allergen-specific T-cell epitopes and have been demonstrated to induce blocking IgG antibodies without stimulation of allergen-specific T-helper cells.[49,54] Furthermore, when therapeutically applied in a murine model of allergic asthma, they could, like reported here for an anti-idiotypic Fab fragment, prevent acute lung hypersensitivity and inflammation upon allergen rechallenge.[57]

Still, to achieve good quality of the immune response towards the relatively short peptide mimotopes, bystander T-cell help was needed. The immunogenic carrier for the mimotopes was in that case KLH providing bystander T-helper cell epitopes. Alternatively, tetanus toxoid (TT) could also be chosen as an immunostimulatory agent because of its promiscuous T-cell epitopes.[58]

Another possibility to enhance antigenic density is the attachment of linear peptides to tyrosine backbones. The so called multiple antigenic peptides (MAPs) can be synthesized in a straightforward manner and allow a dense display, most often as tetra- or octameric constructs.[59] Taken up by antigen presenting cells (APC), they are processed to T-cell epitopes and can activate T-cells too. However, controversial studies have raised the question whether or not the addition of a promiscuous T-helper epitope to a MAP is needed for achieving a sufficient immune response upon MAP-vaccination.[60]

Another option to overcome the limitations of antibody libraries are designed ankyrin repeat proteins (DARPins). These DARPin libraries are a source of β-hairpin structures, which mimic naturally occurring repeat proteins, and play an important role in innate immunity[61]; reviewed in.[62] In a study by Vogel et al, DARPin libraries were used to isolate ligands against the variable region of the nonanaphylactogenic anti-IgE antibody BSW17. Confirming the high specificity of the identified anti-idiotypic DARPins, they successfully prevented binding of BSW17 to IgE-sensitized rat basophils expressing human FcεRIα.[63] Recent molecular studies indicated that hairpin scaffold libraries render mimetics, which closely mimic the crystal structure of a native protein.[64] We are convinced that these novel developments will open up new avenues for anti-idiotypic strategies in allergy research.

SYNOPSIS

From several studies in the past it has emerged that natural anti-ids take part in the regulation of the immune response including immediate-type hypersensitivity. Obviously, each antibody response to an allergen is accompanied by the production of anti-idiotypic antibodies and anti-antibodies, together composing an idiotypic regulatory network. As predicted by Jerne, several studies could indeed show on the molecular level that anti-ids function as internal images of allergens. Thus, it may be hypothesized that anti-ids for allergen-specific IgE potentially nourish IgE memory in periods of allergen absence, like for instance outside the pollen season. In experimental studies, anti-ids have been able to specifically down-regulate, or vice versa, boost allergen-specific immune responses. This can on the one hand be because of the fact that they act on B- and T-lymphocyte antigen receptors in either a nonproductive (monovalent) manner or in a productive way by cross linking B cell receptors, or crosslinking B with T-lymphocytes, thereby forcing cellular crosstalk. Furthermore, anti-ids have been found in nonallergic individuals, pointing toward a protective regulatory mechanism.

Despite controversial in vitro results of the influence of anti-ids on the allergic response, levels of anti-ids usually get elevated during the course of specific immunotherapy (SIT). Therefore, anti-ids that mimic the relevant structural epitopes of an allergen are possibly attractive candidates for immunotherapy. For vaccination, mimotopes or DARPins represent novel anti-idiotypic alternatives. We propose that with anti-idiotypic tools, being synthetic or generated from the patient's antibody repertoire, immunologic disorders like allergies could be manipulated.

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CHAPTER II

The cutting edge between allergen-specific asthma versus nonspecific provocation by indirect calorimetry in BALB/c mice

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ABSTRACT

Besides immunologically mediated specific hyperresponsiveness towards the allergen, hyperreactivity against nonspecific triggers is a problem of great clinical relevance in patients with allergic asthma. We applied indirect calorimetry combined with blood cell and gas analysis for studying physiological and immunological differences between specific and nonspecific events in a mouse asthma model of grass pollen allergy.

Acute asthma towards allergen Phl p 5 was induced in BALB/c mice using an intraperitoneal prime and aerosol boost regimen. Allergen Phl p 5 or irrelevant antigen ovalbumin were used fro nebulization. Respiratory gas exchange and physical activity were measured before and after aerosol challenge by indirect calorimetry. Mice nebulized with Phl p 5 showed significant consumption of O_2 and significant production of CO_2, whereas physical activity was significantly reduced in both groups. Blood pH was also significantly decreased in both groups, accompanied by reduced bicarbonate levels in Phl p 5 challenged mice only. Phl p 5 rather than OVA challenge resulted in substantially decreased white blood cell counts associated with lymphoid infiltration into lungs. In both challenge groups, however, red blood cell counts decreased. The results illustrate that indirect calorimetry in combination with blood cell and blood gas analysis explains the difference of pathophysiological changes in acute allergic asthma versus nonspecific airway irritation. We suggest that this mouse model renders novel insights contributing to the understanding and diagnosis of human disease.

INTRODUCTION

Allergic asthma in humans is defined as a chronic inflammatory disorder of the airways and is characterized by airway inflammation, persistent airway hyperresponsiveness (AHR) and intermittent acute, reversible airways obstruction (Bousquet et al., 2000). Typically, the IgE-mediated, immediate obstruction is followed by a late phase reaction triggered by the release of inflammatory mediators like leukotrienes from cells recruited to the lungs. This results in respiratory and, consequently, physical deficits of patients for a considerable time after an asthma attack. Additionally, in the absence of allergen, even exposure to unrelated antigens may trigger short-breathlessness and wheezing, again limiting lung function and thus, seriously affecting the patients' quality of life.

Although mouse models of allergic asthma exhibit some limitations compared to the human disease, they have provided important information about the conditions necessary for allergen sensitization. The common basis for the induction of allergic asthma in mice is systemic sensitization followed by pulmonary challenges with the allergen. The asthma phenotype in mice mirrors the setting in human patients and is characterized by eosinophilic lung inflammation, increased Th2-type antibody isotype levels, mucus hypersecretion and airway hyperresponsiveness. However, the expression of each of these parameters can vary within the disease, dependent on the sensitization protocol used (Epstein, 2006).

We have previously adapted an acute asthma model in BALB/c mice for proof of concept studies of the therapeutic efficacy of Phl p 5 mimotope vaccines. Although the established airway inflammation was profoundly counterbalanced and protection from an allergen rechallenge could be achieved (Wallmann et al., 2010), the question remained how significant this asthma model would mimic the clinical setting. Therefore, in this study we evaluated this mouse model for significant effects on respiratory gas exchange.

Allergen-specific experimental asthma was induced in BALB/c mice, which included aerosol challenges with the specific allergen in one group, and added ovalbumin (OVA) from chicken

egg white as an irrelevant control antigen to compare and distinguish between specific allergen-induced asthma and nonspecifically triggered hyperreactivity in the hypersensitive animals. We used for the first time an open-circuit indirect calorimetry system (Pospisilik et al., 2010), which enabled us to analyze respiratory gas exchange of allergic mice before, immediately after and 48 hrs after aerosol challenges. By the use of an integrated infrared beam, we recorded the number of movements of mice simultaneously. Additionally we measured arterial blood parameters and evaluated blood cell counts in all naive and test groups of mice. To morphologically and immunologically describe differences in airway inflammation as a consequence of aerosol provocations with either specific or unspecific allergen/antigen, histochemistry and cytokine profiling were performed.

MATERIALS AND METHODS

Immunization of mice

Female BALB/c mice (n=20) were immunized intraperitoneally (i.p.) with rPhl p 5a (10 µg/100 µl PBS) on days 0 and 21. Ten days later, mice (n=10) were aerosol challenged with nebulized rPhl p 5a (protein and plasmid kindly provided by Arnd Petersen, Research Institute Borstel, Germany) (0.25 mg rPhl p 5a/100 ml PBS/challenge or Ovalbumin from chicken egg white 1%/100 mL PBS/challenge) with an ultrasonic nebulizer (Kendall, Aerodyne X, MTE Medizintechnik, Oldendorf, Germany) for 60 min twice daily with a four hours interval, on two consecutive days (day 31 and 32). Control groups were sensitized either with rPhl p 5a (n=5) or PBS (n=5) on days 0 and 21 and aerosol challenged 10 days later with PBS. One mouse group (n=5) remained naive. Measurements were performed on day 31 before, immediately after (day 32) and 48 hrs after aerosol challenges (day 34).

Respiratory gas exchange measurements

Mice (n=10) were put individually into Oxylet system chambers (Bioseb, France) separately) connected to an LE400 airflow unit (Bioseb, France) providing constant airflow. In brief, room air was pumped into the chamber housing a single, unrestrained mouse. The rate of the air supply (l/min) was adjusted for each individual chamber according to the animal's weight multiplied by 10 (i.e. 10ml air/g body weight /min). Oxygen (O_2) and carbon dioxide (CO_2) concentrations of automatically and continuously sampled air were measured using a highly sensitive and very fast response O_2/CO_2 sensor unit (LE 405 O_2/CO_2 gas analyzer, Bioseb, France). Data were recorded and analyzed using the Metabolism 2.1. software package (Panlab, Spain).

Assessment of activity

Activity was evaluated using InfraRed Actimeter frames equipped with infrared beam and detector units (Bioseb, France). Activity measurements were performed in parallel with respiratory gas analysis; on day 31 before aerosol challenges, on day 32 and on day 34.

Arterial blood gas analysis and differential blood cell counts

Arterial blood samples from the tails of mice were taken and measured by a radiometer ABL800 FLEX (Wiener Neudorf, Austria). Differential blood cell counts were performed simultaneously with an Abbott Cell Dyn CD 3500 CS Analyzer, Vienna, Austria. Measurements were performed with naive mice on day 0 and immediately after sensitized mice were aerosol challenged with either Phl p 5 or OVA on day 31. In addition to blood pH, carbon dioxide partial pressure (pCO2) and bicarbonate levels ($CHCO3^-$), concentration of hemoglobin (HGB) and hematocrit (HCT) were determined.

Beside red blood cells (RBC) and white blood cells (WBC), HGB and HCT were confirmed by differential blood cell counts.

Histopathology

On day 35, mice were anesthetized (200 µl 2% Rompun (Bayer, Leverkusen, Germany) and 5 ml Ketanest S (5mg/ml); 600 µl per mouse)) before fixation procedures were initiated. Lungs were fixed by intratracheal instillation which was performed with 4% paraformaldehyde in 0.1 M Sörensen's phosphate buffer (12.0 mM NaH_2PO_4, 69.0 mM Na_2HPO_4) (pH 7.4), as described (Forkert, 1995). Lungs were embedded into paraffin as described earlier (Wegmann et al., 2005). Systematic uniform random samples (SURS) of lung tissue were analyzed according to standard methods. Paraffin sections of 2 µm in thickness were stained with periodic-acid-Schiff (PAS) for airway epithelial mucus. The surface area of mucus-secreting goblet cells (S_{gc}) per total surface area of airway epithelial basal membrane (S_{ep}) and the

volume of PAS-stained epithelial mucus (V_{muc}) per S_{ep} were determined using a computer-assisted Olympus BX61 microscope equipped with the newCAST stereology tool box (Visiopharm, Hoersholm, Denmark) according to following formulas:

$S_{gc}/S_{ep}=\sum I_{gc} / \sum I_{ep}$

$V_{muc}/S_{ep}=L_P \times \sum P_{muc} / 2 \times \sum I_{ep}$

$\sum I_{gc}$ = sum of intersections of test-lines with goblet cells

$\sum I_{ep}$ = sum of all intersections of test-lines with epithelial basal membrane

$\sum P_{muc}$ = sum of all test-points hitting stored mucus

L_P = test-line length per test-point at final magnification (490x)

The volume of lymphoid follicles (V_{fol}) per total surface area of airway epithelial basal membrane (S_{ep}) was determined according to the formula:

$V_{fol} /S_{ep} = L_P \times \sum P_{fol} / 2 \times \sum I_{ep}$

$\sum I_{ep}$ = sum of all intersections of test-lines with epithelial basal membrane

$\sum P_{fol}$ = sum of all test-points hitting mucus

L_P= test-line length per test-point at final magnification (490x)

Eosinophilic infiltration

Paraffin sections of 2 µm in thickness were stained with Congo Red, according to (Pali-Scholl et al., 2008), for the assessment of airway wall infiltration with eosinophilic granulocytes. The degree of infiltration within each field of view collected according to SURS, was ascribed to one of the three scores; (A) no (B) one or (C) two ore more eosinophils in the airway wall.

RNA isolation and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the Trizol Reagent from Invitrogen (Invitrogen, Lofer, Austria) according to the manufacturer's instructions. RNA was quantified by measuring the absorption at 260/280 nm using a NanoDrop spectrophotometer (Nanodrop Technologies Montchanin, DE, USA). 3 µg of total RNA from each sample were reverse transcribed in a total volume of 20 µl using the high capacity cDNA reverse transcription kit (Applied Biosystems, Vienna, Austria), according to the manufacturer's instructions. 2 µl of cDNA were used for amplification by qRT-PCR which was performed with POWER SYBR®Green PCR Master Mix on an ABI StepOnePlus Realtime PCR System (Applied Biosystems, Vienna, Austria). IL-4, IL-5 and IL-13 mRNA levels were quantified by the comparative $\Delta\Delta C_T$ method using the SDS software (Applied Biosystems, Foster City, CA, USA).

For each of the samples, relative expression was normalized to the housekeeping gene Hypoxanthine-phophoribosyl-transferase (HPRT). Pooled cDNA of naive tissue was used as a calibrator and set the value 1; the relative expression levels of the other samples were compared hereto. Each sample was applied in duplicates. Following qRT-PCR program was used : 50°C for 2 min, 94°C for 2 min, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. Primers were designed using Primer Express (Applied Biosystems) and were located on different exons to prevent amplification of potentially contaminating genomic DNA. Following primer sequences were used:

IL-4: fwd primer: ccatgcttgaagaagaactctagtgt

rev primer: attcatggtgcagcttatcgatg

IL-5: fwd primer: gaggcttcctgtccctatcataa

rev primer: tacccccacggacagtttga

IL-13: fwd primer: ccatctacaggacccagaggat

rev primer: gggaggctggagaccgtagt

Statistical analysis

Statistical comparison between groups was performed by the Mann Whitney-U test, using the software SPSS (version 17.0 for Windows). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Challenging asthma with the specific allergen but not with an unspecific control antigen alters CO_2/O_2 exchange levels

To determine differences between allergic mice after allergen-specific and nonspecific pulmonary challenges, we evaluated respiratory gas measurements using indirect calorimetry. This system allows the determination of the respiratory gas exchange, i.e. CO_2 production and O_2 consumption of single mice.

Mice were primed i.p. with Phl p 5 followed by Phl p 5 aerosol challenges 10 days later to induce allergic asthma. Another group of mice was primed with Phl p 5 but aerosol challenged with irrelevant control antigen chicken egg white ovalbumin (OVA) from chicken egg white. Respiratory gas analysis in metabolic cages was performed before, immediately after and 48 hrs after aerosol challenges (Fig.1; sensitization and challenge protocol).

Atmospheric CO_2 levels increased immediately and significantly when Phl p 5 sensitized mice were nebulized with Phl p 5, but remained unchanged after challenges with OVA (Fig. 2 a), indicating endured and enforced respiration in allergen-, but not in nonspecific airway provocation. O_2 levels dropped in calorimetric cages of both groups immediately after aerosol challenges, indicating increased O_2 consumption in the acute phase probably due to faster and deeper breathing. However, in the late phase (48 hrs after challenge), gas exchange continued to be increased in the Phl p 5 challenged mouse group. At the 48 hr timepoint, O_2 consumption was still significantly enhanced after specific challenges with Phl p 5 but not with OVA (Fig. 2 b) indicating that animals provoked with a nonspecific irritant recovered faster from alteration in gas exchange.

Alteration in gas exchange after challenge with specific allergen and irrelevant antigen results in a significant reduction of motor activity

The physical activity pattern of mice was recorded before and after aerosolizations by counting the rearings (Z-axis movements) at three different timepoints; before, immediately after challenge and 48 hrs later. Rearing of mice could be assessed simultaneously with respiratory gas exchange measurements by the use of an infrared beam attached to the calorimetric cages. Regardless of the allergen/antigen used for provocation of the airways, physical activity was in both mouse groups drastically reduced (Fig. 2 c). Importantly, at the 48 hr timepoint, activity continued to be significantly reduced, irrespective of the agent used.

Blood gas analysis of Phl p 5-challenged allergic mice indicates impaired lung function combined with a metabolic disorder

The results of the gas exchange measurements had indicated that airway challenge with Phl p 5 led to profoundly increased consumption of O_2 and production of CO_2 in the asthma attack. Consequently, arterial blood samples from mice before sensitization and aerosol challenges (with either Phl p 5 or OVA) and right after were subjected to blood gas analysis (Fig. 3). Both challenged mouse groups showed a significant drop in pH (Fig. 3 a), pointing towards acidosis. Blood pCO_2 remained relatively unchanged (Fig. 3 c), whereas $cHCO3^-$ was significantly reduced in Phl p 5-specific, but nonsignificantly in OVA challenged mice (Fig. 3 b). Control mice which were sensitized and challenged with PBS and mice which were sensitized with Phl p 5 but challenged with PBS did not show variations of these parameters (data not shown). Taken together, these results indicate a higher rate of decompensation in the allergen-challenged mouse group.

Peripheral erythrocyte numbers substantially decline after specific and nonspecific aerosol challenges in hypersensitive mice

Besides their function in O_2/CO_2 transport, erythrocytes bind and produce $HCO3^-$, thus representing important buffer systems in the blood. Being effectively recirculated, they balance therefore pH extremes. When evaluating peripheral red blood cell counts (RBC), hemoglobin (HGB) and hematocrit (HTC) before and after aerosol challenge, we observed significant decreases of these parameters in the allergen-challenged groups, suggestive for increased localization of erythrocytes in the lung (Fig. 4).

