Phl p 1-specific human monoclonal IgE and design of a hypoallergenic group 1 grass pollen allergen fragment[1]

Mattias Levin[*], Frida Rydnert[*], Eva Källström[†], Lor Wai Tan[‡], Peter J. Wormald[‡], Malin Lindstedt[*], Lennart Greiff[§] and Mats Ohlin[*]

[*] Department of Immunotechnology, Lund University, Lund, Sweden;

[†] Department of Laboratory Medicine, Skåne University Hospital, Lund, Sweden;

[‡] Department of Surgery-Otorhinolaryngology, Head and Neck Surgery, University of Adelaide, Adelaide, Australia;

[§] Department of Otorhinolaryngology, Head and Neck Surgery, Skåne University Hospital, Lund, Sweden

Running title: Phl p 1-specific human IgE and a hypoallergenic fragment

Corresponding author: Dr. Mats Ohlin, Dept. of Immunotechnology, Lund University, Medicon Village (Building 406), S-223 81 Lund, Sweden. Telephone: +46-46-2224322. Telefax: +46-46-2224200. e-mail: mats.ohlin@immun.lth.se

Originally published in *The Journal of Immunology*. Levin M, Rydnert F, Källström E. Tan LW. Wormald PJ, Lindstedt M, Greiff L, Ohlin M. 2013. Phl p 1-specific human monoclonal IgE and design of a hypoallergenic group 1 grass pollen allergen fragment. *J. Immunol.* 191: 551-560 (DOI: 10.4049/jimmunol.1202051).

Abstract

Detailed understanding of how antibodies of the IgE isotype interact with allergen at the onset of an allergic reaction is of great importance for the deciphering of mechanisms involved in the development of disease and may aid in design of hypoallergenic variants. Here, we have utilized a set of human monoclonal IgE antibodies derived from the repertoires of allergic individuals, specific for the major timothy grass pollen allergen Phl p 1, to gain detailed information on the interaction between antibodies and allergen. These allergen-specific IgE are to varying degrees cross-reactive towards both different allergen isoforms and various group 1-allergens originating from other grass species. The usage of human monoclonal IgE, as an alternative to polyclonal preparations or mouse antibodies, allowed us to locate several important IgE-binding epitopes on the C-terminal domain of Phl p 1, all clustered to an IgE-binding "hot-spot". By introducing three mutations in the IgE-binding area of the C-terminal domain we were able to significantly reduce its reactivity with serum IgE. In conclusion, our study shows the great potential of using human monoclonal IgE as a tool for studies of the molecular interactions taking place during allergic responses. Further, we present a novel IgE-hyporeactive fragment with the potential to be used as a safer hypoallergenic alternative in specific immunotherapy than the pollen extracts used today.

Keywords: Antibodies; Epitopes; Allergy; Vaccination; Molecular Biology

Introduction

Type I allergy is an increasing, worldwide health issue currently affecting as much as 30% of the population of many countries [1]. At the initiation of an allergic response antigen-specific antibodies of the IgE isotype, bound to the surface of mast cells and basophils via the high affinity IgE receptor (FcεRI), are cross-linked by allergen. Such cross-linkage causes activation of these effector cells triggering them to degranulate and to release a range of biologically active compounds such as histamines, proteases, cytokines and chemokines, which eventually gives rise to symptoms associated with an allergic response [2, 3].

Grass pollen is a major cause of allergic disease and may affect as many as 20 % of the general population [4], severely affecting the quality of life of these patients and causing huge costs to society [5, 6]. Today the only disease-modifying treatment method used against grass pollen allergy, with demonstrated long-term clinical efficacy [7], is specific immunotherapy. For over a century, this treatment has been carried out using natural pollen extracts [8]. However, such natural extract may be of varying quality and potential pit-falls that must be considered include variations in allergen quantity, low stability and poor immunogenicity of certain important allergen components as well as the presence of contaminations [9]. In addition, there is a risk of inducing life-threatening side effects when using these extracts in immunotherapy [10, 11]. Recently, on account of the significant progress during the last two decades in characterization of the molecular nature of important allergens, safer alternatives to the natural extracts have been introduced, such as the use of recombinant allergens and so called natural or recombinant hypoallergens. Hypoallergens are allergen variants with a reduced IgE-reactivity, while the immunogenicity and T cell reactivity are kept intact. They are thus able to induce production of allergen-specific protective IgG antibodies and to modify T cell responses, without provoking a detrimental systemic IgE-mediated allergic

response. Altogether, they may reduce the sensitivity to the allergen of interest with minimal side effects [12, 13, 14, 15].

Among the many different allergen groups present in grass pollen, the group 1 allergen is the most common sensitizer and as many as 90 % of all grass pollen allergic individuals have group 1-reactive IgE [16]. Although the molecular nature of many different group 1 allergens as well as their reactivity with human polyclonal IgE has been quite thoroughly investigated [16, 17, 18, 19, 20, 21], we still lack detailed knowledge of the interaction of these allergens with human IgE at a clonal level. Only recently have a few studies been published were human monoclonal IgE have been used as tools to gain a detailed understanding of the molecular interaction between IgE and allergen, so crucial for the initiation of an allergic response [18, 22, 23, 24, 25, 26, 27].

Seeking to exploit the information content contained in clonally resolved human IgE, this study present a detailed characterization of a set of human monoclonal IgE derived from four different allergic individuals, in terms of their interaction with the major timothy group 1 pollen allergen, Phl p 1, and other group 1 allergens. The study enabled us to identify IgE-binding epitopes on the surface of Phl p 1, all closely located at an IgE-binding “hot-spot”. Based on this knowledge, we designed, produced and evaluated an IgE-hyporeactive fragment to be used as a safer alternative to natural extracts in specific immunotherapy.

Methods

Recombinant allergens, mouse monoclonal antibodies and patient sera

Recombinant allergens Phl p 1.0102, Phl p 2, Phl p 5, Phl p 6 and Phl p 7 were purchased from Biomay (Vienna, Austria). Phl p 1.0101 was purchased from INDOOR Biotechnologies (Wiltshire, United Kingdom). These recombinant allergens had all been produced in *Escherichia coli.* Three mouse mAb (1.8, 1.10, 1.21) [17] specific for Phl p 1 were kindly provided by Dr. D. Barber (ALK-Abelló, Madrid, Spain). Sera were obtained from grass pollen-positive allergic patients. These studies were approved by the local ethical committee.

Production of the C-terminal domain of Phl p 1 and mutant versions thereof

Codon optimized genes encoding the C-terminal domain of Phl p 1.0102, mutant versions thereof and a mutant version of the C-terminal domain of Phl p 1.0101 were purchased from GeneArt and cloned into the pGEX-6P-1 expression vector (GE Healthcare, Piscataway, NJ) to allow for production of GST-fusion proteins. The sequence of Phl p 1.0102 and description of the C-terminal domain and mutated residues can be found in Figure 1. To achieve an initial small scale production of protein, vectors carrying the inserted genes were transformed into T7 Express competent *E. coli* (New England Biolabs, Ipswich, MA) and grown in 2xYT-medium (supplemented with 100 µg/ml carbencillin) until OD_{600}=0.4, at which time induction of protein production was achieved by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM. The production was allowed to continue for 3 hours at 37°C before cells were harvested and treated with lysozyme (Sigma-Aldrich, St. Louis, MO). Soluble GST-fusion proteins were purified using affinity chromatography with GSTrap FF columns (GE Healthcare). Purified proteins were analyzed by SDS-PAGE to ensure that a pure product had been obtained (Figure 1). The GST-tagged C-terminal domain of Phl p

1.0102 carrying the K8A, N11A and D55A mutations was also produced and purified in larger scale in *E. coli* TUNER(DE3) cells at the Lund Protein Production Platform (LP3) (Lund University, Lund, Sweden). The cells were for this purpose grown at 18°C for 18 h following induction of protein production. A portion of purified protein was treated with PreScission protease (GE Healthcare) for 1-3 days at 4°C to remove the GST-tag. The untagged product was separated from the GST-tag using size exclusion chromatography on a 329 ml HiLoad 26/600 Superdex 200 pg gel filtration column (GE Healthcare) connected to an ÄKTA Purifier system. Purified proteins, both before and after protease treatment, were analyzed by SDS-PAGE to ensure that a pure product had been obtained (Figure 1).

Phl p 1-specific human antibody fragments

Phl p 1-specific single chain antibody fragments $(scFv)^2$ p1-15 and p1-20 have previously been isolated [28] by selection on Phl p 1.0102 from a phage display library established using heavy chain variable (VH) domain-encoding sequences of the IgE-encoding transcriptome of an allergic patient [22]. The sequences encoding Phl p 1-specific antibody fragment clone 10, selected on Phl p 1.0102 from a phage displayed IgE library [18], were collected from GenBank (accession numbers AJ512649 and AJ512646 (http://www.ncbi.nlm.nih.gov/genbank/)). A codon-optimized gene encoding clone 10 in scFv format was obtained from GeneArt. Four N-terminal codons (encoding EVQL as defined by the IGHV3-9*01 gene), not found in the published sequence, were added to the construct.

The construction of the two combinatorial scFv libraries used in this study, from mRNA derived from individuals sensitized to grass pollen, has previously been described [29]. To isolate Phl p 1-specific binders from these libraries, phage display selections were performed on recombinant Phl p 1.0102. Three selection rounds were performed in Immunotubes (Nunc, Roskilde, Denmark) coated with allergen diluted to 5 μg/ml in PBS. After a pre-selection

performed in tubes incubated with selection buffer (1% w/v BSA, 0.05% Tween 20 v/v in PBS) the phage library was applied to the allergen-coated tubes and incubated for 2 hours. Unbound phages were washed away and bound phages were eluted by addition of trypsin, which cleaves a trypsin sensitive site between the scFv and phage protein III. After the third selection round clones were picked at random and phage stocks produced for specificity analysis.

Construction of vectors for production of scFv and scFv-CHε2-4 fusion proteins

To enable production of soluble scFv, genes encoding scFv were transferred in a single cloning step into a production vector [24]. Ligated DNA was transformed into chemically competent One Shot Top10 *E. coli* (Invitrogen, Carlsbad, CA).

To allow for production of scFv-CHε2-4 fusion proteins the pFUSE-hIgG-Fc2 vector [30] was modified to produce scFv fused to the IgE CH2-4 domains. A codon-optimized gene encoding the IgE CH2-4 domains, with codon 2 in CHε2 mutated to alanine, was purchased from GeneArt and cloned into the pFUSE-hIgG-Fc2 vector between the *Not*I and *Nhe*I restriction sites. In a second cloning step genes encoding scFv were cloned into the vector using *Nco*I and *Not*I. The ligated DNA was transformed into chemically competent XL1-Blue *E. coli* (Agilent Technologies, Santa Clara, CA).

Production of scFv in *E. coli*

Cells carrying the vector encoding scFv were grown in 2xYT-medium (supplemented with 100 μg/ml carbencillin). At OD_{600}=0.9 protein production was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM. Production was allowed to proceed for 16 hours at 30°C. Cells were harvested and treated with lysozyme

(Sigma-Aldrich, St. Louis, MO). Soluble scFv were finally purified using affinity chromatography with Ni-NTA agarose columns (Qiagen, Hilden, Germany).

Production of scFv-CHε2-4 fusion proteins in HEK293 cells

For production of scFv-CHε2-4 fusion proteins HEK293 cells were grown in Minimum Essential Medium (Invitrogen) containing 2mM L-glutamine and 10% HyClone Fetal Bovine Serum (Hyclone Laboratories, South Logan, UT) in 5% CO_2 at 37°C until 90% confluency was reached. Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were grown for 72 hours at which time the supernatant containing scFv-CHε2-4 was collected and sterilized by filtration (0.45 μm) prior to analysis.

ELISA binding assays

The ability of the investigated recombinant proteins, either displayed on phage, as soluble scFv or as scFv-CHε2-4 fusion proteins, to bind intact allergens, the C-terminal domain of Phl p 1.0102 or mutant versions thereof was determined using ELISA. Antigens were coated in microtiterplates at a concentration of 5 μg/ml. Either BSA or GST was used as negative control. Blocking was performed with 1% w/v milk, 0.05% v/v Tween 20 in PBS. Bound phages were detected with an HRP-labeled anti-M13 mAb (GE Healthcare), scFv with an HRP-labeled anti-FLAG M2 mAb (Sigma Aldrich), scFv-CHε2-4 fusion proteins with an HRP-labeled anti-IgE antiserum (KPL, Guildford, UK) and mouse mAb with an HRP-labeled polyclonal rabbit anti-mouse immunoglobulin antiserum (Dako, Glostrup, Denmark) using 1-Step Ultra TMB - ELISA Substrate (Pierce, Rockford, IL) as chromogen. Absorbance was measured at 450 nm.

The IgE-reactivity of serum derived from 11 Phl p 1-postive donors to Phl p 1.0102, the C-terminal domain of Phl p 1.0102, mutant versions of this domain, with or without the GST-tag, and GST was analyzed using ELISA. Antigens were coated and wells were blocked as described above. Bound IgE was detected using HRP-labeled anti-IgE antiserum. Detection was performed as described above.

ELISA blocking assay

A blocking assay was performed to identify the presence, or not, of multiple epitopes on the C-terminal domain of Phl p 1.0102. Briefly, microtiterplates were coated with the Phl p 1 C-terminal domain or BSA at a concentration of 0.2 μg/ml. Blocking was performed as described above. Wells were pre-incubated with Phl p 1-specific soluble scFv or Phl p 1-specific mouse antibodies for 1 hour before addition of scFv-CHε2-4 fusion proteins. Bound scFv-CHε2-4 fusion proteins were detected using the HRP-labeled anti-IgE antibody and a chemiluminescent substrate (SuperSignal ELISA Femto Maximum Sensitivity Substrate, Pierce).

To determine the ability of soluble antigen to block the binding of Phl p 1-specific scFv-CHε2-4 fusion proteins to immobilized antigen, the antibody was pre-incubated with the soluble antigen for 1 h prior to addition to microtiterplate wells coated with Phl p 1.0102. Bound recombinant scFv-CHε2-4 fusion proteins were detected as described above.

Genetic analysis

Inserts encoding Phl p 1-specific binders made in this study were sequenced by GATC Biotech (Konstanz, Germany). The genetic origin of sequences encoding VH and variable light (VL) domains and mutational status of the sequences included in this study was determined using the IMGT/V-QUEST web tool (program version 3.2.20; reference directory

release: 201135-3) [31]. Genes were annotated according to the IMGT nomenclature [32]. Sequence alignments were performed using MacVector 12.0.3 (MacVector, Cory, NC).

Statistical analysis

Differences in lengths of the third complementarity determining region of the heavy chain (CDRH3) between the set of group 1 allergen-specific IgE included in this study and previously described polyclonal IgE repertoires were assessed by a Mann-Whitney Test (http://vassarstats.net). Differences in IgE-reactivity of serum samples against mutant versions of the C-terminal fragment of Phl p 1.0102 and wild-type allergen or allergen fragment were confirmed using a Two-Sample t-Test (http://vassarstats.net).

ImmunoCAP analysis

Crude, diluted supernatants containing scFv-CHε2-4 were assessed for binding to allergens found in pollen extracts using the standard ImmunoCAP system (Phadia, Uppsala, Sweden). All samples were assessed in technical duplicates.

Basophil degranulation assay

Heparinized peripheral blood was collected from three timothy grass pollen allergic donors at the Department of Otorhinolaryngology at Lund University hospital. All donors were positive in a skin prick test and had circulating timothy allergen-specific IgE levels (9-17.3 kU/L), as determined by ImmunoCAP (Thermo Fisher Scientific Inc.). They were also positive in immunoassays for recombinant Phl p 1-specific IgE. The study was approved by the local Ethics Committee. PBMC were isolated through density gradient centrifugation (Lymphoprep, Axis-Shield PoC, Oslo, Norway) immediately after blood sampling. Cells were resuspended in RPMI 1640 (without L-glutamin, Thermo Fisher Scientific, MA) with 0.5% (w/v) BSA.