Allergen-triggered acute asthma but not nonspecific respiratory challenge is associated with increased airway inflammation

Systemic sensitization combined with nebulization with Phl p 5 renders acute allergic asthma characterized by airway inflammation and mucus hypersecretion (Wallmann et al., 2010). Here, we determined to what extent aerosol challenge of Phl p 5-allergic mice with an irrelevant antigen, i.e. control antigen OVA, would induce airway inflammation as compared to the original allergen. For this purpose, systematic uniform random samples (SURS) were collected from the entire lung and analyzed for goblet cell metaplasia, mucus production, lymphoid follicles and infiltration of the airways with eosinophils 64 hrs after aerosol challenge (Fig. 5). In contrast to mice challenged with the allergen Phl p 5, mice immunized with Phl p 5 but challenged with OVA exhibited hardly any goblet cell metaplasia (Fig. 5 a) or mucus production (Fig. 5 b and c-e).

When we assessed white blood cell counts (WBC) in arterial blood of Phl p 5-asthmatic mice, peripheral leukocyte numbers were substantially decreased in the allergen-exposed group but remained more or less unchanged in the group nebulized with control antigen OVA (Fig. 6 a). On the contrary, leukocytes seemed to infiltrate the airways where significant numbers of lymphoid follicles were found when mice were challenged with the specific allergen, slightly

less upon OVA challenge (Fig. 6 b and d-f). To determine whether these infiltrates are associated with eosinophilic immigration, we determined the percentage of those FOVs (field of views) exhibiting airway walls containing eosinophils. Eosinophilic infiltration was significantly increased in both, OVA and Phl p 5 challenged allergic mouse groups compared to naive controls (Fig. 6 c).

Allergic mice aerosolized with the specific allergen exhibited higher Th2 cytokine mRNA production in splenocytes and lungs.

To understand the events leading to the profound physiological and immunological differences between allergen- and nonspecific challenge, we next analyzed the expression of Th2 associated cytokines. As a measure for systemic immune deviation, spleen cells were investigated and compared to local effects in lungs. The spleens and the left lung of each mouse were isolated 64 hrs after the last treatment. Relative expression of IL-4, IL-5 and IL-13 mRNA levels was quantified by qRT-PCR.

Aerosol challenges of Phl p 5-allergic mice with OVA did neither considerably elevate Th2 cytokine mRNA in spleens nor lungs as compared to completely naive animals. As expected, challenges with the specific allergen Phl p 5 elevated Th2 cytokine mRNA production in lungs and even more significantly in spleens (Fig. 7, a, b and c). These data indicate that significant transcription of IL-4, IL-5 and IL-13 is turned on as a consequence of specific allergen challenge and that it is a systemic event.

DISCUSSION

In allergy research, innovative mouse models and methods of acute allergic asthma are urgently needed for proof of concept studies (Mojtabavi et al., 2002). Using major grass pollen allergen Phl p 5 as a model allergen, we have recently tested the treatment efficacy of a peptide mimotope vaccine with respect to airway inflammation and cytokine secretion in a memory model of asthma (Wallmann et al.,2010).

In addition to the former approach, the present study was designed to actually evaluate the pathophysiological changes in acute allergic asthma of mice. In addition, we introduced a mouse group being aerosol challenged with an irrelevant antigen to define the cutting edge between pathophysiological changes in allergen-specific asthma versus nonspecific airway provocation. Today, a number of invasive as well as non-invasive tests are available for the experimental determination of lung function (Drazen et al., 1999). For the acute mouse model, we took here for the first time advantage of a highly sensitive open-circuit indirect calorimetric system able to record, besides parameters like food and drink intake, activity and respiratory metabolism (Dominguez et al., 2009).

After development of acute allergic asthma of Phl p 5 hypersensitive mice due to aerosol challenges with the specific allergen, O_2 and CO_2 levels in the ambient air were monitored.

Independent whether specific allergen or irrelevant antigen was used for the provocation, the O_2 levels in the ambient air decreased significantly, indicating enhanced oxygen consumption. 48 hrs after challenge, the allergen-challenged animals had not recovered yet. Simultaneously, CO_2 levels in the air increased significantly in the allergen-exposed group only, indicating enhanced expiration in the acute phase, possibly an attempt for compensating the observed acidosis. Multiple potential causes of the acidosis are discussed, among them lactic production by respiratory muscles or tissue hypoxia during respiratory distress, at least in humans (Mountain et al., 1990). We failed to determine lactic acid in the samples, even though the pH values in the blood had dropped significantly. Further, significantly decreased

bicarbonate levels combined with slightly reduced pCO2 levels were recorded in arterial blood upon specific challenges with Phl p 5, reminding to human reports on metabolic acidosis in severe asthma (Meert et al., 2007, Mountain et al., 1990, Roncoroni et al., 1976) . This level of severity is not reached in our model, but on the other hand it has not been investigated so far which intensity of asthma in humans would lead to metabolic effects (Rashid et al., 2008). Interestingly, metabolic acidosis was recently associated with a bronchodilatory effect and thus may resolve bronchial obstruction (Brijker et al., 2009). Surprisingly, a substantial decrease of red blood cells, hematocrit and hemoglobin levels right after allergen challenges of the airways, with twofold significance upon specific airway provocation with Phl p 5 was observed. To the best of our knowledge, no reports on effects of asthma on red blood cells have been made so far, but sporadic evidence suggests that human asthmatics may experience a decrease of hematocrit during recovery from acute episodes (Gopalan et al., 1983). In mouse models of allergic asthma, the degree of overall airway inflammation, the cellular composition and location of infiltrates very much depends on the experimental disease induction protocol used (Epstein, 2006). In the memory model of acute asthma analyzed here, immunization and aerosol challenges with Phl p 5 rendered an overall modest induction of mucus production, lymphoid follicle formation and eosinophilic infiltration in the airways. The simultaneous drop of white blood cells in the periphery may illustrate the dynamics of leukocytes immigrating into the lung. Most prominent inflammation was observed in the proximal parts of the lung, perfectly representing the phenotype of acute asthma.

Nebulization with an irrelevant antigen rendered milder overall lung inflammation, characterized by less and smaller lymphoid infiltration than following a challenge with the specific allergen. Eosinophilic infiltration of the airways occurred to a similar extent as in the allergen challenged mouse group.

In the sensitization process with an allergen, a number of different Th_2-associated cytokines are implicated. Among them, IL-4 plays a pivotal role in the production of cytophilic IgE antibodies, which, upon crosslinkage by the corresponding allergen, induce the release of proinflammatory compounds leading to acute airway obstruction. In concert with IL-13, IL-4 narrows airways by causing epithelial cells to swell and increases the contractibility of airway smooth muscle cells, thereby playing an essential role in AHR (Corry et al., 1996, Galli et al., 2008). IL-5 is primarily engaged in the recruitment of eosinophils, with a possible role in airway injury acting possibly pro-inflammatory, or, in contrast, supporting airway remodeling (Bochner et al., 2005). Thus, the role of eosinophils in the clinical manifestion of asthma such as AHR has been questioned recently (Leckie et al., 2000, Radinger et al., 2009, Tournoy et al., 2000). IL-13 is involved in IgE isotype switching, but also in the effector phase of asthma by supporting goblet cell metaplasia and mucus production (Mitchell et al., 2010).

As expected, IL-4, IL-5 and IL-13 mRNA levels from splenocytes of mice being sensitized *and* challenged with Phl p 5 were significantly increased in comparison to Phl p 5 sensitized, but OVA challenged mice. Interestingly, qRT-PCR measurements of the lungs indicated that similar to the systemic events, locally a significant elevation of IL-4 and an upward trend of IL-5 and IL-13 levels occurred.

In summary, we demonstrate that respiratory exposition of grass pollen allergic mice with the specific allergen induces acute enforced respiration characterized by increased O_2 consumption and CO_2 expiration, combined with metabolic acidosis, decreased motor activity and persistent local Th2-related airway inflammation. The impairment of respiration and tissue inflammation was profound and mice had not recovered 48 hrs after allergen challenge. In contrast, challenges with irrelevant antigen produced increased respiration which was, however, less pronounced and could be easier compensated. Further, also Th_2-type lung inflammation was less pronounced upon nonspecific aerosol challenge. In both cases, we found significant impact of the provocation on the re-distribution of white and red blood cells

from the periphery to the lung. Future studies will address whether these phenomena are also relevant in human asthma patients.

Taken together, our study clearly demonstrates that acute allergen-induced asthma versus provocation with a nonspecific trigger can be monitored and precisely categorized by the use indirect calorimetry in metabolic studies. We suggest that this novel application may also open new avenues towards understanding the events which are specifically and nonspecifically affecting the quality of life of asthma patients.

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We would like to thank Franziska Ganzert and Erika Bajna for their excellent technical assistance. This work was supported by SFB project F1808-B13 of the Austrian Science Fund and the German Research Foundation (Cluster of Excellence EXC306). HE was supported by the Vienna Science and Technology Fund (project LS07-058).

FIGURES

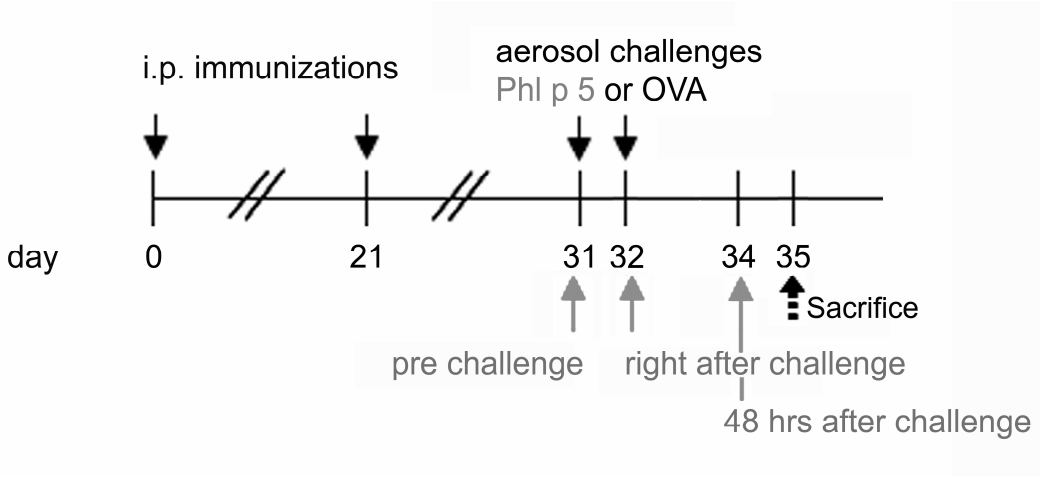

Figure 1. Schematic protocol flow chart for induction and metabolic monitoring of grass pollen-induced acute asthma in mice. Airway inflammation was induced in BALB/c (n=20) sensitized with 10 µg Phl p 5 (days 0 and 21) and airway challenged twice on two consecutive days (day 31 and 32) with Phl p 5 or OVA. Nebulization of mice was performed twice a day with a four hours interval on two consecutive days in a plastic chamber connected to an ultrasonic nebulizer. Atmospheric O_2 and CO_2 levels within metabolic cages housing the nebulized mice were assessed (before, immediately after and 48 hrs after aerosol challenges). Mice were sacrificed (day 35) for tissue harvest. Sensitization and aerosol challenges are indicated with black arrows. Time points of metabolic cage measurements are highlighted in grey. Numbers below the x-axis represent days.

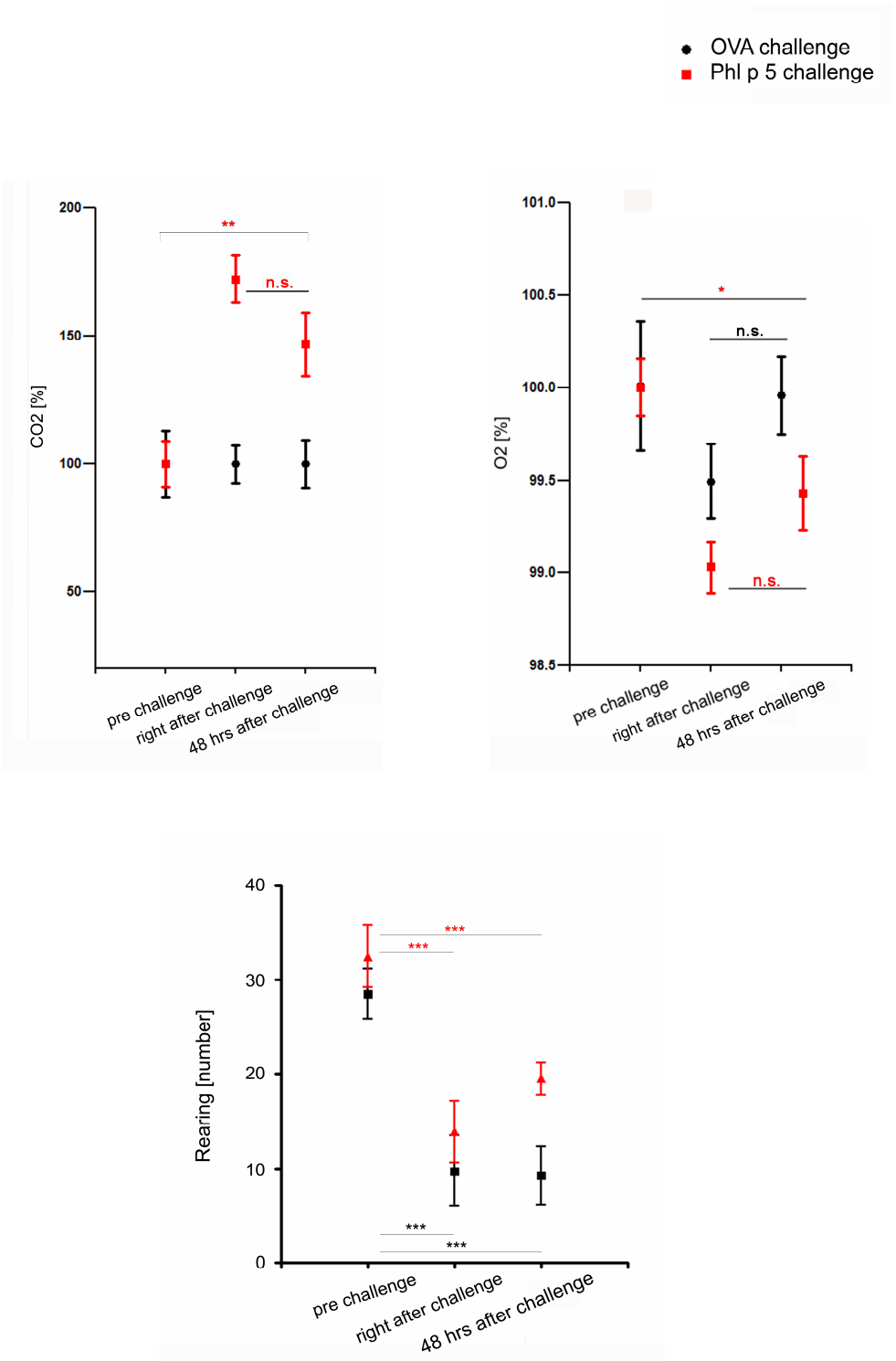

Figure 2. Nebulization of Phl p 5-allergic mice with the allergen, but not with an irrelevant antigen induces acute hyperventilation and decreased motor activity. BALB/c mice were sensitized by two i.p. injections with rPhl p 5a and ten days later nebulized with either aerosolized rPhl p 5a (n=10) or OVA in PBS (n=10). Respiratory gas analysis of mice was performed after they were put in metabolic cages, each cage housing one animal. Measurements were done before, right after and 48 hrs after the challenge nebulization.

Atmospheric CO_2 (a) and O_2 (b) concentrations within metabolic cages housing Phl p 5-allergic, unchallenged mice were set 100% and the values after airway challenges were compared hereto. Simultaneously, mice were analyzed for their motor activity, whereby rearing of each mouse was recorded by an integrated laser (c). Data points of Phl p 5-sensitized mice being subsequently challenged with OVA are represented in black, data points of mice immunized and aerosolized with Phl p 5 in red. Timepoints of measurements are indicated below the x-axis. Data are mean ± SD. n = 5 mice/group. Two independent experiments were performed. *, P < 0.05 and **, P < 0.01 for challenged versus unchallenged mice.

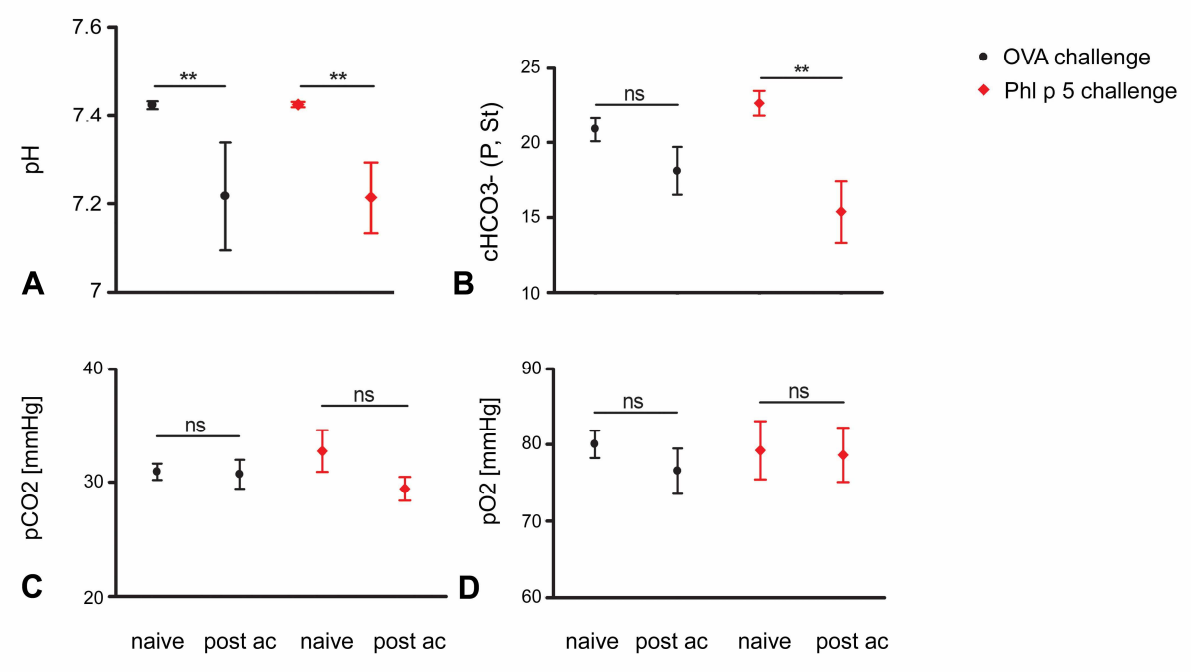

Figure 3. Induction of acute Phl p 5-specific asthma causes an acid-base imbalance in mice. Mice were sensitized with Phl p 5 and subsequently aerosol challenged with either Phl p 5 or OVA. Arterial blood samples, taken from the tail artery of mice before sensitization and after airway challenges were subjected to blood gas analysis.

pH (a) bicarbonate (b) pCO_2 (c) and pO_2 (d) levels in arterial mouse blood before sensitization and immediately after aerosol challenges (timepoints are indicated below the x-axis). Black data points represent the mouse group sensitized with Phl p 5 but challenged with OVA, the red squares represent the mouse group sensitized and aerosol challenged with Phl p 5. Data are mean ± SD. n = 5 mice/group. Two independent experiments were performed. *, P < 0.05 and **, P < 0.01 for challenged versus naive mice.