PBMC were challenged with the C-terminal domain fused to GST at 1, 0.1, 0.01 or 0.001 µg/ml or equimolar concentrations of full-length Phl p 1.0101 (Biomay), the N11A mutant or the K8A, N11A, D55A mutant of the C-terminal domain of Phl p 1 fused to GST. A control with an equimolar concentration of GST relative the highest concentration of the C-terminal domain fused to GST and a positive control with 20 µM fMLF (Sigma-Aldrich) were included. 2 ng/ml human IL-3 (Miltenyi Biotec, Bergisch Gladbach, Germany) was added and after 60 min incubation in a water bath (37˚C, 5% CO2) the degranulation was stopped by addition of 20 mM EDTA. One unstimulated control sample, incubated at 37˚C and treated with EDTA and IL-3, and one untreated control, kept on ice and not treated with EDTA or IL-3, were included. Cells were thereafter washed and resuspended in PBS with 0.5% (w/v) BSA and 2.5 mM EDTA. Non-specific IgG binding was blocked using 6 µg/ml mouse IgG (Jackson Immunoresearch, West Grove, PA) and the cells were stained for 20 min at 4˚C with HLA-DR-PerCPCy5.5 (BioLegend, San Diego, CA), CD203c-APC (Miltenyi Biotec), CD123-PE (BD Pharmingen, San Diego, CA) and CD63-FITC (BD Pharmingen). After washing, the cells were resuspended in PBS with 0.5% (w/v) paraformaldehyde (Thermo Fisher Scientific) and analyzed with a flow cytometer (FACSCanto II, BD Biosciences, San José, CA) and FCS Express 4 (De Novo Software, Los Angeles, CA).

Results

Isolation of novel Phl p 1-specific human monoclonal IgE

To allow for analysis of the IgE immune response against grass pollen group 1 allergens, the available set of specific binders [18, 28] was expanded by phage display selections of antibody fragment libraries on recombinant Phl p 1. These new libraries had been established using VH-encoding sequences of the IgE-encoding transcriptome of patients sensitized to grass pollen [29]. Three rounds of selections were performed and randomly chosen clones were screened for Phl p 1-specificity using phage-ELISA. Two novel specific binders (1p1:8 and 5p3:1) were isolated to complement the three previously available clones (p1-15, p1-20 and clone 10), creating a set of clones derived from 4 different IgE libraries. The two new clones were highly group 1 allergen-specific, not showing any detectable signs of cross-reactivity with other timothy grass allergens (Fig. 2).

Genetic composition of Phl p 1-specific human monoclonal IgE

Analysis of the sequences of five Phl p 1-specific antibody fragments revealed a diversity amongst the set of binders in terms of both heavy and light chain germline gene origin and length of CDRH3 (Table I). Both frequently used (IGHV1-18) and less common (IGHV3-9 and IGHV3-53) variable heavy chain genes [33] were utilized. The length of the product of the gene rearrangement encoding the CDRH3, known to be of substantial importance for antibody specificity [34], also shows variability ranging from 6-14 codons (mean value = 10.2), a range shorter than that described for polyclonal IgE repertoires, such as those reported by Kerzel *et al.* [35] (mean value = 15.9; p=0.006) and Andréasson *et al.* [22] (mean value = 14.8; p=0.027). The five clones, i.e. including those that originate from the same heavy chain germline gene, uses different VDJ rearrangements and have substantially different mutational patterns (Supplementary Fig. 1). One particularly notable exception is a

shared double mutation in codon 32 in the first complementarity determining region of the heavy chain (CDRH1) of clones originating from germline gene IGHV1-18, a set of mutations that results in an S→D substitution (Supplementary Fig. 1).

The set of human IgE is specific for the C-terminal domain of Phl p 1

In an effort to map important binding epitopes of human Phl p 1-specific IgE a 95 amino acids long C-terminal fragment of Phl p 1 (Fig. 1) was produced. This fragment has previously been shown to be immunodominant and to be recognized by a human mAb of IgE isotype [18]. The ability of the five available Phl p 1-specific human antibodies and three mouse antibodies detecting distinct epitopes on Phl p 1 [17] to bind the fragment was determined using ELISA (Fig. 3). All five tested human antibodies did bind the C-terminal fragment, confirming its immunodominance in human IgE responses. In contrast only one (1.8) of the three mouse mAb bound this allergen fragment. The affinity of four of the Phl p 1-specific binders for natural Phl p 1 has been characterized and two of them have been shown to mediate high affinity interactions with dissociation constant below 10 nM (Table I).

Cross reactivity to different isoforms of Phl p 1

Grasses often produce different isoforms of their allergens, a fact that may complicate diagnosis and immunotherapy if the epitopes are not shared between the isoforms. The two isoforms, defined by the Allergome database [36], of the group 1 allergen of timothy, Phl p 1.0101 and Phl p 1.0102, differ in 15 out of 240 residues (94% identity) found in the mature protein and in 9 out of 95 residues (91% identity) of the C-terminal domain. To assess the ability of various antibodies to recognize different isoforms, we tested their binding activity in ELISA (Supplementary Fig. 2). The mouse antibodies, which had been induced by immunization with natural Phl p 1 [17], recognized Phl p 1.0101 equally well or slightly

better than Phl p 1.0102. Human antibodies (in the form of scFv-CHε2-4), which all had originally been isolated for binding to Phl p 1.0102, also recognized Phl p 1.0101, although several of them, in particular clone p1-15, were less reactive towards Phl p 1.0101 as compared to Phl p 1.0102. Epitopes are thus partially shared between the two isoforms of Phl p 1.

Cross reactivity to group 1 allergens

To investigate the cross-reactivity amongst Phl p 1-binding human IgE towards pollen extracts from a range of other species, the five binders in the format of scFv-CHε2-4 fusion proteins were analyzed for reactivity using ImmunoCAP (Fig. 4) with allergens derived from 9 grass species with group 1-allergens carrying high sequence similarity to Phl p 1 (Supplementary Fig. 2). All five clones did to some extent cross-react with several of the analyzed extracts and one of the clones (clone 10) showed reactivity against all tested species except *Zea mays* (maize). This is largely in agreement with previous studies demonstrating a broadly cross-reactive behavior of this clone although ImmunoCAP analysis did not detect binding to maize extracts defined as weakly positive by immunoblot [18]. Nevertheless, several grass extracts, in particular that derived from *Cynodon dactylon* but also those derived from *Triticum sativum* and *Paspalum notatum*, were recognized by only one or a few members in this collection of clones. This is in agreement with the fact that the sequences of the C-terminal domain of representatives of group 1 allergens of these grasses differ extensively from that of Phl p 1 (Supplementary Fig. 2). Also extracts likely containing more closely related group 1 allergens were not recognized by one of the human antibodies (1p1:8). Altogether, these findings indicate that a polyclonal IgE preparation like a serum sample that test positive for several extracts still may be very restricted in terms of repertoire size when targeting some of these allergen sources.

Epitope mapping of Phl p1-specific human IgE

Blocking assays using the available human scFv and the mouse mAb (1.8) reactive with the C-terminal domain of Phl p 1 revealed that this domain holds adjacent, but not overlapping, IgE-binding epitopes (Supplementary Table I). Not all antibody fragments were efficient inhibitors of allergen binding of other antibody fragments and one scFv was not available in soluble form, as it could not be produced. Despite this it is evident that 5p1:3 and p1-20, two binders that are encoded by similar, yet distinct, germline rearrangements (Table I, Supplementary Fig. 1), recognize overlapping sequences. Similarly, clone 10 blocks not only itself but also allergen binding of 1p1:8. These two scFv also originate from an identical IGHV gene. The mouse mAb 1.8 seems to bind a third epitope, overlapping the two epitopes defined above for human scFv originating from IgE repertoires. ScFv p1-15 shows epitope reactivity in-between that of the two other sets of human scFv in this assay setup.

To further pinpoint areas important for IgE-binding on Phl p 1, 12 mutant versions of the C-terminal fragment (Fig. 2), each carrying a single mutation of a surface-exposed amino acid, were produced and the effect of the mutations on antibody binding was evaluated in a direct binding assay. The amino acids chosen for investigation are spread across the surface of the C-terminal part of Phl p 1. Several mutations affected the binding of human antibodies to protein immobilized on microtiter plates. However, three different mutations (K8A, N11A and D55A) that in particular reduced the reactivity with one or several of the tested Phl p 1-binding IgE were identified (Supplementary Fig. 3). To further confirm binding specificity the ability of the mutants in soluble form to prevent binding of antibody specificities to immobilized Phl p 1 was investigated. These studies confirmed the relevance of one or several of the three identified amino acids one by one for the binding of 1p1:8, 5p1:3, p1-20 and clone 10 (Supplementary Fig. 3). Phl p 1-C-N11A failed to inhibit the binding of 5p1:3

and p1:20, binders that had been identified as recognizing overlapping epitopes. This mutant of the C-terminal fragment of Phl p 1 were also partly defective in its recognition of clone 10, a binder that was even less reactive to Phl p 1-C-D55A and Phl p 1-C-K8A. Allergen binding of 1p1:8, a binder with specificity overlapping that of clone 10, was also prevented by the K8A mutation. Specific binding by p1-15 was poorly inhibited by several soluble proteins, but in particular by Phl p 1-C-K87A. In contrast, the only mouse antibody, 1.8, that recognized the C-terminal domain of Phl p 1, was not affected by mutations in any of these residues. Instead its binding to soluble antigen was reduced by the E84A mutation, indicating a different specificity of this antibody.

Identified epitopes constitute a significant part of the IgE-binding epitopes on Phl p 1

To further assess the importance of these amino acids for the IgE reactivity, as found in serum of allergic donors, samples derived from 11 Phl p 1-positive donors were assayed against the 12 mutants by ELISA (Supplementary Fig. 3). This analysis confirmed the particular influence of mutation N11A also on reactivity of polyclonal human serum IgE to the C-terminal immunodominant domain of Phl p1.

The three mutations (K8A, N11A and D55A) identified above as important for the binding of human monoclonal IgE to Phl p 1 are closely located on the surface of the C-terminal fragment (Fig. 5), constituting a potential human IgE-binding hot spot. Based on this novel knowledge of important residues for IgE-binding on Phl p 1 yet another potentially hypoallergenic variant was created, based on the C-terminal domain carrying the three mutations K8A, N11A and D55A. The new combined mutant fused to GST failed to fully inhibit the allergen-binding potential of any of the five Phl p 1-specific human antibodies included in this study (Fig. 6). These findings were further confirmed for IgE clones 1p1:8, 5p1:3, p1-20 and clone 10, representing two distinct epitopes, using a non-GST-tagged

version of the combined mutant, excluding any major effects exerted by the GST-tag itself (Fig. 6). Importantly, just as Phl p 1-C-N11A, the GST-tagged mutant carrying all three mutations as well as its tag-free version showed a significantly ($p<0.0001$) reduced reactivity with human serum IgE (Fig. 7), an indication that a majority of IgE present in these sera targets epitopes affected by the introduced mutations. In contrast, the combined mutant, with or without (Fig. 6) the GST-tag, did bind mouse mAb 1.8, further emphasizing the difference in epitopes targeted by this murine antibody and the five human IgE.

As antibodies cross-react between different isoforms of Phl p 1 we also investigated the effect of introducing the mutations shown to be important for binding to Phl p 1.0102 into the sequence of Phl p 1.0101. This recombinant C-terminal protein domain in fusion to GST also demonstrated a substantially reduced level of binding by human recombinant scFv-CHε2-4, as compared to the wild-type protein sequence (Supplementary Fig. 3). These mutations can thus be used also to render the C-terminal domain of Phl p 1.0101 hyporeactive to IgE.

Mutated variants of Phl p 1 C-terminal domain exhibit reduced ability to induce degranulation of basophils

The basophil activation test was performed on cells from grass pollen allergic donors in order to investigate the capacity of two mutated recombinant versions (N11A and K8A, N11A, D55A) of the C-terminal domain of Phl p 1 to induce degranulation, as compared to wild-type allergens. The mean fluorescence intensities (MFI) of the degranulation marker CD63, as well as the percentage of CD63-positive cells, were assessed after allergen stimulation of basophils. Basophils were stimulated with four different concentrations of wild-type Phl p 1 and GST fusion proteins of the C-terminal fragment of Phl p 1 and mutated (N11A and K8A, N11A, D55A, respectively) variants thereof. The stimulation index, based on changes in MFI of CD63 expression and percent CD63+ cells in comparison to the relevant controls, was

evaluated. This analysis demonstrated that a higher concentration of the mutated variants of the C-terminal domain of Phl p 1 was required to achieve degranulation of basophils in two donors, highlighting their hypoallergenic nature (Fig. 8). The third donor's basophils in contrast were poorly degranulated by the C-terminal domain of Phl p 1 alone suggesting that some features not found in this recombinant protein domain alone were required for efficient degranulation by this donor's IgE.

Discussion

Detailed understanding of how antibodies of the IgE isotype interacts with allergens at the onset of an allergic reaction is of great importance to understanding disease development and may aid in the development of safer alternatives to be used in specific immunotherapy, e.g. hypoallergens. Here we provide a detailed characterization of the interaction between a set of human monoclonal IgE and the major group 1 timothy grass pollen allergen Phl p 1. We also present the design, production and evaluation of a novel fragment with IgE-hyporeactive properties, with potential reduced risks of side effects, in specific immunotherapy.

While previous studies of Phl p 1 have taken use of either mouse mAb produced via immunization or a very limited number of clonally related human antibodies of the IgE isotype derived from combinatorial libraries based on the repertoire of a single allergic individual [17, 18], we in this study include five diverse human IgE antibodies (used as scFv in solution, displayed on filamentous phage or fused to the Fc of human IgE) with origin in four different donors. This allowed us to pinpoint an IgE-binding "hot-spot", containing at least one high affinity IgE epitope that is represented on natural Phl p 1, that was demonstrated to be important for the interaction with serum IgE of several allergic donors. The presence of such clusters of IgE-binding epitopes has previously been described [18, 37, 38] and should be suitable targets for mutagenesis in attempts to create hypoallergenic variants. Indeed, the constructed version of the C-terminal domain of Phl p 1, carrying three mutations (K8A, N11A and D55A), showed significantly reduced reactivity with serum IgE of allergic donors. These results were further confirmed by basophil activation test where up to 100-fold higher levels of the K8A, N11A, D55A mutant than of the wild-type protein were needed to induce degranulation. This clearly demonstrates that a majority of the IgE binding epitopes on Phl p 1 are located at the described hot-spot and establishes the designed fragment

as an IgE-hyporeactive protein with potential use as a safer alternative in specific immunotherapy. This approach to hypoallergen design is only one of many techniques available [11] and hypoallergenic Phl p 1 variants have previously been described both as B cell epitope containing peptides [39, 40] and as a mosaic version of Phl p 1 [41]. There are, however, to our knowledge, no previous reports of potentially hypoallergenic fragments based on group 1-grass pollen allergens based on mutagenesis of IgE-binding epitopes, defined by human monoclonal IgE. Further assessment of this variant's immunological behavior is necessary, such as its ability to activate pre-existing or new timothy allergen-specific T-cell responses and its ability to induce blocking IgG antibodies, important features of for a final vaccine. In this context it is of interest to note that several T-cell epitopes restricted to different HLA-DQ and DR alleles [42] were not modified by the K8A, N11A or D55A mutations. This suggests that at least a part of the T-cell reactivity of the C-terminal domain is unaffected by the mutations. Altogether, in addition to the reduced IgE binding of this mutant it appears as if many of its T-cell epitopes are retained.