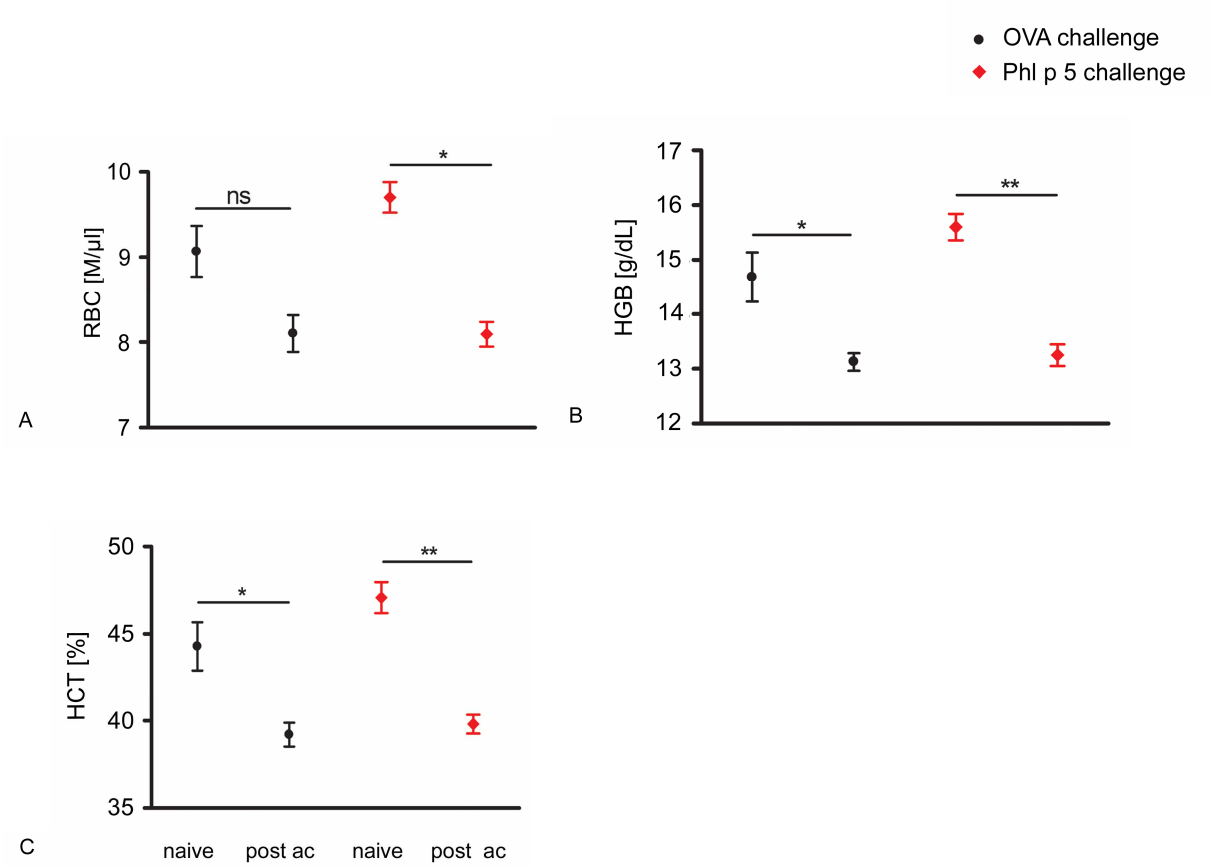

Figure 4. Specific and nonspecific airway provocation of allergic mice impact red blood cells. Mice were sensitized with Phl p 5 and subsequently aerosol challenged with either Phl p 5 or OVA. Arterial blood samples, taken from the tail artery of mice before sensitization and after airway challenges were subjected to differential blood cell counting. RBC (a), HGB (b) and HCT (c) levels of naive, unsensitized mice and after they were sensitized and nebulized are shown. Black points represent the mouse group when sensitized with Phl p 5 but aerosol challenged with OVA, the red squares refer to mice challenged with Phl p 5. Data are mean ± SD. n = 5 mice/group. Two independent experiments were performed. *, $P < 0.05$ and **, $P < 0.01$ for challenged versus naive mice.

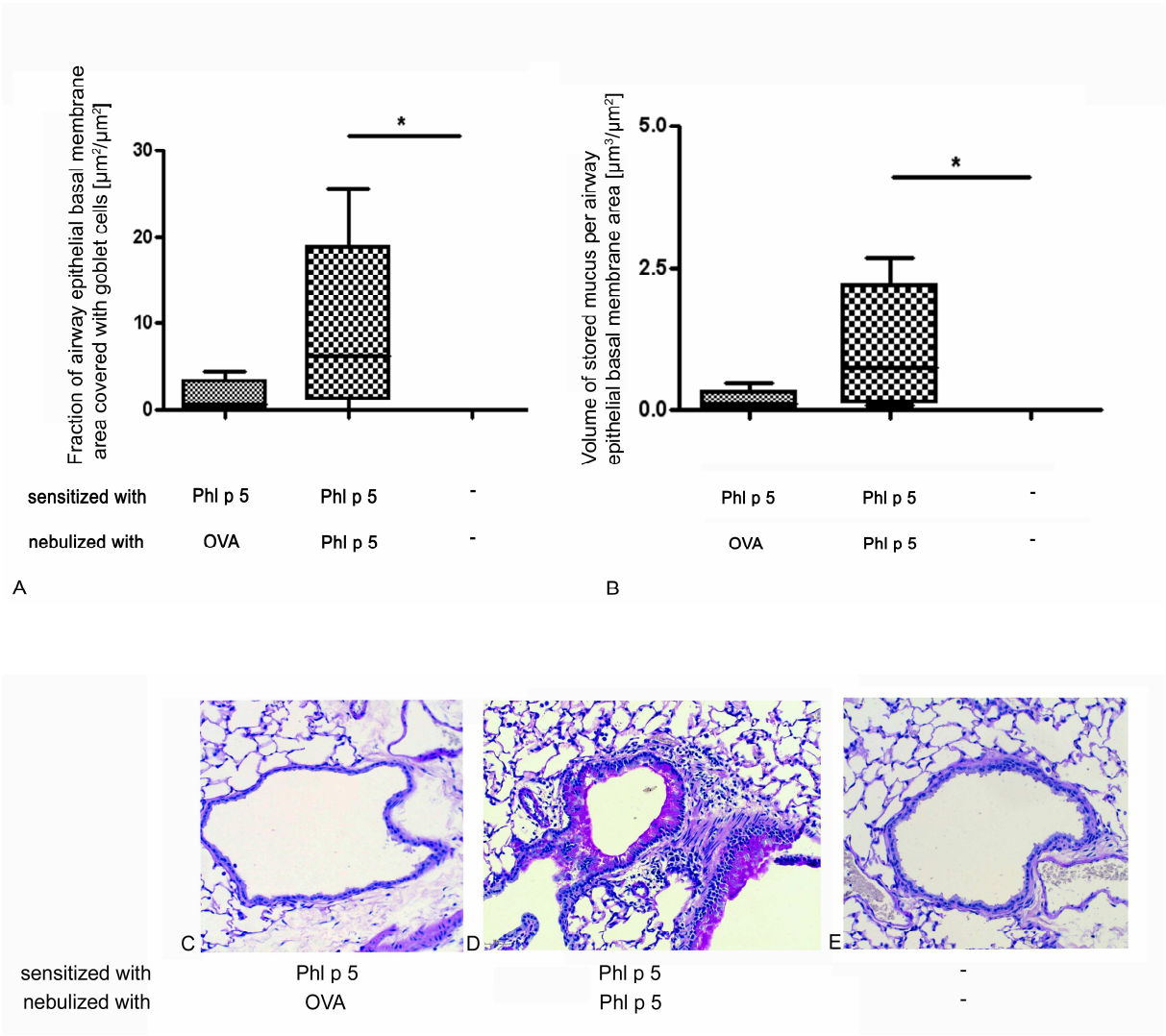

Figure 5. Airway inflammation is induced upon challenge with the specific allergen, and less by the nonspecific antigen. Mice were sensitized with Phl p 5 and aerosol challenged with Phl p 5 or OVA. Three days later mice were anesthetized, lungs were fixated by tracheal installation and vascular perfusion was performed. Paraffin-embedded lungs were sectioned (2 µm) and stained with Periodic Acid Schiff for detection of mucus and mucus-secreting goblet cells (a and b). The volume of PAS-stained epithelial mucus and the area of epithelial basal membrane covered by goblet cells was determined as per total surface area of airway epithelial basal membrane. Representative lung histology from Phl p 5 sensitized and OVA challenged (c), Phl p 5 sensitized and Phl p 5 challenged (d) and naive, unsensitized mice (e). Data are mean ± SD. n = 5 mice/group. Two independent experiments were performed. *, $P < 0.05$ and **, $P < 0.01$ for challenged versus naive mice.

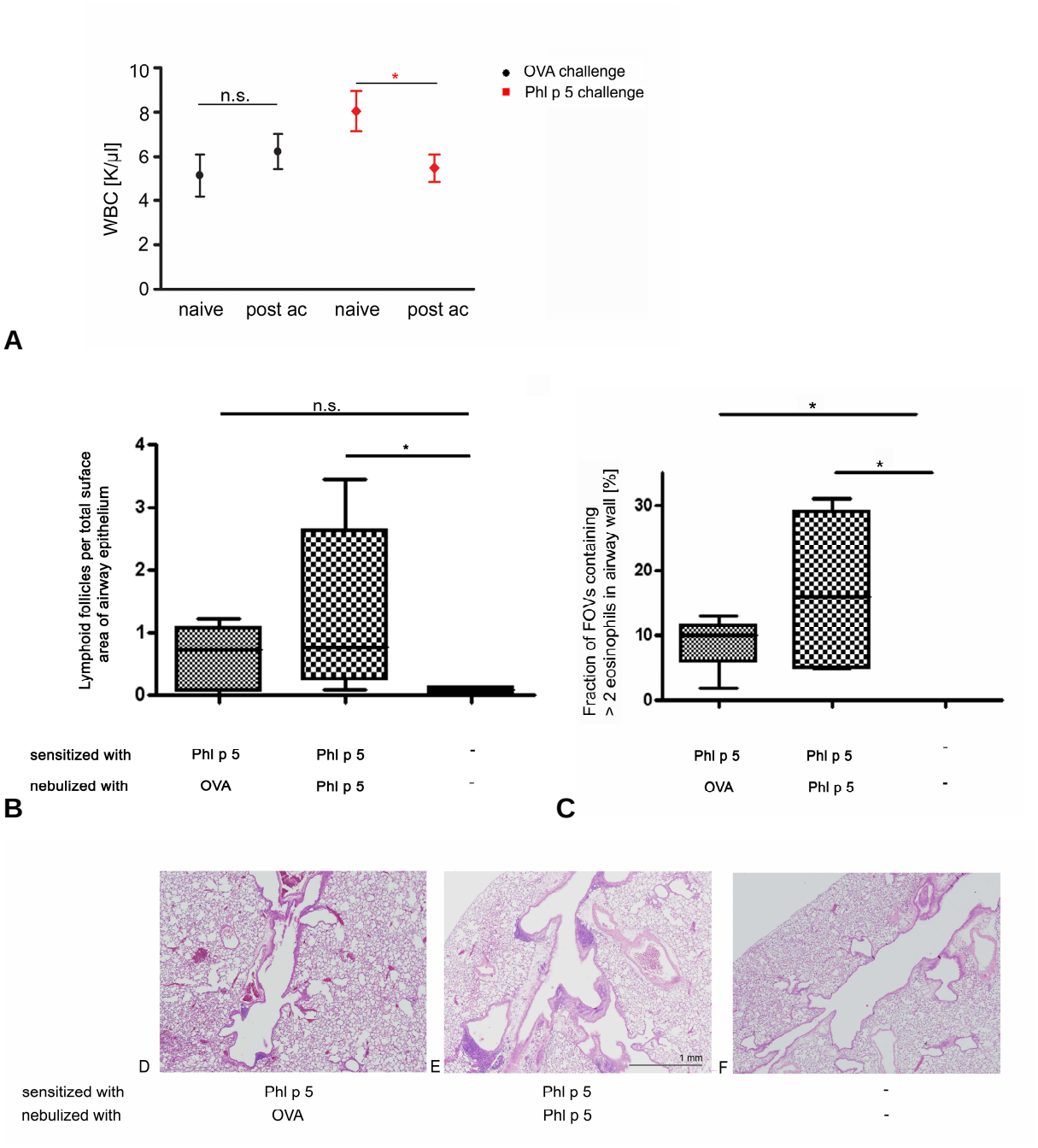

Figure 6. Aerosol allergen challenge specifically attracts leukocytes to lungs. White blood cells were assessed from arterial blood of naive mice and after they were airway challenged. Differential blood cell counts were analyzed from blood of the tail artery of naive mice and after i.p. immunization with Phl p 5 and subsequent nebulization with OVA or Phl p 5 (a). Black points represent the mouse group sensitized with Phl p 5 but aerosol challenged with OVA, the red squares refer to mice challenged with Phl p 5. Mice were anesthetized on day 36 after the final nebulization and lungs were fixed by tracheal installation. Paraffin-embedded lungs were sectioned (2 µm) and stained with congo-red for eosinophils and hematoxylin and eosin (H&E) for lymphoid follicles. The volume of lymphoid follicles per

unit surface area of airway epithelial basal membrane was assessed (b). Eosinophilic infiltration of the airways was semi-quantitatively estimated as percentage of SURS sampled fields of view containing >2 eosinophils in the airway wall (c). The sensitization modality is indicated below the x-axis. Naive mice served as negative controls. Lung histology representatives with respect to lymphoid follicles from Phl p 5 sensitized and OVA challenged (d), Phl p 5 sensitized and Phl p 5 challenged (e) and naive, unsensitized mice (f). Data are mean ± SD. n = 5 mice/group. Two independent experiments were performed. *, $P < 0.05$ and **, $P < 0.01$ for challenged versus naive mice.

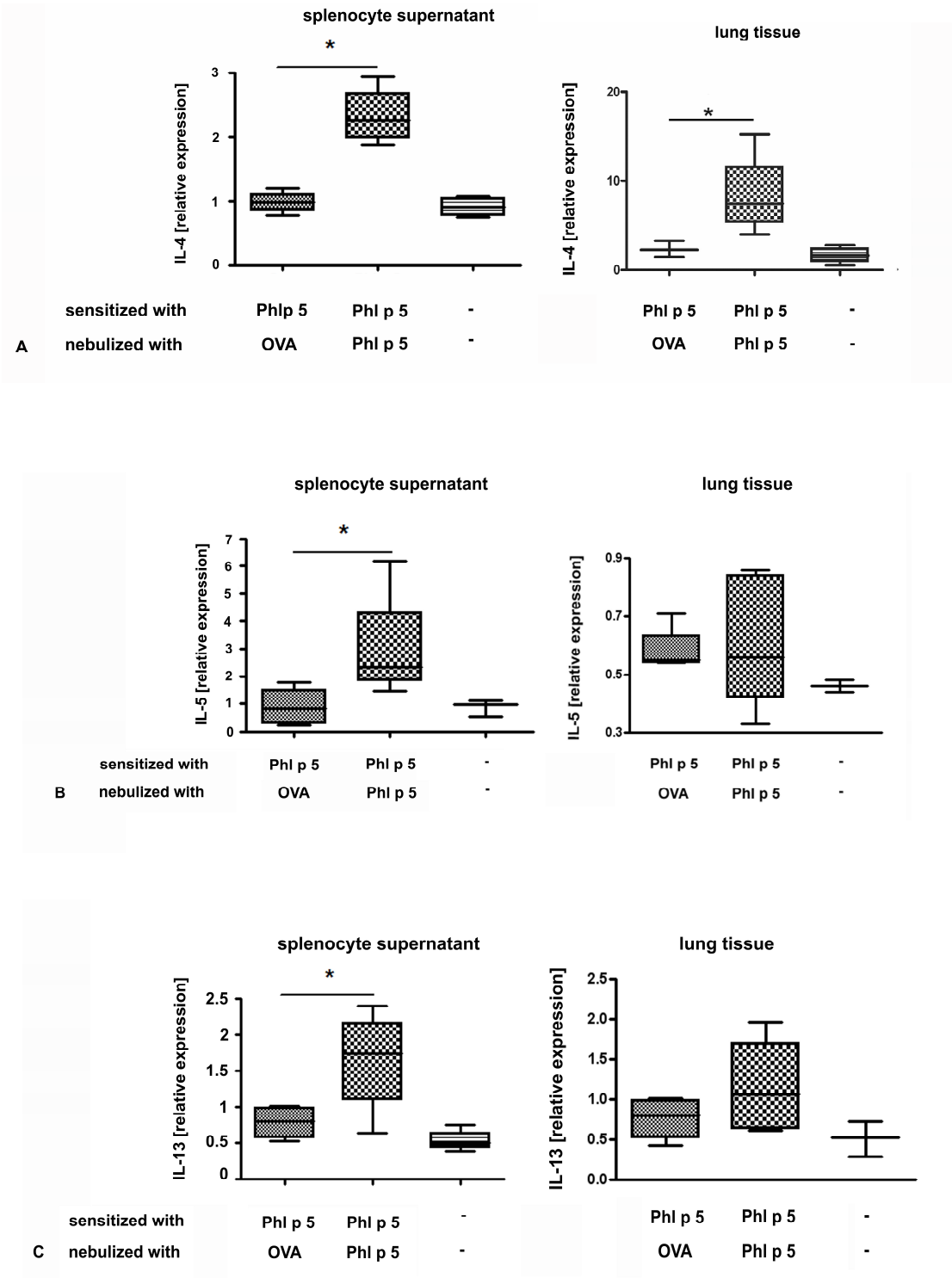

Figure 7. Nebulization of allergic mice with the allergen increases local and systemic Th2 cytokine levels. Mice were sensitized and subsequently aerosol challenged with either the specific allergen or an unspecific control antigen. After the final airway challenges, mice were sacrificed and the spleen and left lung lobe of each mouse were isolated. Organs from naive mice served as negative controls. RNA from spleens and lungs were extracted, reverse

transcribed and the cDNA was used for amplification by qRT-PCR. Pooled cDNA of naive tissue was used as a calibrator and set the value 1; expression levels of the other samples were compared hereto. Th2 associated cytokine mRNA from splenocytes of Phl p 5-allergic mice after Phl p 5 or OVA nebulization was assessed. IL-4 (a), IL-5 (b) and IL-13 (c) mRNA levels from spleens (left) and lungs (right) of challenged mice. Naive organs served as negative controls. The sensitization modality is indicated below the x-axis. Data are mean ± SD. n = 5 mice/group. Two independent experiments were performed. *, $P < 0.05$ and **, $P < 0.01$ for challenged versus naive mice.

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ORIGINAL ARTICLE

Mimotope vaccination for therapy of allergic asthma: anti-inflammatory effects in a mouse model

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Clinical & Experimental Allergy

Summary

Background One of the concerns of allergen-specific immunotherapy is the possible boost of inflammatory allergen-specific T lymphocytes. To address this problem, treatment with B cell epitopes devoid of allergen-specific T cell epitopes would be a promising alternative.

Objective In this study, we examined the therapeutic potency of a single mimotope, mimicking a structural IgE epitope of grass pollen allergen Phl p 5 in an established memory mouse model of acute allergic asthma.

Methods In the experimental set-up, BALB/c mice were primed with intraperitoneal injections of recombinant Phl p 5a (rPhl p 5a) and subsequently aerosol challenged with the nebulized allergen. Mice developed signs of bronchial asthma including hypereosinophilia around bronchi, goblet cell hyperplasia and enhanced mucus production.

Results When the mice were subsequently treated with the grass pollen mimotope coupled to keyhole limpet haemocyanin, bronchial eosinophilic inflammation and mucus hypersecretion decreased. Further, a decrease of Th2 cytokines IL-4 and IL-5 could be observed in the bronchoalveolar lavage (BAL). In contrast to rPhl p 5a, the mimotope was *in vitro* not able to stimulate splenocytes to proliferation or IL-5 production. Despite not affecting the levels of pre-existing IgE, vaccination with the single mimotope thus rendered anti-inflammatory effects in a mouse model of acute asthma.

Conclusion From our data, we conclude that vaccination with a mimotope peptide representing a single IgE epitope of the allergen Phl p 5a and being devoid of allergen-specific T cell epitopes is able to down-regulate inflammation in acute asthma.

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Introduction

To date, specific immunotherapy (SIT) of allergy is the only causative treatment with long-term effects in patients suffering from IgE-mediated allergic disorders. The efficacy of SIT is well documented [1]. It is ascribed to a down-regulation or modulation of the allergen-specific Th2 response and is accompanied by the induction of allergen-specific blocking antibodies [2]. Furthermore, peripheral T cell tolerance is turned on, initiated by the autocrine action of IL-10 and TGF-β which are produced by antigen-specific T cells in health and disease [2, 3].

On the other hand, clinical studies with T cell epitope peptides have indicated that the directed stimulation of allergen-specific regulatory T lymphocytes might be difficult due to the danger of recruitment of inflammatory T cells [4, 5]. Therefore, treatments with B cell epitopes devoid of T cell epitopes could be an attractive option. For this, mimotopes, i.e. small peptides able to imitate structural B cell epitopes by molecular mimicry, could be useful. Indeed, in a recent study, a mimotope cDNA construct has been successfully shown to trigger allergen-specific antibody responses without the activation of allergen-specific T cells [6]. To ensure sufficient immunogenicity of pure B cell epitopes, T cell bystander help is needed and can be provided by the carrier of the peptides [7]. Keyhole limpet haemocyanin (KLH) is an attractive carrier as it allows the dense and rigid display of coupled peptides.

With respect to grass pollen allergens, we have been focusing on major timothy grass allergen Phl p 5a in

previous studies and identified mimotopes from different types of phage libraries [8, 9]. Three-dimensional analysis showed structural, discontinuous surface patches of Phl p 5a as being relevant for IgE binding. The mimotopes were able to interfere with the high-affinity interaction of IgE with recombinant Phl p 5a (rPhl p 5a) ranging in the order of 10^{-11} M [8]. Consequently, the goal of the present study was to examine for the first time the therapeutic efficacy of a decameric peptide mimotope of Phl p 5a, termed here Phl mim 5. In an established memory mouse model of acute allergic asthma [10], we mimicked the human sensitization process with Phl p 5 during the flowering period via inhalative allergen challenges to induce an asthmatic phenotype including inflammation and hypersecretion of mucus. To evaluate whether vaccination with the Phl mim 5-peptide mimotope linked to KLH could protect from acute asthma upon re-exposure to the allergen, mice were, after vaccination therapy, re-challenged with aerosolized allergen.

Materials and methods

Generation of peptide mimotopes

In a previous study, a phage clone encoding the peptide mimotope C-KLGKFGAARV-C (1,12 cyclo), here termed Phl mim 5, could be identified as a specific epitope mimic (mimotope) of the IgE epitope of grass pollen major allergen Phl p 5 [8]. This mimotope [molecular weight (M_w): 1895.1 Da] was synthesized (piCHEM, Graz, Austria) with a purity >95% and coupled to didecameric KLH (Sigma-Aldrich, Sulzfeld, Germany), which has a M_w of approximately 800 kDa with 50–75 potential binding sites. The KLH used in this study is for preclinical purposes and has no GMP quality like its monomeric counterpart used for human clinical trials. The sequence GPGPGK-(S-acetomercaptoacetic acid)-G was used as a linker.

Animals

BALB/c mice (female; 5–6 weeks) were purchased from Charles River Laboratories GmbH (Sulzfeld, Germany) and treated according to European Community rules of animal care [11] with the permission of the Austrian Ministry of Science (number BMBWK-66.009/0233-BrGT/2006).

Immunization of mice

Mice ($n = 5$) were immunized subcutaneously (s.c.) with Phl mim 5-KLH [20 μg/100 μL phosphate-buffered saline (PBS); piCHEM] on days 0, 7, 14 and 28. Control mice ($n = 5$) were immunized s.c. with rPhl p 5a (10 μg/100 μL PBS) (Biomay, Vienna, Austria) on days 0 and 21. Blood samples to be analysed were taken after the last immunization.

For the asthma model, mice ($n = 15$) were immunized intraperitoneally (i.p.) with rPhl p 5a (10 μg/100 μL PBS) on days 0 and 21 or were sham treated with sterile PBS (100 μL). Ten days later, mice were aerosol challenged with nebulized rPhl p 5a (0.25 mg rPhl p 5a/50 mL PBS/challenge) in a plexiglass chamber by an ultrasonic nebulizer (Kendall, Aerodyne Omega, Oldenburg, Schleswig-Holstein, Germany) for 60 min twice daily at a 4-h interval on 2 consecutive days (days 31 and 32). At this stage, physiologically relevant airway hyperreactivity was proven by respiratory metabolism studies using the Oxylet system (Panlab, Barcelona, Spain).

On days 48, 76, 84, 119, 126 and 150, mice ($n = 5$) were immunized s.c. with either KLH (10 μg/100 μL PBS; Sigma-Aldrich), Phl mim 5-KLH (20 μg/100 μL PBS; piCHEM) or rPhl p 5a (2 μg/100 μL). During the entire treatment period with the mimotope vaccine, no local or systemic side-effects could be observed in the animals. Unsensitized control mice ($n = 5$) were sham treated (100 μL PBS). For disease relapse, mice were re-challenged with aerosolized rPhl p 5a on day 172 following treatment (0.25 mg rPhl p 5a/50 mL PBS per aerosol challenge).

Blood samples were drawn on days 0 (preimmune serum), 22 (after systemic sensitization), 47 (after aerosol challenge), 55, 84, 102, 126, 157 (during treatment) and on day 172 (before sacrifice).

Specific serum immunoglobulin G1 antibody detection in immunodot

Analysis of IgG1 from immunized mice was performed in immunodot. Synthetic mimotope Phl mim 5 alone or coupled to KLH, rPhl p 5a, rDer p 2 (kindly provided by Prof. Dr J. M. Saint-Remy, University Leuven, Belgium) as a control allergen and a control peptide (C-AISGGYPV-C cyclo 1–12, a mimotope of profilin [12]) were dotted in triplicates (1 μg/dot) onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Niedersachsen, Germany). Dot strips were air-dried and blocked with TBS/0.5% Tween-20 (TBST) (Merck, Darmstadt, Germany)/5% dry milk powder (DMP) overnight at 4 °C. Pooled mouse sera were diluted 1 : 10 in 0.5% TBST/1% DMP and incubated for 4 h at 4 °C. Bound IgG1 was detected using IgG1-specific rat anti-mouse antibody (PharMingen, San Diego, CA, USA). The reaction was developed using the ECL Plus Western Blotting Detection System (Amersham, Buckinghamshire, UK).

Specific serum immunoglobulin G1 and E antibody detection in enzyme-linked immunosorbent assay

For measurement of Phl p 5-specific IgE antibodies in an ELISA, microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with rPhl p 5a (1 μg per well/100 μL 50 mM $NaHCO_3$, pH 9.6), washed with TBST and blocked for 2 h at room temperature (RT) with TBST/1% bovine serum albumin (BSA) (Sigma-Aldrich).

Sera were diluted 1 : 100 in TBST/0.1% BSA for IgG1 and 1 : 10 for IgE and incubated overnight at 4 °C. Plates were washed and the respective isotype-specific rat anti-mouse antibody (PharMingen) 1 : 500 in TBST/0.1% BSA was added for 2 h at RT. After washing, plates were incubated with horseradish peroxidase-conjugated mouse anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1 : 1000 in 0.05% TBST/0.1% BSA for 2 h at RT. Standard serial dilutions of the respective purified mouse antibody (PharMingen) served as the standard. The reaction was detected by adding 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (Sigma-Aldrich) in citric acid and hydrogen peroxide. The optical density was measured at 405–490 nm in a microplate reader (Molecular Devices, München, Germany).

Recovery of the bronchoalveolar lavage and preparation of cytospin slides

The tracheas of lethally anaesthesized mice (naïve, asthmatic or mimotope-treated) were cannulated (BD Venflon, 0.8×25 mm; Heidelberg, Baden Württemberg, Germany), the supernatants were collected individually and cell pellets were resuspended in 500 μL PBS (2×10^4 cells) after centrifugation at 800 g for 18 min. Total leucocytes were counted with a haemocytometer. Cytospin slides were prepared and stained with May–Grünwald–Giemsa [13] to determine the cell differential. BAL supernatants were individually collected for cytokine measurements.

Counts of inflammatory cells by Tissue FAXs technology

Following BAL recovery, tracheas were perfused with formalin (4%). Paraffin-embedded lung sections of 4 μm were stained with Luna [14] for eosinophil and with periodic acid-Schiff (PAS) for mucopolysaccharide staining. In each mouse, the total eosinophil numbers surrounding 10 bronchioles positioned in a bordered area in parallel to the central airways were counted by laser scanning microscopy and analysed with the HistoQuest software module (Tissuegnostics, Vienna, Austria). Visual grading of mucus was performed in a blinded fashion in comparison with the maximal release in asthmatic mice. This value was set to 100% and the other samples were compared hereto.

Cytokine measurements

IL-4, IL-5 and IFN-γ measurements were performed by ELISA with anti-mouse cytokine antibodies and standards (Bender MedSystems, Vienna, Austria) in non-diluted BAL samples according to the manufacturer's instructions.

Isolation of splenocytes for the interleukin -5 cytokine assay and the ^3H thymidine proliferation assay

Mice ($n = 5$) sensitized and aerosol challenged with rPhl p 5a were killed and the spleens were removed. Spleen cell suspensions of each mouse were prepared by cutting, mincing and filtering the spleens through 70 μm nylon meshes (BD Biosciences, Schwechat, Austria). Cells were resuspended in RPMI medium (Gibco Invitrogen, Lofer, Austria) supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin.

Mononuclear cells from each spleen were isolated by density separation (Lympholyte-M; Cedarlane, Hornby, ON, Canada) according to the manufacturer's instructions. Cells were plated (4×10^5 cells/well) in triplicates in sterile round-bottom 96-well tissue culture plates (Costar, New York, NY, USA). For stimulation, rPhl p 5a, mimotope Phl mim 5, control allergen Ara h 2 (from peanut; vector kindly provided by Prof. Dr H. Sampson, Mount Sinai School of Medicine, New York) and positive control Con A (Sigma-Aldrich) (2.5 μg/mL each) were added. The cells were cultured for 72 h at 37 °C and 5% CO_2. The supernatants were harvested and stored at −20 °C until further

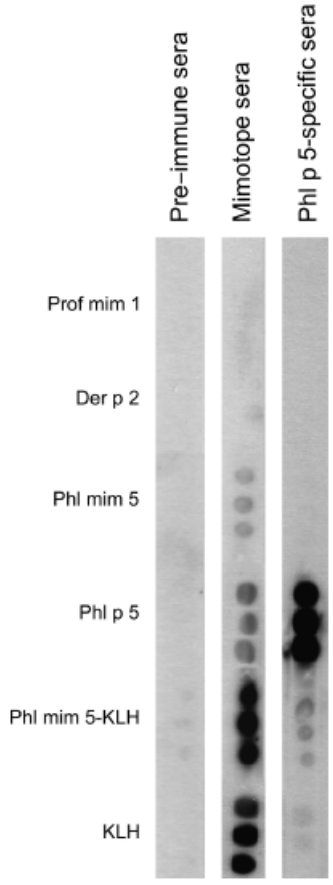

Fig. 1. Antigenic cross-reactivity of mimotopes and recombinant allergen Phl p 5a (rPhl p 5a) in immunodot. Pooled sera of mimotope- or of rPhl p 5a-immunized BALB/c mice were tested for IgG1 antibodies towards various antigens and control antigens (1 μg/dot) indicated on the left-hand side. No reactivity could be detected to control allergen Der p 2 from house dust mite and to control peptide Prof mim 1. Preimmune sera represent the sera of naïve mice. Bound IgG was detected with peroxidase-labelled anti-mouse IgG. KLH, keyhole limpet haemocyanin.

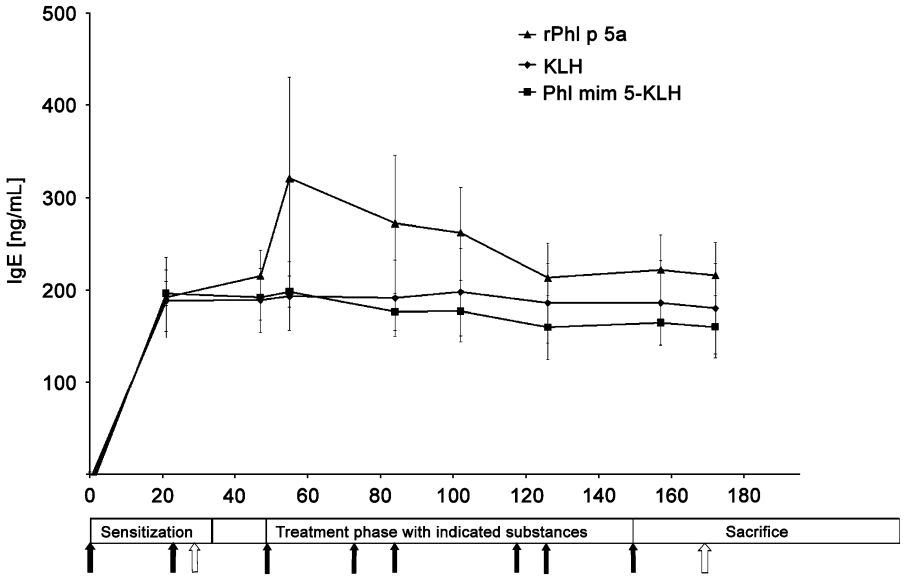

Fig. 2. Recombinant Phl p 5a (rPhl p 5a)-specific IgE titres in sera of mice with grass pollen-specific asthma in ELISA. During treatments, IgE levels in all the groups remained at comparable levels upon treatment with either allergen rPhl p 5a, the keyhole limpet haemocyanin (KLH)-linked mimotope Phl mim 5 or KLH alone. Black arrows represent treatments, i.e. immunizations. White arrows indicate aerosol challenges of mice with the nebulized allergen. Values of sham-treated control animals were below the detection limit.

use for cytokine determination. Single splenocytes were pulsed with 0.5 μCi/well ^{3}H thymidine diluted in 50 μL RPMI medium for 16 h at 37 °C and 5% CO_2. Cells were harvested with a Filter Mate Harvester (Perkin Elmer, Waltham, MA, USA) and radioactivity of DNA was measured in counts per minute and expressed as mean value per mouse.

Measurement of IL-5 was performed by ELISA with anti-mouse cytokine-antibodies and standards (Bender MedSystems) in pooled supernatants of stimulated splenocytes (diluted 1 : 2) according to the manufacturer's instructions.

Statistical analysis

Statistical comparison between groups was performed by the Mann–Whitney *U*-test, using the software SPSS (version 14.0 for Windows). Differences were considered statistically significant at $P < 0.05$.

Results

Immunization with the mimotope induced Phl p 5-specific immune responses in naïve mice

For proof of the mimicry potential, naïve mice were immunized s.c. with the mimotope Phl mim 5 linked to KLH. Testing of pooled sera showed that the mimotope-induced IgG antibodies cross-reacted with the orthologue allergen Phl p 5, but not with the control allergen Der p 2 or an unrelated control mimotope of panallergen profilin, termed Prof mim 1 (Fig. 1). As expected, these sera showed IgG reactivity against the carrier KLH, but also to uncoupled synthetic Phl mim 5. Vice versa, rPhl p 5a immunization yielded predominantly anti-Phl p 5 IgG and only slight crossreactivity towards Phl mim 5, which may have to do with the fact that a mimotope only represents a sub-epitope structure.