The three Phl p 1-specific mouse mAb [17] were distinctly different from human IgE in terms of targeted epitopes on the allergen. Only one of the antibodies recognized the C-terminal fragment of Phl p 1, which is immunodominant in humans, and it recognized an epitope different from those recognized by human IgE, as defined by alanine scanning mutagenesis. This suggests that there may be differences in epitopes targeted by antibodies derived from naturally developed repertoires and antibodies produced by immunization, as previously reported in several other systems [43, 44, 45, 46, 47]. Such differences could potentially be explained by a combination of biological processes and technical variations. It is thus not unlikely that immunization with a fully or partially denatured allergen in an emulsified adjuvant would give rise to antibodies targeting epitopes other than the conformational epitopes bound by IgE. Furthermore, natural sensitization in allergy most

likely occurs via a very weak antigen stimulation, favoring a direct class switch from IgM to IgE [48], while the much stronger stimulation in immunization would mainly trigger a second pathway leading to an initial switch to IgG [48]. As indications that there are differences in epitopes targeted by IgE and IgG has been presented [49], it is possible that antibodies developed via these two different class switch pathways would then also target different epitopes. Taken together, these findings argue for the use of human monoclonal IgE derived from naturally occurring repertoires over mAb produced via immunization in studies like this, in which the aim is to pinpoint biologically relevant IgE-binding epitopes.

As for other available allergen-specific human monoclonal IgE the five IgE antibodies included in this study were selected from combinatorial libraries [22, 23, 29, 37, 50]. The origin of these libraries in an IgE-repertoire relies on the critical role that the heavy chain V domain plays in creation of specificities [34]. There is a tendency, although the total number of investigated clones is small, that individual epitopes on Phl p 1 are often recognized by antibodies of similar genetic origin. This observation agrees with studies of other antibody repertoires [23, 51, 52] and suggests that the heavy chain V domain is important for the creation of particular antigen specificities. The combinatorial feature of such libraries could be argued to favor VH that are promiscuous in regard to their VL pairing, preferentially allowing selection of a subset of antibodies. There is also a risk of creating new specificities that do not match those that develop *in vivo*. However, several of the IgE antibodies in this study have been selected as a number of related variants, using different but clonally related VH (data not shown). Such clonal relationship amongst selected binders would imply that these clones have developed via diversification in *in vivo* selection processes and it is thus unlikely that they are rare novel specificities created in the process of library construction. In addition, several of the VH of the included IgE antibodies were selected paired with different, but highly similar, VL (data not shown), further supporting the hypothesis that these antibody

specificities were not created in the uncontrolled pairing of VH and VL. Thus, establishment of these binding sites is not a random process but relies on relevant combinations of heavy and light chain variable domains. In all, we are confident that the five antibodies critical for this study represent a set of biologically relevant Phl p 1-specific IgE and that they are good clonal representatives of IgE responses as they occur *in vivo*.

Not only is the access to human monoclonal IgE with defined allergen-specificities advantageous in the process of finding biologically relevant epitopes to target in design of IgE-hyporeactive proteins, but it also allows for additional studies, such as a comparative analysis of the complexity of the response against different isoforms of the allergen and of cross-reactivity profiles against numerous related allergens from different allergen sources. We performed a profiling of the cross-reactivity of the included five IgE antibodies against both the two major isoforms of Phl p 1 (Phl p 1.0101 and Phl p 1.0102) and a set of 9 different allergens derived from grass species with group 1-allergens carrying high sequence homology to Phl p 1. We observed cross-reactivity to Phl p 1.0101, despite the fact that the binders all had originally been isolated through their ability to bind Phl p 1.0102. In contrast, different IgE clones recognized group 1 allergens found in extracts of pollen of different species to varying degrees. The fact that all extracts but mays (*Zea mays*) were recognized by at least one recombinant group 1-specific IgE argues against extensive variability of such allergens, as reported by others [53, 54], in the different extracts used to establish the assays. It is notable that even a small pool of IgE antibodies, such as the one tested here, could have the potential to provoke allergic responses against such a wide range of different grass species. Although the clonal size of allergen-specific IgE is positively correlated to biological effect, i.e. basophil degranulation, it is enough to have a single pair of IgE antibodies, targeting different epitopes (like 5p3:1 and clone 10, both of which recognize pollen allergens of several different species) or dimeric allergen structures, to achieve a strong degranulation [25].

Taken together, these results suggest that even an allergen source that is only able to induce production of a restricted number of clones in a single individual specific for its allergens may still be able to cause severe responses not only against the sensitizing specie, but also against a wide range of other species carrying homologous allergens.

In summary, this study presents the hitherto most detailed characterization of a set of human monoclonal IgE and the interaction with their targeted allergen, in this case the major timothy grass pollen allergen, Phl p 1. The results demonstrate the usefulness of human monoclonal IgE antibodies in studies of molecular interactions taking place between antibody and allergen in the course of an allergic response. We were able to locate several biologically relevant IgE-binding epitopes, all closely located on the surface of the C-terminal domain of Phl p 1. We also demonstrate the presence of these epitopes on both the two major Phl p 1-isoforms and a wide range of group 1-allergens from other grass species. In addition, we designed, produced and performed promising evaluation of a hypoallergenic fragment of the group 1 grass pollen allergen, based on binding characteristics of a set of diverse human recombinant IgE. If successful in future validation studies, such a hypoallergenic fragment or variants thereof could be of great use as a safer alternative to the extracts currently used in specific immunotherapy.

Acknowledgements

We are grateful to Dr. Svante Bohman (Thermo Fisher Scientific Inc, Uppsala, Sweden) for providing data on the affinity of Phl p 1-specific binders for natural Phl p 1 and to the Lund Protein Production Platform (LP3) (Lund University, Lund, Sweden) for production of GST-free protein.

References

1 Finkelman F.D and D. Vercelli. 2007. Advances in asthma, allergy mechanisms, and genetics in 2006. *J. Allergy Clin. Immunol.* 120: 544-550.

2 Gould H.J., B.J. Sutton, A.L. Beavil, R.L. Beavil, N. McCloskey, H.A. Coker, D. Fear and L. Smurthwaite. 2003. The biology of IgE and the basis of allergic disease. *Annu. Rev. Immunol.* 21,:579-628.

3 Gould H.J. and B.J. Sutton. 2008. IgE in allergy and asthma today. *Nat. Rev. Immunol.* 8**:** 205-217.

4 Andersson K. and J. Lidholm. 2003. Characteristics and immunobiology of grass pollen allergens. *Int. Arch. Allergy. Immunol.* 130: 87-107.

5 Kiotseridis H., C.M. Cilio, L. Bjermer, M. Aurivillius, H. Jacobsson and A. Tunsäter. 2010. Swedish translation and validation of the Pediatric Allergic Disease Quality of Life Questionnaire (PADQLQ). *Acta. Paediatr.* 100: 242-247.

6 Hellgren J., A. Cervin, S. Nordling, A. Bergman and L.O. Cardell. 2010. Allergic rhinitis and the common cold – high cost to society. *Allergy* 65: 776-783.

7 Durham S.R., S.M. Walker, E.M. Varga, M.R. Jacobson, F. O'Brien, W. Noble, S.J. Till, Q.A Hamid and K.T. Nouri-Aria. 1999. Long-term clinical efficacy of grass-pollen immunotherapy. *N. Engl. J. Med.* 341: 468-475.

8 Noon L. 1911. Prophylactic inoculation against hay fever. *Lancet* 1: 1572-1573.

9 Focke M., I. Swoboda, K. Marth and R. Valenta. 2010. Developments in allergen-specific immunotherapy: from allergen extracts to allergy vaccines bypassing allergen-specific immunoglobulin E and T cell reactivity. *Clin. Exp. Allergy* 40: 385-397.