Immunoglobulin E antibody titres of asthmatic BALB/c mice were not affected by mimotope and control treatments

For the induction of acute allergic asthma, mice were sensitized i.p. with rPhl p 5a, followed by an aerosol challenge. The allergen induced increases of specific IgE in all sensitized mice (Fig. 2). Consecutively, groups of mice were treated s.c. either with Phl mim 5 coupled to KLH, with KLH alone or with rPhl p 5a, for control. During the entire treatment period, the IgE (and IgG1, data not shown) antibody titres of mimotope- and control-treated mice remained elevated without a statistical difference (Fig. 2).

Reduced eosinophil numbers in bronchoalveolar lavage and peribronchiolar tissue of mimotope-treated mice

As serology did not reveal any differences between the treated mouse groups, we aimed to analyse inflammatory parameters in the lungs. BAL cytology revealed that mimotope vaccination significantly prevented eosinophil infiltration in the airways compared with mice treated with KLH alone ($P = 0.032$) (Fig. 3). The effect was more pronounced than treatments with the recombinant allergen molecule rPhl p 5a (not significant).

Accordingly, we evaluated the eosinophil numbers in peribronchiolar regions of the paraffin-embedded lungs by Luna-staining highlighting the eosinophil granules in red.

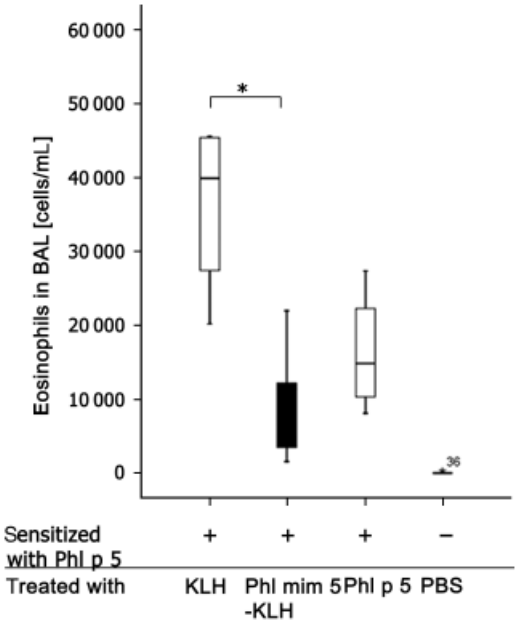

Fig. 3. Mimotope treatments of BALB/c mice with grass pollen-specific asthma are associated with a decreased number of eosinophils in bronchoalveolar lavage (BAL) cytospins. Mice were intraperitoneally and aerosol sensitized with rPhl p 5a (+) and subsequently vaccinated with substances indicated on the *x*-axis. Finally, mice were aerosol re-challenged with rPhl p 5a. Calculation of eosinophils was performed by BAL cytometry. $P < 0.05$. BAL samples showing more than a threefold deviation from the end of the box were defined as extremes and marked with asterisks.

The results indicate that Phl mim 5-KLH vaccination, but not KLH treatment alone, protected mice from eosinophilic infiltration ($P = 0.029$), similar to treatments with rPhl p 5a ($P = 0.024$) (Figs 4a–c).

Mimotope treatments reduce T-helper type 2 cytokine levels in bronchoalveolar lavage

The influence of the mimotope vaccine on the cytokines IFN-γ, IL-4 and IL-5 in BAL was investigated by ELISA. As depicted in Fig. 5, treatment with Phl mim 5-KLH before re-challenge with the allergen did not affect IFN-γ production (Fig. 5a), but slightly reduced IL-4 ($P = 0.3$) (Fig. 5b), although no significance could be achieved. With respect to IL-5 levels in BAL, the effect of the mimotope vaccine was comparable to treatment with rPhl p 5a, again without a significant difference ($P = 0.4$) (Fig. 5c).

Mimotope treatments diminish mucus hypersecretion within bronchioles

To determine whether mucus hypersecretion could be modulated by mimotope vaccination, lung sections were stained with PAS. Mucus production as a direct indicator for goblet cell numbers was graded for each mouse group, showing that Phl mim 5-KLH and rPhl p 5a but not KLH vaccinations, induced a reduction of the goblet cell numbers (Fig. 6a). Also, less mucus was visible in mice immunized with the Phl mim 5-KLH and rPhl p 5a in comparison with mice receiving KLH alone (Fig. 6b) or PBS (data not shown).

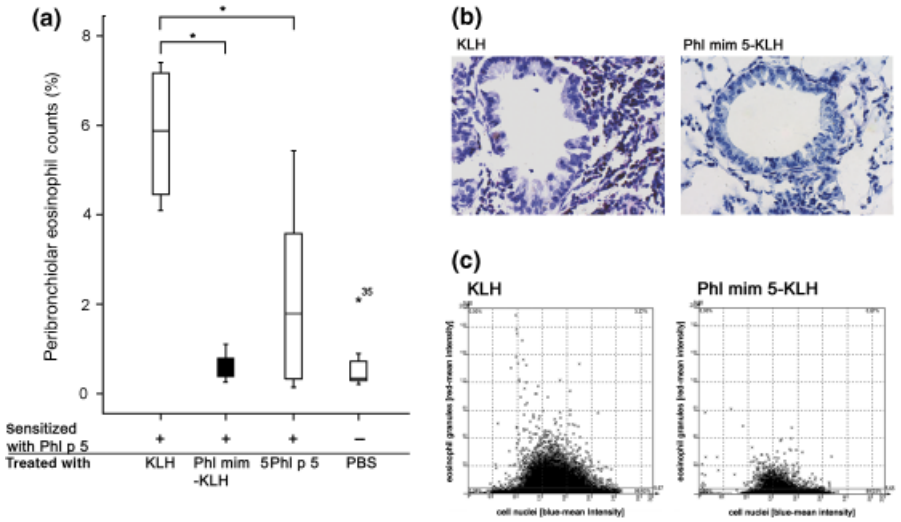

Fig. 4. Peribronchiolar eosinophil counts in mimotope-vaccinated mice. Mice were sensitized with recombinant Phl p 5a (rPhl p 5a) (+) and treated with substances indicated on the *x*-axis. After treatment, they were aerosol re-challenged with rPhl p 5a (a). Peribronchiolar eosinophil counts were determined and samples lying more than threefold away from the end of the box were defined as extremes and marked with asterisks (b). Representative histological Luna stainings of lungs from mice treated with keyhole limpet haemocyanin (KLH) or Phl mim 5-KLH (c). Respective scattergram by Tissue FAXs; *x*-axis: counted cells according to their size and the staining of their nuclei; *y*-axis: the number of eosinophils according to their granules stained in red. PBS, phosphate-buffered saline.

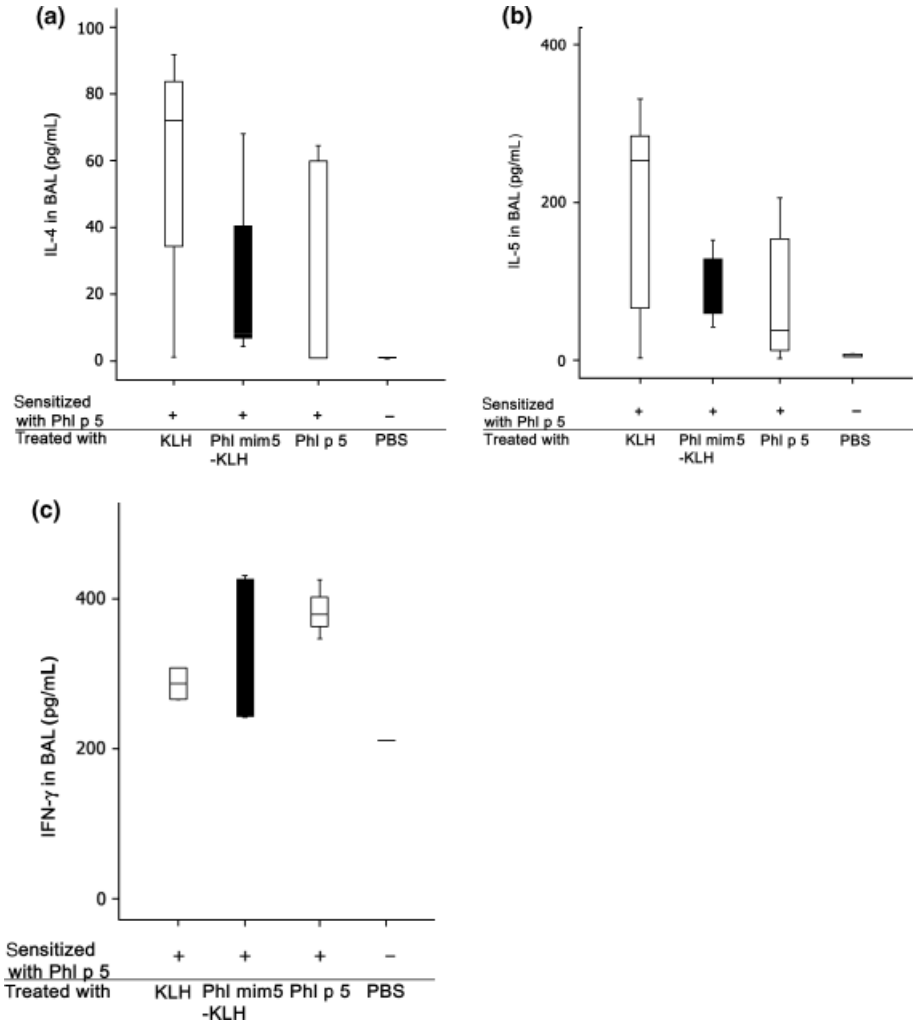

Fig. 5. Th2 cytokines in the bronchoalveolar lavage (BAL) fluid of mimotope-treated asthmatic mice in ELISA. Mice were sensitized either with rPhl p 5a (+) or treated with substances indicated below the *x*-axis. After aerosol re-challenge with rPhl p 5a, ELISA measurements revealed that the IL-4 (a) and IL-5 (b) levels in BAL were reduced in Phl mim 5-treated mice, whereas the IFN-γ (C) levels remained unchanged. PBS, phosphate-buffered saline; rPhl p 5a, recombinant Phl p 5a.

Proliferation and interleukin-5 production of splenocytes upon allergen or mimotope stimulation

To prove whether a mimotope, being devoid of allergen-specific T cell epitopes, is able to activate allergen-specific T cells, specific proliferation of splenocytes was analysed in a ^{3}H thymidine incorporation assay. Stimulation of murine splenocytes with rPhl p 5a resulted in more specific proliferation than treatment with mimotope Phl mim 5 ($P = 0.4$) or irrelevant allergen Ara h 2 ($P = 0.1$) (Fig. 7a). In parallel, IL-5 production was monitored in pooled supernatants of these stimulated splenocytes. In accordance with the proliferation assay, more IL-5 production could also be observed in the supernatants of rPhl p 5a-stimulated splenocytes, whereas there was hardly any response to Phl mim 5 (Fig. 7b).

Discussion

In a previous study, decameric peptides have been defined as IgE-epitope mimics (mimotopes) of major grass pollen allergen rPhl p 5a [8]. The mimotope with the highest specificity and binding capacity to rPhl p 5a-specific IgE (termed here Phl mim 5) was chosen for synthesis and coupling to KLH as an antigenic carrier in the present study. Although previously less effective for immunization, due to the low copy number of peptides when displayed by phage protein pIII, we demonstrate here that the same mimotope induced rPhl p 5a-reactive IgG in naïve BALB/c mice when displayed at a high density on KLH. When manufacturing the KLH-mimotope conjugate, we intended to approach an equimolar dose with rPhl p 5a. According to our calculation, the mimotope in this

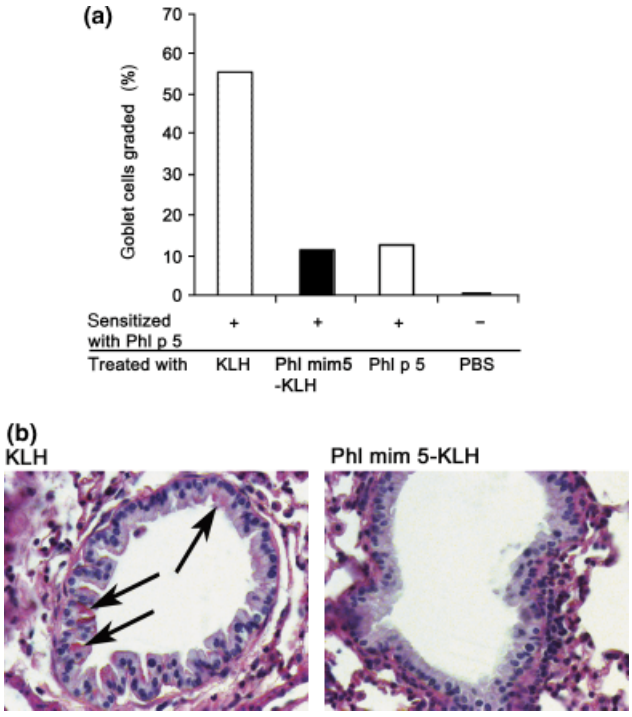

Fig. 6. Mimotope-treated mice show diminished mucus secretion within bronchioles. Mice sensitized with recombinant Phl p 5a (rPhl p 5a) (+) were treated with the substances indicated. Following an aerosol re-challenge with rPhl p 5a, mucus secretion in bronchioles was evaluated by periodic-acid-Schiff staining. (a) Diagram showing the relative percentage of mucus production in treated mice as compared with the level in untreated control, which was set to 100%. (b) Representative lung stainings from mice treated with keyhole limpet haemocyanin (KLH) or Phl mim 5-KLH (arrows: mucus-positive cells). PBS, phosphate-buffered saline.

conjugate exceeded the molarity of rPhl p 5a per applied dose by a factor of 5. Still, the levels of the mimotope-induced IgG were lower and the immunodot showed that it exhibited a higher degree of cross-reactivity with the allergen than vice versa. Both findings may have to do with the fact that the mimotope represents only a subunit of the original allergen epitope.

As a continuation of our previous efforts, the present study was designed to evaluate for the first time the therapeutic capacity of Phl mim 5, taking advantage of a memory mouse model of acute allergic asthma [15]. The induction of allergic asthma in mice is characterized by airway hyperreactivity, eosinophilia, increased IgE levels and mucus hypersecretion, which makes it a relevant model of human allergic disease and useful for testing novel therapeutics. In the BALB/c mouse model, a sustained elevation of allergen-specific IgE can be induced by rPhl p 5a sensitization; however, aerosol challenge with the grass pollen allergen is needed to induce the asthmatic phenotype in the bronchi. To determine whether immunological memory was generated, mice were allowed to recover from acute disease before a re-challenge, which should mimic re-exposure in the pollen season. As intermittent exposure to seasonal allergens already induces allergic symptoms, the rapid reaction to these allergens in sensitized human patients or in mice refers to immunological memory [10]. It has been demonstrated previously that up to 800 days after the onset of acute disease, mice were still susceptible to the development of characteristic features of allergic asthma by a secondary aerosol challenge using ovalbumin as a specific antigen [15]. One predominant parameter of acute allergic asthma in mice is the eosinophilic airway inflammation that resolves within 11 days after onset of acute disease. Indeed, we observed recurrent eosinophilic inflammation in BAL fluids 48 h upon re-challenge with the allergen rPhl p 5a. Even though representing a subunit vaccine as outlined above, the mimotope vaccination inhibited peribronchiolar eosinophilic infiltration and exsudation of eosinophils into BAL, indicating the anti-inflammatory capacity of this intervention. This effect seemed to be antigen-specific, as treatments with the carrier KLH alone had no effect.

IFN-γ is described to promote cell-mediated immune responses, and in case of asthma, inhibits the development of airway hyperreactivity in the lungs induced by Th2 responses [16]. In our model, IFN-γ levels in the BAL were not changed; however, the Th2 cytokines IL-4 and IL-5 showed a downward trend in the mimotope-treated group, pointing towards an immunomodulation. IL-4 plays a major role in the induction of IgE, including the IgE switch and airway hyperreactivity [17]. Similarly, IL-5 plays an important role in airway hyperresponsiveness and is a central factor mediating eosinophil expansion, priming, recruitment and prolonged tissue survival in response to allergic stimuli [18]. IL-4 and IL-5 are thus key regulators of airway inflammation and hyperreactivity in asthma. In our study, the relatively lower IL-5 levels observed after mimotope therapy were in accordance with lower counts of eosinophils. However, the somehow reduced IL-4 levels in the mimotope-treated group did not parallel IgE antibody titres, which remained unchanged during the whole experiment. This may possibly be due to long-lived plasma cells [19] and/or 'tissue-memory' as receptor-bound IgE prolong the survival time of effector cells in the periphery (reviewed in [20]). Under healthy conditions, the columnar epithelial surface in the bronchi comprises a small percentage of goblet cells and a majority of ciliated cells, whereas in chronic airway diseases like asthma, 20–25% of airway epithelial cells transform mucus-producing goblet cells [21]. After an acute onset of asthma, mucus within goblet cells of allergic mice normally decreases to baseline levels within 30 days after acute disease. In contrast, upon repeated allergen exposure, mucus production can be readily restored and visualized on PAS-stained lungs sections. The fact that mucus hypersecretion by goblet cells was abrogated in the mimotope-treated animals supports our

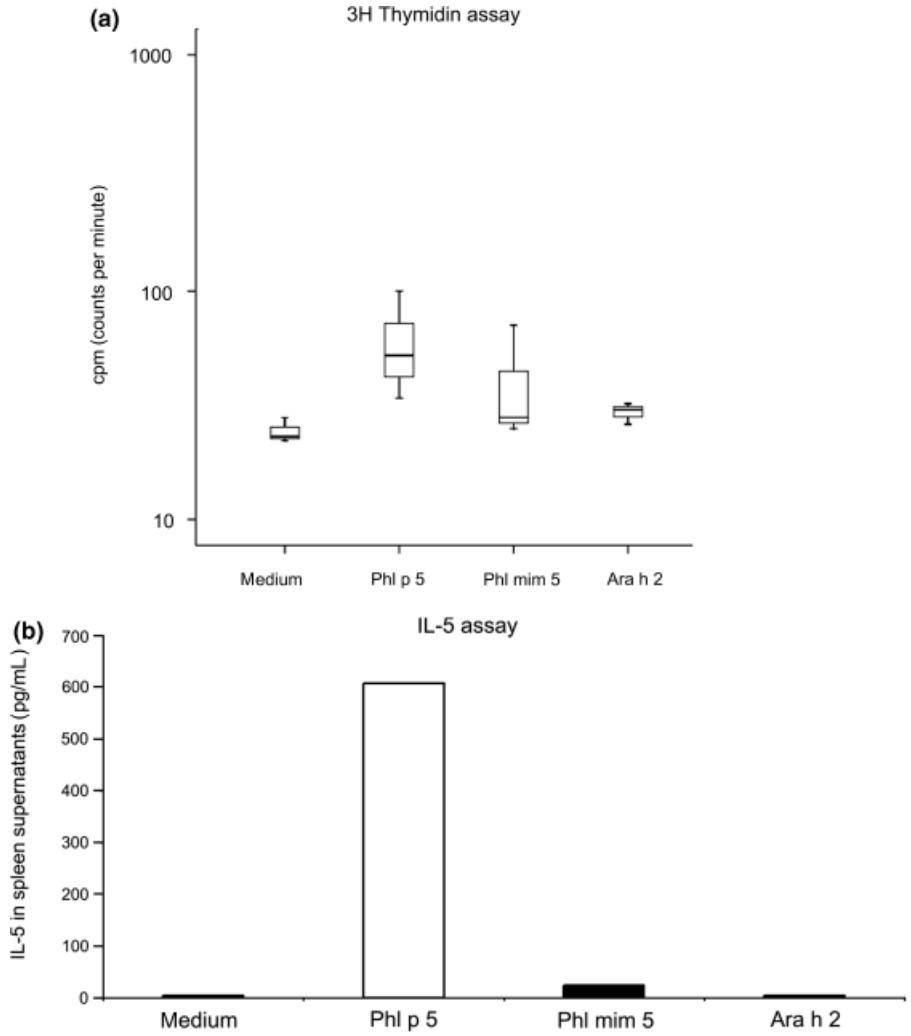

Fig. 7. Mice were sensitized and nebulized with recombinant Phl p 5a (rPhl p 5a) to induce allergic asthma. Splenocytes of these mice were isolated and stimulated with either rPhl p 5a, peptide mimotope Phl mim 5 or control allergen Ara h 2. (a) Less proliferation of splenocytes was observed in response to stimulation with mimotope Phl mim 5 and irrelevant allergen Ara h 2 than after addition of rPhl p 5a. The *y*-axis indicates the amount of ^{3}H thymidine incorporated into DNA of stimulated splenocytes. Splenocytes were stimulated with substances indicated below the *x*-axis. (b) Stimulation with rPhl p 5a, but not with control allergen Ara h 2 leads to IL-5 synthesis. The concentration of IL-5 levels is given in pg/mL, indicated on the *y*-axis. Substances used for stimulation are indicated below the *x*-axis.

hypothesis that B cell epitope mimics could have a potency in abating allergic asthma. In this context, the fact that the Phl mim 5 mimotope used here is devoid of T cell epitopes may be advantageous [6] because allergen-specific T lymphocytes cannot be re-activated through the treatment. We believe that the absence of allergen-specific T cell epitopes might have contributed to the observed anti-inflammatory effects of the mimotope vaccine in our mouse model. The duration of the vaccine effect remains to be determined, but might be comparable to the effect of other subunit vaccines. Another advantage is that mimotope peptides can be easily produced synthetically on a large scale and at relatively low costs.

Taken together, the results from this study suggest that there are several arguments in favour of the presented mimotope vaccine. It down-regulates bronchial inflammation including Th2 cytokines, eosinophil counts and mucus production without the activation of allergen-specific T cells, and may thus relieve the symptoms of grass pollen-induced asthma.

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A mimotope gene encoding the major IgE epitope of allergen Phl p 5 for epitope-specific immunization

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ABSTRACT

A gene vaccine based on a mammalian expression vector containing the sequence of a peptide mimotope of Phl p 5 was constructed. To test whether mimotope gene vaccines can induce allergen-specific antibody responses via molecular mimicry, BALB/c mice were immunized using the mimotope construct with or without a tetanus toxin T-helper epitope. Moreover, intradermal injection was compared to epidermal application via gene gun immunization.

Immunization with both mimotope gene constructs elicited allergen-specific antibody responses. As expected, gene gun bombardment induced a Th2-biased immune response, typically associated with IgG1 and IgE antibody production. In contrast, intradermal injection of the vaccine triggered IgG2a antibody expression without any detectable IgE levels, thus biasing the immune response towards Th1. In an RBL assay, mimotope-specific IgG antibodies were able to prevent cross-linking of allergen-specific IgE by Phl p 5. A construct coding for the complete Phl p 5 induced T-cell activation, IFN-γ and IL-4 production. In contrast, the mimotope-DNA construct being devoid of allergen-specific T-cell epitopes had no capacity to activate allergen-specific T cells.

Taken together, our data show that it is feasible to induce blocking IgG antibodies with a mimotope-DNA construct when applied intradermally. Thus the mimotope-DNA strategy has two advantages: (1) the avoidance of IgE induction and (2) the avoidance of triggering allergen-specific T-lymphocytes. We therefore suggest that mimotope gene vaccines are potential candidates for epitope-specific immunotherapy of type I allergy.

1. Introduction

Phage display libraries can be screened with allergen-specific IgE antibodies to identify mimics of allergenic B-cell epitopes. The selected mimics resemble IgE binding epitopes of the allergen surface by virtue of their physico-chemical properties [1]. In peptide form, such epitope-mimics (mimotopes) have repeatedly been shown to induce epitope-specific, allergen-specific immune responses [2–6]. Due to the fact that mimotopes do not contain allergen-specific T-cell epitopes, the immune responses towards mimotopes depend on the recruitment of bystander Th-cells. In a previous study we could show that mimotopes *per se* were not cross-reactive with the allergen at the T-cell level and that only phage-displayed mimotopes could activate T-cells to generate a mimotope-specific antibody response [5]. This unspecific bystander T-cell help can be provided by the phage coat protein pIII, which is composed of 406 amino acids [7]. The mimotope-induced IgG antibodies are then directed not only against the mimotopes, but co-recognize the 3-dimensional allergen epitope via molecular mimicry. Therefore, they are able to prevent the high-affinity interaction between allergen and specific IgE antibodies [3,8] and thus can be called blocking antibodies [9,10]. In several studies we and others have already characterized peptides and anti-idiotypic Fab fragments being mimotopes of different allergens and antigens [2,6,11–13].

Besides attempts to improve protein-based allergen immunotherapy by generation of allergen mutants, hypoallergens or peptides, immunization experiments with allergen-encoding DNA have yielded promising results. DNA vaccination does not only prevent allergic sensitization, but is also capable to modulate already ongoing Th2 responses [14–17]. Genetic immunization

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approaches with mimotope genes have hitherto been restricted to tumor antigens [18]. Therefore, we investigated in the present study whether this strategy could also be useful in the context of allergy therapy. For this purpose, we designed the construct "pCMV-F1", a gene vaccine composed of a mimotope of the grass pollen allergen Phl p 5 and phage coat protein pIII, the latter serving as (i) a "non-allergenic" carrier protein, (ii) a source of T-helper epitopes and (iii) a stabilizer of the three-dimensional exposure of the mimotope. In a complementary strategy to further enhance the immunogenicity of the mimotope construct, a promiscuous tetanus toxin T-helper epitope from *Clostridium tetani* [19] was additionally introduced into a second construct, designated pCMV-F1/Tet.

As the route of the DNA application might critically affect the cytokine milieu and thus the outcome of immunizations [20], we aimed to compare head to head two different routes of gene vaccine administration. Whereas gene gun immunization using only minute amounts of DNA has been demonstrated to trigger a predominant Th2 type immune response, intradermal application is known to recruit efficiently Th1 cells, possibly also due to the delivery of higher amounts of DNA [21,22].

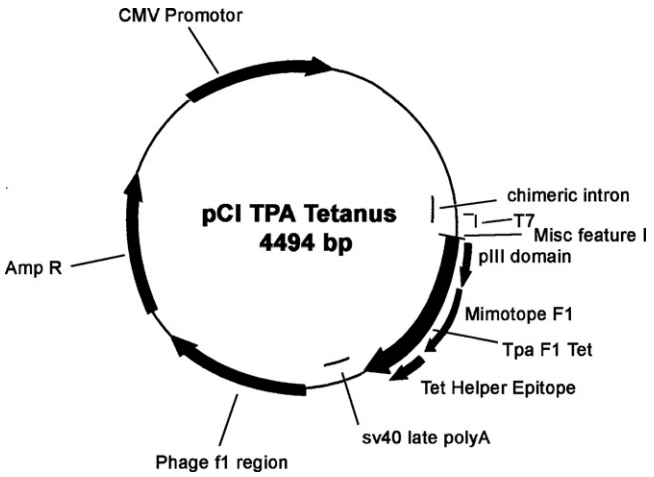

Fig. 1. Map of the eukaryotic expression vector pCI. The universal expression vector contains among other features a CMV immediate early promoter-enhancer region, an optimized chimeric intron, a multiple cloning site (MCS) and the SV40 late polyadenylation site. The inset shown here comprises the phage coat protein pIII, the mimotope F1 and an additional tetanus toxin helper epitope (pCMV-F1/Tet).

2. Materials and methods

2.1. Construction of the DNA immunization vectors pCMV-F1 and pCMV-F1/Tet

In a previous study, peptide mimotope F1 was identified as a specific epitope-mimic (mimotope) of grass pollen major allergen Phl p 5 by screening a phage display library with Phl p 5-specific IgE [3]. The peptide library consisted of decameric peptides presented on minor phage coat protein pIII of filamentous phage M13. Clone F1 (SRLGRSSAWV), showing the highest binding capacity to the antibodies, was chosen for expression in the high copy number plasmid pCI (Promega, Madison, WS, gb:CVU471199). The expression cassette in pCI contains the CMV immediate-early promotor and can provide three independent multiple cloning sites for the individual in-frame cloning of, e.g. an ER-targeting leader sequence, the gene of interest and a heterologous helper epitope [23]. For cloning of pCMV-F1, a PCR reaction using the filamentous phage DNA (M13) plus mimotope insert (F1) as a template was performed. To amplify the sequence of the pIII phage coat protein together with the mimotope sequence, the following primers were used, with F1-M13 DNA serving as a template: upstream primer pIII fwd1 EcoRI 5′-CACCGAATTCATGGCTGAATGCAGTCGTCT-3′ and reverse primer pIII rv1 XbaI 5′-GGGGTCTAGATTACTCATTTTCAGGGATAG-3′. The resulting 272 bp (base pair) fragment was digested with EcoRI–XbaI and ligated into the pCMV vector being digested with the same restriction enzymes.

For the construction of the pCMV-F1/Tet vector (see Fig. 1), inclusion of a foreign 63 bp long toxin helper epitope (fnnftvsfwlrvpkvsashle), here named "Tet" from *C. tetani* was performed [19]. A PCR reaction using the filamentous phage DNA (M13) as a template was performed (upstream primer pIII fwd1 EcoRI 5′-CACCGAATTCATGGCTGAATGCAGTCGTCT-3′ and the reverse primer pIII rv2 MluI 5′-GGGGACGCGTCTCATTTTCAGGGATAGCAA-3′). The resulting construct was digested with EcoRI–MluI and ligated into a pCMV TPA Tet vector (constructed from pCI, see [23]) digested with the same enzymes. For control, pCMV-mock, representing the vector without phage coat protein pIII and insert, and pCMV-P5 encoding the recombinant Phl p 5a (rPhl p 5), were used [24]. Successful cloning was controlled by sequencing all constructs in an Abi PrismTM Genetic Analyzer (MWG Biotech, Germany).

The plasmids were propagated in *E. coli* strain XL-1 Blue and large-scale purified with an Endo Free Plasmid Giga Kit (MWG Biotech, Germany). Plasmid DNA was then analyzed for size by agarose gel electrophoresis and quantified by spectrophotometry (OD260 nm/OD280 nm). Vectors were stored in endotoxin-free water at −20 °C.

2.2. Animals

BALB/c mice (female; 6–10 weeks) were purchased from Charles River Laboratories (Sulzfeld, Germany GmbH) and treated according to European Community rules of animal care [25] with the permission of the Austrian Ministry of Science (BMBWK-66.012/0015-BrGT/2005 and BMWF-66.012/0015-CIGT/2007).

2.3. Mice and immunizations

The detailed immunization schemes are depicted in Fig. 2. Mice ($n = 6$) were immunized either by particle bombardment using a Helios gene gun (Bio-Rad, Munich, Germany) or by intradermal (i.d.) needle injection with pCMV-F1, pCMV-F1/Tet, pCMV-mock or pCMV-P5 into the shaved abdomen. For gene gun immunizations, plasmids were coated onto 1.6 μm gold particles and a helium discharge pressure of 400 psi was used to deliver a total of 4 μg per immunization by two shots at two ventral sites. Particle bombardment was performed on days 2, 16, 30 and finally four weeks later at day 59 according to Scheiblhofer et al. [20]. Blood samples were drawn on days 0, 15, 29, 44 and 73.

i.d. immunizations were performed using a slightly different protocol, according to Hartl et al. [23] with 100 μg plasmid in a volume of 200 μl PBS on days 2, 10, and 16 into 4–6 sites at the shaved abdomen. Blood samples were taken on days 0, 9, 15 and 22. According to an established immunization protocol [16], a control group of mice was immunized subcutaneously (s.c.) with 5 μg rPhl p 5 adsorbed to 100 μl $Al(OH)_3$ in a total volume of 150 μl sterile endotoxin-free PBS.

2.4. Serum immunoglobulin detection in ELISA

For the measurement of Phl p 5-specific IgG, 96-well high-bind, flat-bottomed immunoplates (FluoroNunc, Roskilde, Denmark) were coated overnight at 4 °C with rPhl p 5 (0.1 μg per well/100 μl PBS). Plates were washed with PBS/0.1% Tween 20 and blocked for 1 h at room temperature with 200 μl/well PTB (PBS/0.1% Tween-20/0.5% bovine serum albumin (BSA)).

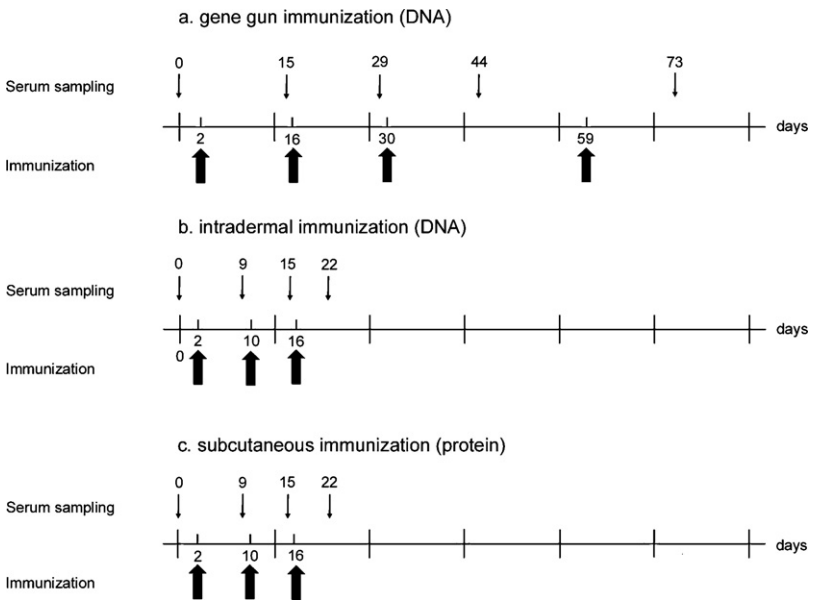

Fig. 2. Schemes of the immunization protocols. The axis indicates 14 days intervals. Gene gun (a) and intradermal immunization protocol (b) using the constructs pCMV-F1, pCMV-F1/Tet, PCMV-mock and pCMV-P5. Subcutaneous application protocol (c) using rPhl p 5 as the immunization substance.

Sera were added at a dilution of 1:100 in 100 μl PTB for total IgG, IgG1 and IgG2a and incubated for 1 h at RT. Plates were washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (BioRad, Germany), HRP-conjugated rabbit anti-mouse IgG1 (Zymed, San Francisco, USA) or HRP-conjugated rabbit anti-mouse IgG2a (Zymed), each diluted 1:1000 in PBT for 1 h at RT.

The reaction was detected by adding Luminol (Boehringer-Mannheim, Germany) 1:2 in aqua bidest. Chemiluminescence was measured at 405–490 nm by a LucyII ELISA-plate luminometer (Anthos Labtec, Salzburg, Austria) and fluorescence was calculated in photon counts × 1000 (kilo photon counts, KPC).

2.5. β-Hexosaminidase release from rat basophil leukemia cells

RBL-2H3 cells were plated into 96-well, flat-bottomed tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 4×10^4 cells/well in 100 μl of RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U penicillin and streptomycin/ml, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES and 100 μM of 2-mercaptoethanol. Plates were incubated o.n. at 37 °C, 95% relative humidity, 7.5% CO_2.

Subsequently, 50 μl of supernatant was removed. RBL cells were passively sensitized by adding pooled sera from either DNA-s.c. rPhl p 5-immunized mice or preimmune sera in a dilution of 1:100 for 2 h at 37 °C.

RBL cells were washed twice with 200 μl/well Tyrode's buffer [23]. Cross-linking of FcεRI-bound IgE was induced by addition of rPhl p 5 (0.3 μg/ml in Tyrode's buffer). For maximal release, 10 μl 10% Triton X-100 was added. Sera from untreated mice served as negative control. Cells were incubated for 30 min at conditions indicated above. Thereafter, plates were centrifuged for 5 min at 1200 rpm at RT, 50 μl of supernatants were transferred to fresh plates and incubated with 50 μl 4-methyl-umbelliferyl-N-acetyl-β-D-glucosaminide (4-MUG) (Sigma, Deisenhofen, Germany) in citrate buffer (0.1 M, pH 4.5) for 1 h at indicated conditions. The reaction was stopped by adding 100 μl glycine buffer (0.2 M glycine and 0.2 M NaCl, pH 10.7) and fluorescence was measured at 465–360 nm using a microplate reader (Spectrafluor, Tecan, Austria). Results were calculated in relation to the percentage of total β-hexosaminidase released after addition of Triton X-100 (100%). The release value obtained by Triton treatment was set 100% and the other results compared hereto.