10 Mellerup M.T., G.W. Hahn, L.K. Poulsen and H. Malling. 2000. Safety of allergen-specific immunotherapy. Relation between dosage regimen, allergen extract, disease and systemic side-effects during induciton treatment. *Clin. Exp. Allergy* 30: 1423-1429.

11 Valenta R., K. Niespodziana, M. Focke-Tejkl, K. Marth, H. Huber, A. Neubauer and V. Niederberger. 2011. Recombinant allergens: What does the future hold? *J. Allergy Clin. Immunol.* 127: 860-864.

12 Valenta R., T. Ball, M. Focke, B. Linhart, N. Mother, V. Niederberger, S. Spitzauer, I. Swoboda, S. Vrtala, K. Westritsching and D. Kraft. 2004. Immunotherapy of allergic disease. *Adv. immunol.* 82,:105-153.

13 Wachholz P.A., N.K. Soni, S.J. Till and S.R. Durham. 2003. Inhibition of allergen-IgE binding to B cells by IgG antibodies after grass pollen immunotherapy. *J. Allergy Clin. Immunol.* 112: 915-922.

14 Mothes N., M. Heinzkill K.J. Drachenberg, W.R. Sperr, M.T. Krauth, Y. Majlesi, H. Semper, P. Valent, V. Niederberger, D. Kraft and R. Valenta. 2003. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clin. Exp. Allergy* 33: 1198-1208.

15 Larche M. 2006. Immunoregulation by targeting T cells in the treatment of allergy and asthma. *Curr. Opin. Immunol.* 18: 745-750.

16 Laffer S., S. Vrtala, M. Duchêne, R. van Ree, D. Kraft, O. Scheiner and R. Valenta. 1994. IgE-binding capacity of recombinant timothy grass (Phleum pratense) pollen allergens. *J, Allergy Clin, Immunol,* 94: 88-94.

17 Duffort O., J. Quitana, H. Ipsen, D. Barber and F. Polo. 2008. Antigen similarity among group 1 allergens from grasses and quantitation ELISA using monoclonal antibodies to Phl p 1. *Int. Arch. Allergy Immunol.* 145: 283-290.

18 Flicker S., P. Steinberger, T. Ball, M.T. Krauth, P. Verdino, P. Valent, S. Almo and R. Valenta. 2006. Spatial clustering of the IgE epitopes on the major timothy grass pollen

allergen Phl p 1: importance for allergenic activity. *J. Allergy Clin. Immunol.* 117: 1336-1343.

19 Laffer S,, M. Duchene, I. Reimitzer, M. Susani, C. Mannhalter, D. Kraft and R. Valenta. 1996. Common IgE-epitopes of recombinant Phl p I, the major timothy grass pollen allergen and natural group I grass pollen isoallergens. *Mol. Immunol.* 33: 417-426.

20 Tamborini E., S. Faccini, J. Lidholm, M. Svensson, A. Brandazza, R. Longhi, H. Grönlund, A- Sidoli and P. Arosio. 1997. Biochemical and immunolochemical characterization of recombinant allergen Lol p 1. *Eur. J. Biochem.* 249: 886-894.

21 Smith P.M., A. Avjiouglu, L.R. Ward, R.J. Simpson, R.B. Knox and M.B. Sing. 1994. Isolation and characterization of group I isoallergens from Bermuda grass pollen. *Int. Arch. Allergy Immunol.* 104: 57-64.

22 Andréasson U., S. Flicker, M. Lindstedt, R. Valenta, L. Greiff, M. Korsgren, C.A. Borrebaeck and M Ohlin. 2006. The human IgE-encoding transcriptome to assess antibody repertoires and repertoire evolution. *J. Mol. Biol.* 362: 212-227.

23 Persson H., S. Flicker, M. Karbalaei Sadegh, R. Valenta, L. Greiff and M. Ohlin. 2008. A common idiotype in IgE and its relation to recognition of the grass pollen allergen Phl p 2. *Mol. Immunol.* 45: 2715-20.

24 Persson H., H. Wallmark, A. Ljungars, J. Hallborn and M. Ohlin. 2008. In vitro evolution of an antibody fragment population to find high-affinity hapten binders. *Protein Eng. Des. Sel.* 21: 485-493.

25 Christensen L.H., J. Holm, G. Lund, E. Riise and K. Lund. 2008. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. *J. Allergy Clin. Immunol.* 122: 298-304.

26 Christensen L.H., E. Riise, L. Bang, C. Zhang and K. Lund. 2010. Isoallergen variations contribute to the overall complexity of effector cell degranulation: effect mediated through differentiated IgE affinity. *J. Immunol.* 184: 4966-4972

27 Holm J., N. Willumsen, P.A. Würtzen, L.H. Christensen and K. Lund. 2011. Facilitated antigen presentation and its inhibition by blocking IgG antibodies depends on IgE repertoire complexity. *J. Allergy Clin. Immunol.* 127: 1029-1037.

28 Persson H., M. Karbalaei Sadegh, L. Greiff and M. Ohlin. 2007. Delineating the specificity of an IgE-encoding transcriptome. *J. Allergy Clin. Immunol.* 120: 1186-1192.

29 Levin M., L.W. Tan, L. Baker, P.J. Wormald, L. Greiff and M. Ohlin. 2011. Diversity of immunoglobulin E-encoding transcripts in sinus mucosa of subjects diagnosed with non-allergic fungal eosinophilic rhinosinusitis. *Clin. Exp. Allergy* 41: 811-820.

30 Moutel S., A.E. Marjou, O. Vielemeyer, C. Nizak, P. Benaroch, S. Dübel and F.A. Perez. 2009. A multi-Fc-species system for recombinant antibody production. *BMC Biotechnology* 9:14.

31 Brochet X., M.P. Lefranc and V. Giudicelli. 2008. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic Acids Res.* 36: W503-508.

32 Lefranc M.P. 1997. The IMGT unique numbering for Immunoglobulins, T cell receptors and Ig-like domains. *The Immunologist* 7: 132-136.

33 Boyd S.D., B.A. Gaëta, K.J. Jackson, A.Z. Fire, E.L. Marshall, J.D. Merker, J.M. Maniar, L.N. Zhang, B. Sahaf, C.D. Jones, B.B. Simen, B. Hanczaruk, K.D. Nguyen, K.C. Nadeau, M. Egholm, D.B. Miklos, J.L. Zehnder and A.M. Collins. 2010. Individual variation in the germline Ig gene repertoire inferred from variable region gene rearrangements. *J. Immunol.* 184: 6986-6992.

34 Xu J.L. and M.M. Davis. 2000. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* 13: 37-45.

35 Kerzel S., T. Rogosch, B. Struecker, R.F. Maier and M. Zemlin. 2010. IgE transcripts in the circulation of allergic children reflect a classical antigen-driven B cell response and not a superantigen-like activation. *J. Immunol.* 185: 2253-2260.

36 Mari A., C. Rasi, P. Palazzo and E. Scala. 2009. Allergen databases: current status and perspectives. *Curr. Allergy Asthma Rep.* 9: 376-383.

37 Flicker S., P. Steinberger, L. Norderhaug, W.R. Sperr, Y. Majlesi, P. Valent, D. Kraft and R. Valenta. 2002. Conversion of a grass pollen allergen-specific human IgE into a protective IgG(1) antibody. *Eur. J. Immunol.* 32: 2156-2162.

38 Van Ree R. And R.C. Aalberse. 1995. Rabbit IgG directed to a synthetic C-terminal peptide of the major grass pollen allergen Lol p 1 inhibits human basophil histamine release induced by natural Lol p 1. *Int. Arch. Allergy Immunol.* 106: 1336-1343.

39 Ball T., T. Fuchs, W.R. Sperr, P. Valent, L. Vangelista, D. Kraft and R. Valenta. 1999. B cell epitopes of the major timothy grass pollen allergen, phl p 1, revealed by gene fragmentation as candidates for immunotherapy. *FASEB J.* 13: 1277-1290.