For the inhibition assay, RBL cells were passively sensitized with sera of s.c. rPhl p 5-immunized mice in a dilution of 1:100 for 2 h. Sera of mice to be tested for presence of blocking IgG antibodies were pooled and heated at 56 °C for 1.5 h to destroy the β-hexosaminidase being present in the serum. Subsequently, these sera were incubated with 0.005 μg rPhl p 5 in dilutions 0, 1:50 and 1:20 for 2 h prior to incubation with RBL cells.

2.6. Isolation of splenocytes and detection of cytokines

Mice were sacrificed and spleens were aseptically removed. Spleens were minced in 1 ml minimal essential culture medium (MEM) and aggregated cells were sedimented for 10 min. Lysis of erythrocytes was performed with 5 ml ACK (ammonium chloride lysis buffer): (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA dissolved in aqua bidest., pH 7.2–7.4) for 5 min at RT. Cells were resuspended in 7 ml MEM and centrifuged for 5 min at 300 × *g*. After repetition of the last step, cells were plated into 96-well, flat bottom tissue culture plates (BD-Falcon, NJ, USA) (2×10^5 cells/well). For stimulation, rPhl p 5 (20 μg/ml), ovalbumin (20 μg/ml) or medium were added for 48 h at 37 °C, 95% relative humidity, and 7% CO_2. IFN-γ and IL-4 measurements of supernatants were performed by a Fluorescent Bead Immunoassay (Bender MedSystems, Vienna, Austria) of pure spleen supernatants according to the manufacturer's instructions.

2.7. Detection of cytokine producing cells by ELISPOT

Anti-IFN-γ (4 μg/ml) coated ELISPOT plates (Millipore, Bedford, MA, USA) were washed with PBS and blocked with PBS/2% dry milk powder (DMP) for 2 h at RT.

At the day of sacrifice, spleens were harvested and cells were cultured at a density of 2×10^5 cells/well with rPhl p 5 (20 μg/ml) at 37 °C, 7% CO_2 o.n. Plates were washed with PBS/0.1% Tween-20.

Biotinylated anti-IFN-γ antibodies (2 μg/ml in PBS/1% BSA) were added and the plates were incubated at RT for 2.5 h. Plates were washed again and incubated with streptavidin-PE (1:1000 I PBS/1% BSA) for 2 h at RT. Cytokine-producing cells per 2×10^5 seeded cells per well were detected by adding 3-amino-9-ethylcarbazole (AEC) substrate according to the manufacturer's instructions.

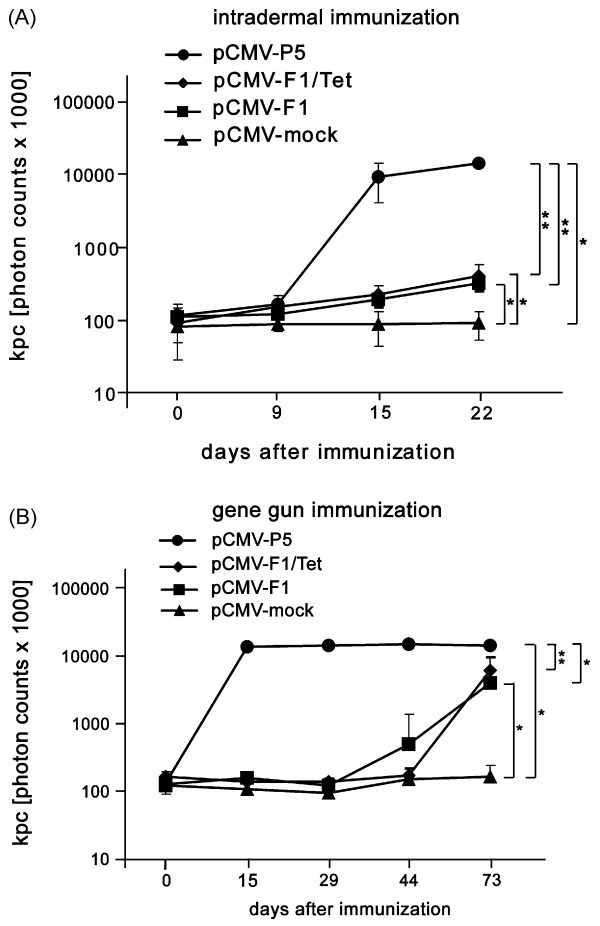

Fig. 3. Phl p 5-specific IgG antibody responses after i.d. and gene gun immunization. (A) i.d. immunization of DNA mimotope constructs pCMV-F1 and pCMV-F1/Tet induced significantly higher Phl p 5-specific IgG responses after the third immunization (day 22) compared to injection of negative control pCMV-mock. Immunization with pCMV-P5 served as positive control. (B) Gene gun immunization with both DNA mimotope constructs after the fourth immunization (day 73) revealed 10-fold higher IgG levels than i.d. administration.The induction of IgG was highly specific in comparison to treatment with pCMV-mock. $^{*}P<0.05$, $^{**}P<0.01$.

2.8. Statistical analysis

Statistical comparison between groups was performed by the Mann Whitney-*U* test, using the software SPSS (version 14.0 for Windows). Differences were considered statistically significant at *P*-values < 0.05.

3. Results

3.1. Mimotope-DNA induces allergen-specific immune responses in i.d. and gene gun immunized BALB/c mice

To prove the mimicry potential of the mimotope constructs pCMV-F1 and pCMV-F1/Tet, mice were immunized—either intradermally (i.d.) or by biolistic gene transfer. Sera of mice immunized with the mock vector, i.e. a construct lacking the mimotope as well as the Tet gene (pCMV-mock), served as negative control. Intradermal immunization with the gene of the entire Phl p 5-allergen (pCMV-P5) elicited a significant increase of the allergen-specific total IgG response already after two injections, as observed from sera of day 15 ($P<0.01$). Three injections with both mimotope constructs were needed to induce a significant antibody response ($P<0.05$), as analyzed in sera from day 22, although with 100-fold lower titres than with the allergen-encoding construct. Coexpression of the tetanus toxin helper epitope did not increase the immunogenicity of the mimotope DNA vaccine (Fig. 3A).

As depicted in Fig. 3B, four gene gun immunizations with the mimotope constructs rendered a tenfold higher titre of Phl p 5-specific IgG antibodies than i.d. treatment. However, a single shot of the Phl p 5 DNA via gene gun (serum from day 15) was already sufficient to reach a significantly elevated level of allergen-specific IgG compared to all other groups.

3.2. Intradermal injection or epidermal gene gun application differently polarize T-helper cell responses against the mimotopes

The induced antibody response was analyzed in more detail with regard to IgG1 and IgG2a levels (Fig. 4). Intradermal immunization with the vector encoding the entire gene of Phl p 5, pCMV-P5 was most effective in inducing Phl p 5-specific IgG1 and IgG2a (Fig. 4A) as seen in sera taken at day 22. The mimotope-DNA constructs pCMV-F1 and pCMV-F1/Tet induced comparable levels of anti-Phl p 5 IgG1 and IgG2a (Fig. 4A) with the IgG1 titre being lower in the pCMV-F1/Tet treated mice.

Four gene gun immunizations with all three constructs induced significantly higher IgG1 compared to IgG2a levels indicating a bias towards a Th2 response (Fig. 4B). Mice immunized via gene gun with pCMV-F1/Tet rendered significantly higher IgG2a levels compared to pCMV-F1.

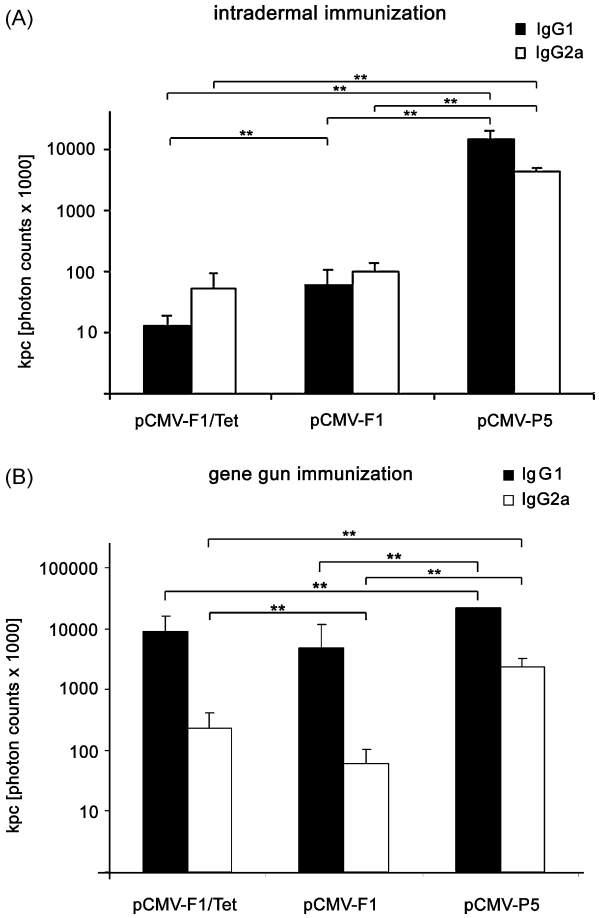

Fig. 4. Phl p 5-specific IgG1 and IgG2a antibody subclass responses after vaccinations. (A) Sera of mice from day 22 after three intradermal immunizations with all three constructs (pCMV-F1/Tet, pCMV-F1, or pCMV-P5) rendered similar tires of IgG1 and IgG2a antibodies. (B) Mice receiving four times particle bombardment by gene gun showed higher IgG1 levels in all three DNA immunized groups (B). $^{**}P<0.01$.

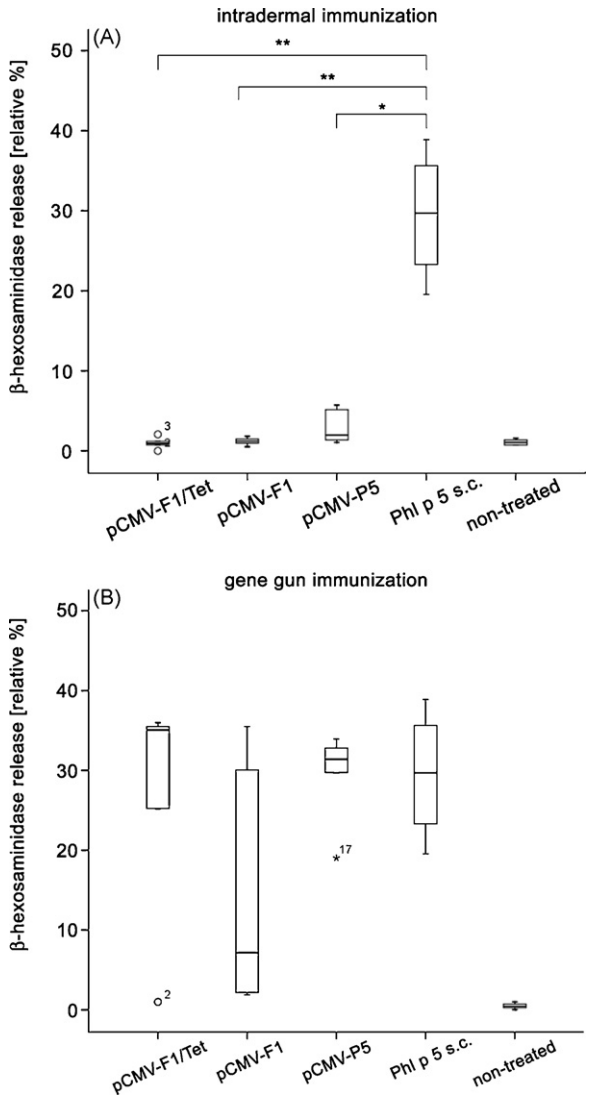

Fig. 5. Functional IgE antibodies measured by RBL cell mediator release assay. (A) rPhl p 5 protein, but none of the mimotope-DNA constructs pCMV-F1 and pCMV-F1/Tet induced significant Phl p 5-specific IgE synthesis after i.d. immunization. (B) In contrast, gene gun injection triggered IgE induced β-hexosaminidase release in all experimental groups. Results are presented as relative percentage of total β-hexosaminidase released after addition of 10% Triton X-100. Samples showing more than 3-fold deviation from the end of the box were defined as extremes and marked with numbers and circles. Numbers represent sample numbers in the order given by the statistical program used. $^{*}P < 0.05$, $^{**}P < 0.01$.

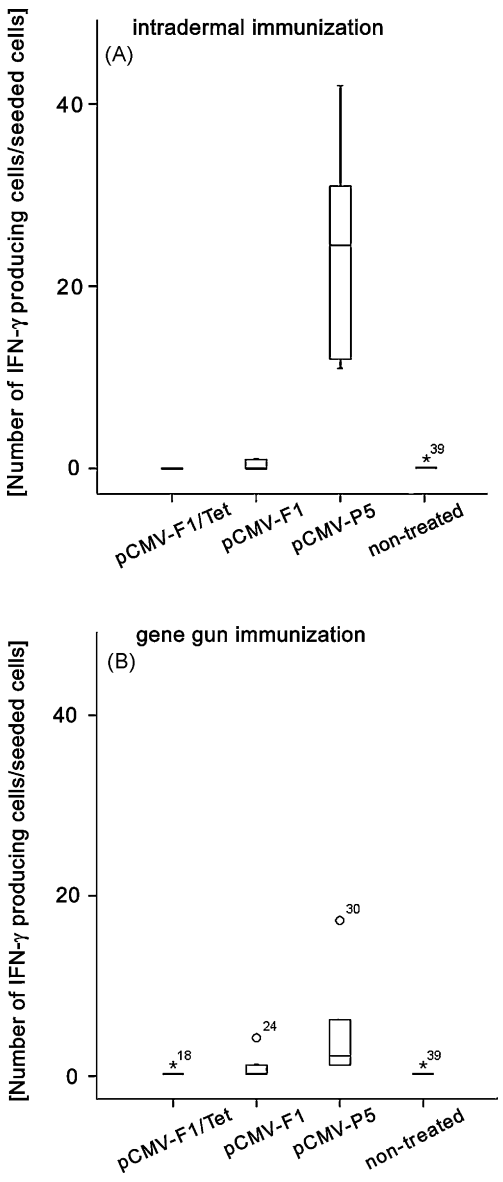

Fig. 6. Number of IFN-γ producing cells. Spleen cells of immunized mice were cultured on ELISPOT plates coated with anti-IFN-γ antibodies and stimulated with rPhl p 5. IFN-γ secreting cells could not be detected in the mimotope groups pCMV-F1 and pCMV-F1/Tet, neither after i.d. (A) nor after gene gun immunization (B). Only i.d. immunization with the construct encoding the entire gene pCMV-P5 resulted in IFN-γ production upon rPhl p 5-stimulation. In the gene gun treated group, only a few IFN-γ expressing cells were detected, probably due to the Th2-biased immune response after the immunization. Samples with signals showing more than 1.5-fold interquartile range deviation from the end of the box were defined as outliers and marked as circles. Numbers represent sample numbers in the order given by the statistical program used.

3.3. Gene gun but not i.d. immunization with mimotope genes triggers production of functional IgE

To investigate whether genetic immunization with mimotopes induces IgE antibodies which can be cross-linked by the recombinant allergen, a rat basophilic leukemia cell release assay was performed. RBL-2H3 cells were passively sensitized with sera diluted 1:100 from mice immunized either i.d. or by particle bombardment. Subsequent cross-linking was performed with rPhl p 5. IgE-containing sera of mice immunized s.c. with rPhl p 5 served as positive control [24], whereas. i.d. injection of both mimotope gene vaccines or the entire gene of Phl p 5 did not induce detectable IgE antibody levels (Fig. 5A). In contrast, gene gun immunization induced functional, although variable IgE antibody levels in all groups (Fig. 5B).

3.4. Allergen mimotope gene vaccines do not stimulate allergen-specific T-cells

The capacity of the DNA constructs to stimulate allergen-specific T-cells was tested by measuring the production of IFN-γ and IL-4 from splenocytes of immunized mice. To determine the number of IFN-γ producing spleen cells of the animals, an ELISPOT assay was carried out. Neither i.d. nor gene gun immunization with the mimotope constructs resulted in T-cell stimulation and IFN-γ release upon allergen stimulation. Significantly increased numbers of IFN-γ-producing cells (Fig. 6A and B), and increased levels of IFN-γ in the supernatants of stimulated splenocytes (Fig. 7A and B) were only detected in mice immunized with the construct encoding the entire

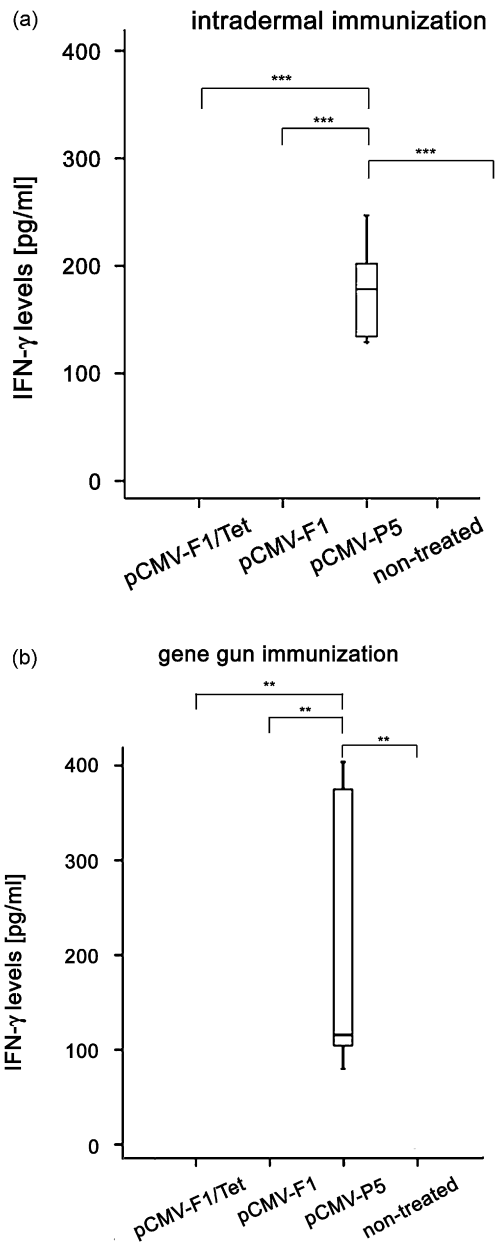

Fig. 7. IFN-γ levels in supernatants of stimulated spleen cells. Spleen cells of immunized mice were stimulated with rPhl p 5 for 48 h. IFN-γ secretion was only observed in spleen cell supernatants from the group immunized with the gene vaccine encoding the allergen (pCMV-P5) after i.d. (A) and gene gun immunization (B). $^{**}P < 0.01$, $^{***}P < 0.001$.

Phl p 5, which contained all relevant T-cell epitopes of the allergen ($P < 0.001$ to the i.d. group, $P = 0.002$ to the gene gun group).

IL-4 production could also be detected in mice immunized with the DNA encoding the recombinant allergen Phl p 5, treated by gene gun bombardment. Levels of all other groups were below the detection limit (Fig. 8).

3.5. Antibodies induced by allergen mimotope DNA act as blocking antibodies

Due to their ability to bind allergen in a specific manner (as demonstrated in Fig. 3), the anti-mimotope antibodies should be able to block the binding of allergen to allergen-specific IgE on mast cells or basophils. This was tested in an RBL-inhibition assay using pooled sera from mice immunized intradermally with mimotope

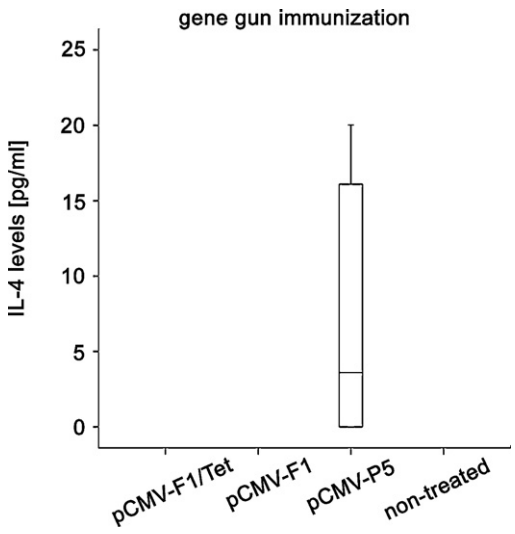

Fig. 8. IL-4 levels in supernatants of stimulated spleen cells. Spleen cells of immunized mice were stimulated with rPhl p 5 for 48 h. IL-4 secretion was only induced in the group immunized with the gene vaccine encoding the allergen (pCMV-P5) after gene gun immunization. No IL-4 production was observed after intradermal immunization with all of the constructs.

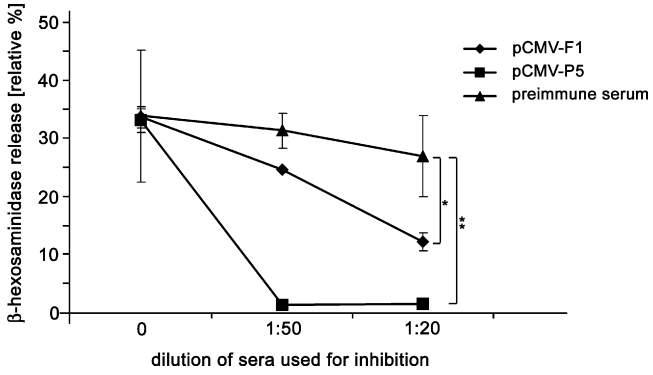

Fig. 9. Allergen-specific blocking antibodies. Antibodies with the potential to bind rPhl p 5 and thus inhibit the cross-linking of sensitized mast cells were measured by a β-hexosaminidase release inhibition assay. Cell release was significantly reduced by preincubation of rPhl p 5 with anti-mimotope (raised i.d. with pCMV-F1) or anti-Phl p 5 sera (raised i.d. with pCMV-P5) diluted 1:20 before addition to the sensitized RBL cells. $^{*}P < 0.05$, $^{**}P < 0.01$.

gene vaccine pCMV-F1, a gene vaccine encoding the recombinant allergen Phl p 5 (pCMV-P5), or preimmune sera. The results in Fig. 9 show that preincubation of allergen with pooled sera of mice immunized with Phl p 5 DNA or mimotope-DNA significantly inhibited ($P < 0.01$ and $P < 0.05$, respectively) the IgE cross-linking capability of the allergen.

4. Discussion

Previously, we were able to define decameric peptides as IgE-epitope mimics (mimotopes) of grass pollen allergen Phl p 5. In these studies, the potential of protein-based mimotopes for allergy immunotherapy was demonstrated by the induction of anti-mimotope antibodies in BALB/c mice which were co-reactive with Phl p 5 [3].

The similarity between mimotopes and B-cell epitopes concerns the three-dimensional structure and the distribution of the electrostatic charges, but mimotopes do rarely share sequential homologies with the antigen they mimic [26]. In the context of allergy treatment, this feature might be interesting, as a mimotope vaccine could promote the stimulation and expansion of

pre-existing allergen-specific B-cells, but simultaneously prevent activation of allergen-specific T-cells. This might be advantageous as it is known that allergen-specific T-cells may act proinflammatory in late phase reactions [27]. Indeed, in the present study the *in vitro* analysis of Th1 associated IFN-γ production and Th2 associated cytokine IL-4 from murine spleens confirmed this hypothesis, because immunization with mimotope gene vaccines did neither stimulate proliferation nor cytokine production of allergen-specific T-lymphocytes.

The safety concerns with respect to possible anaphylactic risks for protein-based immunotherapies of allergy are an issue that might be addressed by applying mimotopes. Mimotopes induce IgG antibodies which are B-cell epitope-specific [5], thereby avoiding sensitization against neo-epitopes and an amplification of the IgE response during immunization. Moreover, the inhibition of the IgE allergen interaction by virtue of their blocking capacity has been shown [8,28]. On the other hand the potency of DNA vaccines to counteract the IgE class switch by inducing IFN-γ producing $CD4^+$ or $CD8^+$ T-cells has yielded promising results in the field of allergy [15]. Consequently, we attempted here to combine the specific features of genetic immunization with the beneficial effects of mimotopes.

Gene vaccines have been introduced into animal tissue by several different routes, the most abundant being intradermal, intramuscular or biolistic gene transfer. The latter has been suggested to induce a Th1 milieu with 100–1000 times less amount of DNA than intramuscular injection [29]. However, gene gun bombardment was questioned for allergen immunotherapy by several studies [22,30–33].

The aim of this study was to incorporate the sequence of a previously identified peptide mimotope of Phl p 5 into a eukaryotic expression vector, the whole construct being designated pCMV-F1. The introduced minigene encoding the mimotope peptide was fused to the pIII phage molecule relevant for the display of mimotopes during their selection. This strategy intended to stabilize the three-dimensional structure of the mimotope exactly in the way it was selected during the biopanning procedure in context with phage protein pIII [34]. PIII could further act as a carrier protein supporting T-cell bystander help, which has been postulated already in the 1980s [35]. As the mimotope per se is composed of only 10 amino acids and has been shown so far only to induce immune responses when coupled to a carrier [5], further T-cell help had to be provided. The results actually demonstrated that the size of the mimotope-pIII construct being 416 amino acids was sufficient to induce an immune response, which could not be further enhanced by the addition of a promiscuous T-cell epitope from tetanus toxin. Intradermal but not gene gun immunization with mimotope-DNA induced blocking IgG antibodies without triggering IgE production, which was demonstrated in a functional test, the RBL-assay. Gene gun-mediated transfer delivers DNA more efficiently into cells by depositing DNA coated beads within the cytoplasm, thereby leading to a higher transfection rate than does direct needle injection [36]. How this may impact IgE synthesis, remains to be answered. Our data confirm results questioning the applicability of gene gun bombardment for allergen immunotherapy by several studies [20–22]. Thus, intradermal injection only eliciting IgG1 and IgG2a, but no IgE antibodies, is the preferred route also for the mimotope gene vaccine. The induced IgG antibodies also proved to have blocking activity by preventing allergen mediated cross-linking of IgE bound via FcεRI to effector cells. The type of the immune response induced by different application routes thus gives an important hint for future applications, e.g. administration of mimotope-DNA in therapeutic settings.

From our data we conclude, that the mimotope gene-vaccine has mimicry potential of a major IgE epitope of the grass pollen allergen Phl p 5. More importantly, the Th1-biased intradermal application of the mimotope-DNA induced an efficient immune response comprising blocking IgG antibodies, simultaneously avoiding IgE expansion and stimulation of allergen-specific T-cells. Based on our data we suggest that the mimotope technology in context with gene vaccination represents a promising novel option for epitope-specific allergen immunotherapy.

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SUMMARY

Allergen immunotherapy is today still associated with undesired side effects. This is in part due to the induction of anaphylactogenic antibodies directed against irrelevant molecules or even irrelevant epitopes within the applied allergens. This could be avoided by epitope-specific immunotherapy using mimotope peptides or anti-idiotypic Fab fragments with mimicry potential (reviewed in Chapter I) instead of allergens. This thesis aimed to further optimize mimotope immunotherapy against grass pollen allergy, and to set up and perform the necessary proof of concept studies.

Therefore, in the first experimental approach (Chapter II), a mouse model of allergic asthma was adapted to sensitization with grass pollen major allergen Phl p 5. Acute asthma was induced via systemic sensitization followed by inhalative allergen challenge rendering mucus secretion, lymphoid follicle formation and eosinophilic inflammation of the airways. To discriminate allergic asthma from non-specifically triggered airway hyperreactivity, we added ovalbumin (OVA) being an irrelevant control antigen for aerosolization of Phl p 5-allergic mice. In an innovative approach we performed respiratory gas exchange measurements by indirect calorimetry before and after challenges with aerosolized antigens. Enhanced respiration was found up to 48 hrs after provocation in the Phl p 5-nebulized mouse group only, characterized by significantly increased consumption of O_2 and release of CO_2 into exhaled air. Although airway inflammation of the OVA challenged group was overall milder than in mice with allergic asthma, both groups showed significantly less motor activity and exhibited acidosis combined with a sharp decline of white and red blood cells shown to be redistributed to the lung. This mouse asthma model therefore proved to be of pathophysiological relevance, being suitable not only for exploring the mechanisms of allergen- induced asthma versus non-specific airway provocation, but importantly also for proof of concept studies for mimotope vaccines.

Consequently, Chapter III shows an approach using in such a way sensitized Phl p 5-asthmatic mice for therapeutical vaccination with a synthetic peptide mimotope linked to carrier KLH. When mimotope-treated mice were then re-exposed with the aerosolized Phl p 5 to mimic allergen re-exposure of humans in the pollen season, they exhibited significantly lower eosinophil numbers in the bronchoalveolar lavage and peribronchiolar tissue as well as an impressive downregulation of mucus production as compared to sham-treated animals or animals treated with KLH only.

To further improve mimotope technology, we constructed in Chapter IV a minigene vaccine by translating the same peptide mimotope as used in the asthma model into a DNA vaccine. We compared head to head two different administration routes for immunization with the gene vaccine. Whereas gene gun bombardment was found to be associated with a Th2 biased immune response, intradermal injection did not induce IgE production but rendered blocking IgG antibodies able to inhibit allergen-mediated crosslinking of effector cell-bound IgE. In addition, no proliferation and cytokine production of allergen-specific T cells were observed.

The pre-clinical evidence collected in this thesis work suggests that mimotopes in peptide or DNA form have therapeutic potency for the treatment of allergic diseases. This is possibly due to the fact that they represent pure B cell-epitope mimics and completely avoid the activation of any pro-inflammatory T-lymphocytes.

ZUSAMMENFASSUNG

Die Immuntherapie mit Allergenen ist heute noch immer mit unerwünschten Nebenwirkungen assoziiert. Zum Teil ist dies bedingt durch die Induktion von anaphylaktogenen Antikörpern, die gegen irrelevante Moleküle oder gar irrelevante Epitope der eingesetzten Allergene gerichtet sind. Dies könnte durch epitopspezifische Immuntherapie verhindert werden, bei der Mimotope oder anti-idiotypische Antikörper mit Mimikry-Potential (Review in Chapter I) anstelle von Allergenen eingesetzt werden. Das Ziel dieser Doktorarbeit war es, die Mimotoptherapie gegen Gräserpollenallergie weiter zu optimieren und die hierfür notwendigen proof of concept Experimente zu etablieren und durchzuführen.

Im ersten experimentellen Ansatz (Chapter II), wurde ein Mausmodell für allergisches Asthma für die Sensibilisierung mit dem Graspollen-Hauptallergen Phl p 5 adaptiert. Akutes Asthma wurde durch systemische Sensibilisierung, gefolgt von inhalativer Exposition mit dem Allergen induziert, wodurch Mukussekretion, Lymphfollikelbildung und eosinophile Entzündung der Atemwege ausgelöst wurden. Um zwischen allergischem Asthma und un-spezifisch getriggerter Hyperreagibilität zu unterscheiden, fügten wir noch Ovalbumin (OVA) als ein irrelevantes Kontrollallergen für die Aerosolisierung von Phl p 5-allergischen Mäusen hinzu. In einem neuartigen Ansatz führten wir Analysen des Austausches der Atemgase mithilfe indirekter Kalorimetrie vor und nach Provokationen mit aerosolisierten Antigenen durch. Verstärkte Atmung konnte nach 48 Stunden nur in der mit Phl p 5 nebulisierten Mausgruppe festgestellt werden, charakterisiert durch erhöhte O_2 Konsumation und CO_2 Freisetzung in die Ausatemluft. Obwohl die Atemwegsentzündung der mit OVA provozierten Gruppe generell schwächer als in Mäusen mit allergischem Asthma war, zeigten beide Gruppen eine signifikante Abnahme der motorischen Aktivität und wiesen Azidose kombiniert mit einem prägnanten Abfall von roten und weißen Blutkörperchen in der Peripherie auf, gepaart mit deren Umverteilung in die Lunge. Daher ist die pathophysiologische Relevanz dieses Mausmodells erwiesen. Es dient aus diesem Grund

nicht nur der Erforschung der Mechanismen von allergeninduziertem Asthma versus nicht-spezifischer Atemwegsprovokation, sondern eignet sich auch für proof of concept Studien von Mimotopvakzinen.

Folglich zeigt Chapter III einen Ansatz, bei dem solchermaßen sensibilisierte Phl p 5-asthmatische Mäuse zur therapeutischen Vakzinierung mit einem synthetisch hergestellten Mimotop gekoppelt an KLH, eingesetzt wurden. Als Mimotop-behandelte Mäuse mit dem aerosolisierten Phl p 5 re-exponiert wurden um die Allergenexposition in der Pollensaison beim Menschen nachzuahmen, wiesen diese eine signifikant geringere Zahl an Eosinophilen in der Bronchoalveolarlavage und im peribronchiolaren Gewebe, sowie eine beeindruckende Herunterregulation der Mukusproduktion im Vergleich zu den „sham"-behandelten Tieren oder zu Mäusen, die nur mit KLH behandelt wurden, auf.

Um die Mimotoptechnologie weiter zu verbessern, konstruierten wir in Chapter IV eine Minigen Vakzine, indem wir das im Asthmamodell verwendete Peptidmimotop in eine DNA Vakzine übersetzten. Wir verglichen parallel zwei verschiedene Verabreichungswege für die Immunisierung mit der Genvakzine. Die Applikation mit der *Gene gun* war mehr mit einer Th2 –ausgerichteten Immunantwort assoziiert. Im Gegensatz dazu wurde durch intradermale Injektion des Minigenkonstruktes keine IgE Antwort induziert, sondern die Produktion blockierender IgG Antikörper ausgelöst, welche das allergenvermittelte Crosslinking von Effektorzell-gebundenem IgE inhibieren konnten. Darüber hinaus konnte keine Proliferation und Zytokinproduktion von allergen-spezifischen T-Zellen beobachtet werden.

Die präklinischen Befunde welche in dieser Doktorarbeit erhoben wurden deuten darauf hin, dass Mimotope in Peptid oder DNA Form therapeutisches Potential für die Behandlung von allergischen Erkrankungen, im besonderen Asthma, besitzen. Dies ist wahrscheinlich auf die Tatsache zurückzuführen, dass Mimotope ausschließlich Allergen- B-Zellepitope repräsentieren, jegliche Aktivierung pro-inflammatorischer T-Lymphozyten jedoch ausgeschlossen werden kann.

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Original articles

1. Pali-Schöll I., Herzog R., Wallmann J., Szalai K., Brunner R., Lukschal A., Karagiannis P., Diesner SC. and Jensen-Jarolim E. 2010. Antacids and dietary supplements with an influence on the gastric pH increase the risk for food sensitization. *Clin Exp Allergy [Epub ahead of print]*

2. Wallmann J., Epstein MM., Singh P., Brunner R., Szalai K.., El-Housseiny L., Pali-Schoell I. and Jensen-Jarolim E. 2010. Mimotope vaccination for therapy of allergic asthma: anti-inflammatory effects in a mouse model. *Clin. Exp. Allergy; 40(4):650-8.* .

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1. Wallmann J., Nittke T., Wegmann M., Kiefer FW., Klein J., Esterbauer H., Epstein MM., Fehrenbach H. and Jensen-Jarolim J. 2010. The cutting edge between allergen-specific asthma versus nonspecific provocation by blood analysis and indirect calorimetry in BALB/c mice.

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1. Hantusch B., Szalai K., Knittelfelder R., Krieger S., Wallmann J., Vogel M., Stadler BM., Boltz-Nitulescu G. and Jensen-Jarolim E. 2005. Anti-idiotypic Fab fragments structurally mimic IgE epitopes of major grass pollen allergen Phlp 5: revival of the network. XIX World Allergy Organization Congress / XXIV Congress of European Academy of Allergology and Clinical Immunology. *Allergy Clin Immunol Internat.* Suppl1 (Nr. 1301): 468.

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Congress abstracts

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