40 Focke M., V. Mahler, T. Ball, W.R. Sperr, Y. Majlesi, P. Valent, D. Kraft and R. Valenta. 2001. Nonanaphylactic synthetic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1, for allergy vaccination. *FASEB J.* 15: 2042-2044.

41 Ball T., B. Linhart, K. Sonneck, K. Blatt, H. Herrmann, P. Valent, A. Stoecklinger, C. Lupinek, J. Thalhamer, A.A. Fedorov, S.C. Almo and R. Valenta. 2009. Reducing allergenicity by altering allergen fold: a mosaic protein of Phl p 1 for allergy vaccination. *Allergy* 64: 569-580.

42 Oseroff C., J. Sidney, M.F. Kotturi, R. Kolla, R. Alam, D.H. Broide, S.J. Wasserman, D. Weiskopf, D.M. McKinney, J.L. Chung, A. Petersen, H. Grey, B. Peters and A. Sette.

2010. Molecular determinants of T cell epitope recognition to the common Timothy grass allergen. *J. Immunol.* 15: 943-945.

43 Axelsson F., S.P. Adler, A. Lamarre and M. Ohlin. 2007. Humoral immunity targeting site I of antigenic domain 2 of glycoprotein B upon immunization with different cytomegalovirus candidate vaccines. *Vaccine* 26: 41-46.

44 Ohlin M., B. Plachter, V.A. Sundqvist, P.G.A. Steenbakkers, J.M. Middeldorp and C.A.K. Borrebaeck. 1995. Human antibody reactivity against the lower matrix protein (pp65) produced by cytomegalovirus. *Clin. Diagn. Lab. Immunol.* 2: 325-329.

45 Sutherland J.N., C. Chang, S.M. Yoder, M.T. Rock and J.A. Maynard. 2011. Antibodies recognizing protective pertussis toxin epitopes are preferentially elicited by natural infection versus acellular immunization. *Clin. Vaccine Immunol.* 18: 954-62.

46 Trible B.R., M. Kerrigan, N. Crossland, M. Potter, K. Faaberg, R. Hesse and R.R. Rowland. 2011. Antibody recognition of porcine circovirus type 2 capsid protein epitopes after vaccination, infection, and disease. *Clin. Vaccine Immunol.* 18: 749-57.

47 Cheng C., J.G. Gall, M. Nason, C.R. King, R.A. Koup, M. Roederer, M.J. McElrath, C.A. Morgan, G. Churchyard, L.R. Baden, A.C. Duerr, M.C. Keefer, B.S. Graham and G.J. Nabel. 2010. Differential specificity and immunogenicity of adenovirus type 5 neutralizing antibodies by natural infection or immunization. *J. Virol.* 84: 630-638.

48 Sudowe S., A. Rademaekers and E. Kolsch. 1997. Antigen dose-dependent predominance of either direct or sequential switch in IgE antibody responses. *Immunology* 91: 464-472.

49 Van Milligan F.J., S. Craig, B.L. Rogers, P. van Swieten and R.C. Aalberse. 1995. Differences between specificities of IgE and IgG4 antibodies: studies using recombinant chain 1 and chain 2 of the major cat allergen Felis domesticus (Fel d) I. *Clin. Exp. Allergy* 25: 247-251.

50 Steinberger P., D. Kraft and R. Valenta. 1996. Construction of a combinatorial IgE

library from an allergic patient. Isolation and characterization of human IgE Fabs with specificity for the major timothy grass pollen allergen, Phl p 5. *J. Biol. Chem.* 271: 10967–10972.

51 Gorny M.K., J. Sampson , H. Li, X. Jiang, M. Totrov, X.H. Wang, C. Williams, T. O'Neal, B. Volsky, L. Li, T. Cardozo, P. Nyambi, S. Zolla-Pazner, X.P. Kong. 2011. Human anti-V3 HIV-1 monoclonal antibodies encoded by the VH5-51/VL lambda genes define a conserved antigenic structure. *PLoS One* 6: e27780.

52 Lingwood D., P.M. McTamney, H.M. Yassine, J.R. Whittle, X. Guo, J.C. Boyington, C.J. Wei, G.J. Nabel. 2012. Structural and genetic basis for development of broadly neutralizing influenza antibodies. *Nature* (in press) (DOI: 10.1038/nature11371.)

53 Chabre H., B. Gouyon, A. Huet, V. Baron-Bodo, E. Nony, M. Hrabina, F. Fenaille, A. Lautrette, M. Bonvalet, B. Maillère, V. Bordas-Le Floch, L. Van Overtvelt, K. Jain, E. Ezan, T. Batard and P. Moingeon. 2011. Molecular variability of group 1 and 5 grass pollen allergens between Pooideae species: implications for immunotherapy. *Clin. Exp. Allergy* 40: 505-519.

54 Fenaille F., E. Nony, H. Chabre, A. Lautrette, M.N. Couret, T. Batard, P. Moingeon and E. Ezan. 2009. Mass spectrometric investigation of molecular variability of grass pollen group 1 allergens. *J. Proteome Res.* 8: 4014-4027.

Footnotes

1 This work was supported by: The Swedish Research Council (grants number 521-2008-3614, 521-2009-2419 and 521-2011-3282), the Alfred Österlund Foundation, Stiftelsen Olle Engkvist Byggmästare and the Signhild Engkvist Foundation.

2 Abbreviations used in this article: CDRH3, third complementarity determining region of the heavy chain; scFv, single chain fragment variable; VH, heavy chain variable; VL, light chain variable;

Figure legends

Figure 1. Sequence of Phl p 1.0102 (A, gray + black), highlighting its C-terminal domain (A, black). Twelve mutant versions of the C-terminal domain were produced, each carrying a single mutation in one of the underlined positions (A), and tested for IgE binding. All mutated amino acids were replaced with an alanine. Purity of produced proteins, after production in small (B-D) and large scale (E, F), was confirmed by SDS-PAGE analysis (GST-tagged wild-type C-terminal domain of Phl p 1.0102 (B); GST (C); GST-tagged (D, E) and GST-tag-free (F) mutant version of the C-terminal domain of Phl p 1.0102 (carrying mutations K8A, N11A and D55A)).

Figure 2. Binding specificity of novel scFv (displayed on phage) selected on Phl p 1.0102 as determined by ELISA on different recombinant timothy grass allergens and BSA. All clones are highly specific for Phl p 1, with no detectable cross-reactivity. All samples were run in duplicates and error bars represent one standard deviation.

Figure 3. Binding of Phl p 1-specific IgE (presented in the form of scFv displayed on phage) to a GST-tagged C-terminal fragment of Phl p 1.0102 was confirmed by ELISA. All five human IgE (1p1:8, 5p1:3, p1-15, p1-20 and clone 10) do bind the C-terminal domain at levels comparable to their ability to bind Phl p 1. In contrast, out of the three mouse mAb (1.8, 1.10 and 1.21) only one (1.8) reacts with the C-terminal domain. All samples were run in duplicates and error bars represent one standard deviation.

Figure 4. Reactivity of Phl p 1-specific human monoclonal scFv-CHε2-4 against protein extracts of 10 grass species with group 1 allergens with high sequence identity to Phl p 1 as determined by ImmunoCAP. All samples were assayed in duplicates with an average coefficient of variation of 4.4 %. Values are normalized and compared to *Phleum pratense* (100%).

Figure 5. 3D visualization of the C-terminal part of Phl p 1.0102 (PDB: 1N10) (green) with mutations K8A, N11A and D55A, shown to be important for IgE-binding, marked in blue. Amino acids in red represent those that failed to affect IgE-binding when mutated.

Figure 6. Inhibition of antibody binding to immobilized allergen by soluble allergen variants. Inhibition of binding of five human IgE (as scFv-CHε2-4 fusion proteins) (1p1:8 (A); 5p1:3 (B); p1-15 (C); p1-20 (D); clone 10 (E)) and one mouse mAb (1.8 (F)) to immobilized Phl p 1.0102 using soluble proteins was determined by ELISA. Inhibition with the GST-tagged C-terminal domain of Phl p 1.0102 (diamonds), the GST-tagged mutant version of the C-terminal domain (carrying mutations K8A, N11A and D55A) (squares) and GST (triangles) was evaluated. The GST-tagged combined mutant failed to inhibit binding of human IgE (A-E), while it did inhibit the binding of the mouse mAb (F) to a level comparable to that of the unmutated GST-tagged C-terminal domain. To ensure that the GST-tag itself did not exert any major effects on IgE binding activity, the binding of clones 1p1:8 (G), 5p1:3 (H), p1-20 (I) and clone 10 (J) (as scFv-CHε2-4 fusion proteins) and the mouse mAb 1.8 (K) to immobilized Phl p 1.0102 was also inhibited using soluble wildtype recombinant Phl p 1.0102 (diamonds), the non-GST-tagged mutant (carrying mutations K8A, N11A and D55A) (squares), and GST (triangles) was evaluated. The combined mutant version of the C-terminal domain of Phl p 1.0102 largely failed to fully inhibit binding of human IgE also

after removal of the GST-tag. The mouse mAb (1.8) was partially inhibited by the mutated version without the GST-tag. This mouse antibody did bind the immobilized GST-tag-free protein (light grey bars) (but not GST (white bars)), to a similar degree as intact Phl p 1.0102 (dark grey bars) in a direct ELISA at two different dilutions (1:10 000 and 1:100 000) (L). All samples were run in duplicates and error bars represent one standard deviation.

Figure 7. Reactivity of serum IgE from 11 Phl p 1-positive donors against GST-fusion proteins of the C-terminal domain of Phl p 1.0102, the potentially hypoallergenic variant thereof and GST, as determined using ELISA (A). The introduction of three mutations (K8A, N11A and D55A) significantly ($p<0.0001$) reduces the reactivity by polyclonal IgE derived from human serum compared to the corresponding wild-type protein sequence. To ensure that the GST tag itself did not exert any significant effects on serum IgE binding, a non-tagged mutant, carrying the above-described mutations, was also studied. Serum IgE reactivity to wild-type Phl p 1.0102 the mutant (K8A, N11A and D55A) of the C-terminal domain with or without the GST-tag, and GST alone was assessed for this purpose (B). Also the non-tagged mutant showed a significantly ($p<0.0001$) reduced recognition by human serum IgE as compared to the recognition of wild-type Phl p 1.0102 by serum IgE, excluding any major effects exerted by the GST-tag itself. The lower and upper margins of the box correspond to quartiles (i.e. percentiles 25 and 75), the bold line corresponds to the median and the whiskers correspond to maximum and minimum values. All samples were run in duplicates.

Figure 8. Patterns of changes in basophil activation in three individual grass pollen allergic donors (D1-3). Basophils were stimulated with the wild-type protein Phl p 1.0102 (filled diamonds) and the C-terminal domain of Phl p 1.0102 fused to GST (filled squares), as well

as with N11A (open triangles) or K8A, N11A, D55A (open circles) mutated variants of the C-terminal domain of Phl p 1.0102 fused to GST. Changes in CD63 expression upon degranulation were assessed. Stimulation index is calculated from the mean fluorescence intensity (MFI) of CD63 expression levels and % CD63+ cells from the stimulated samples as compared to the unstimulated control samples.

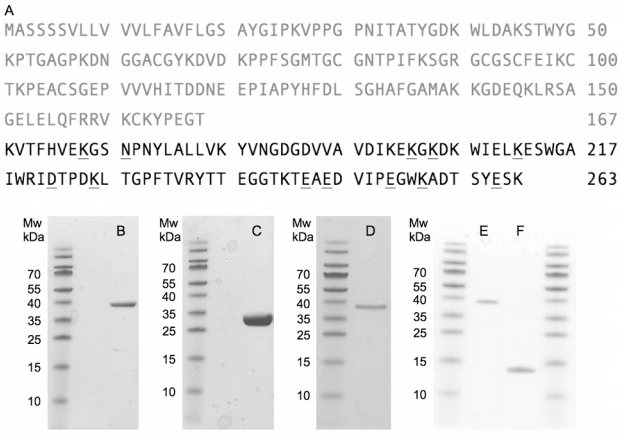
A
MASSSSVLLV VVLFAVFLGS AYGIPKVPPG PNITATYGDK WLDAKSTWYG 50
KPTGAGPKDN GGACGYKDVD KPPFSGMTGC GNTPIFKSGR GCGSCFEIKC 100
TKPEACSGEP VVVHITDDNE EPIAPYHFDL SGHAFGAMAK KGDEQKLRSA 150
GELELQFRRV KCKYPEGT 167
KVTFHVEKGS NPNYLALLVK YVNGDGDVVA VDIKEKGKDK WIELKESWGA 217
IWRIDTPDKL TGPFTVRYTT EGGTKTEAED VIPEGWKADT SYESK 263
Mw kDa
B
C
D
E
F
70
55
40
35
25
15
10

Figure 1

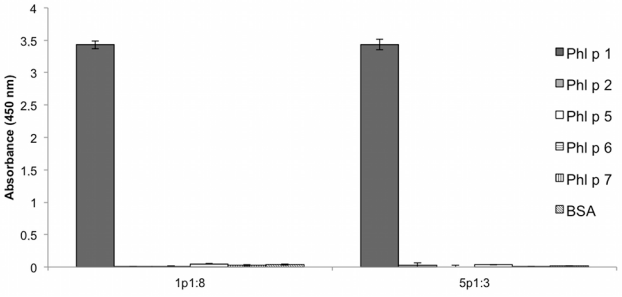
Absorbance (450 nm)
4
3.5
3
2.5
2
1.5
1
0.5
0
1p1:8
5p1:3
Phl p 1
Phl p 2
Phl p 5
Phl p 6
Phl p 7
BSA

Figure 2

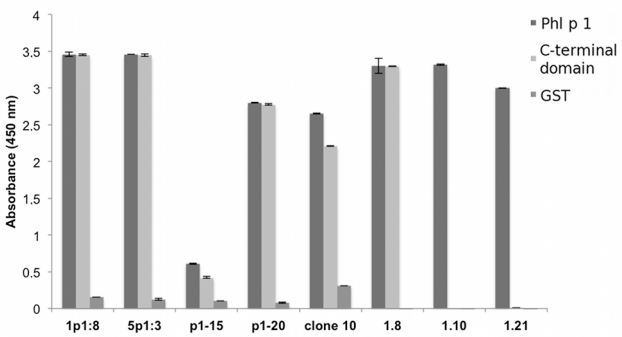
Absorbance (450 nm)
4
3.5
3
2.5
2
1.5
1
0.5
0
1p1:8
5p1:3
p1-15
p1-20
clone 10
1.8
1.10
1.21
Phl p 1
C-terminal domain
GST

Figure 3

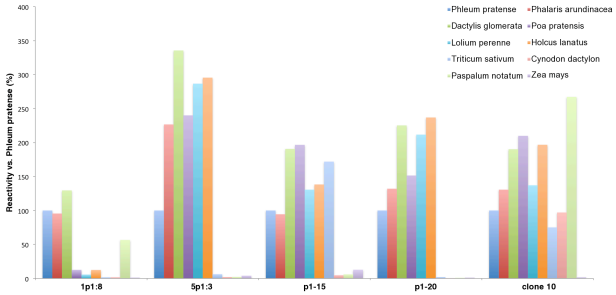
Phleum pratense
Phalaris arundinacea
Dactylis glomerata
Poa pratensis
Lolium perenne
Holcus lanatus
Triticum sativum
Cynodon dactylon
Paspalum notatum
Zea mays
Reactivity vs. Phleum pratense (%)
400
350
300
250
200
150
100
50
0
1p1:8
5p1:3
p1-15
p1-20
clone 10

Figure 4

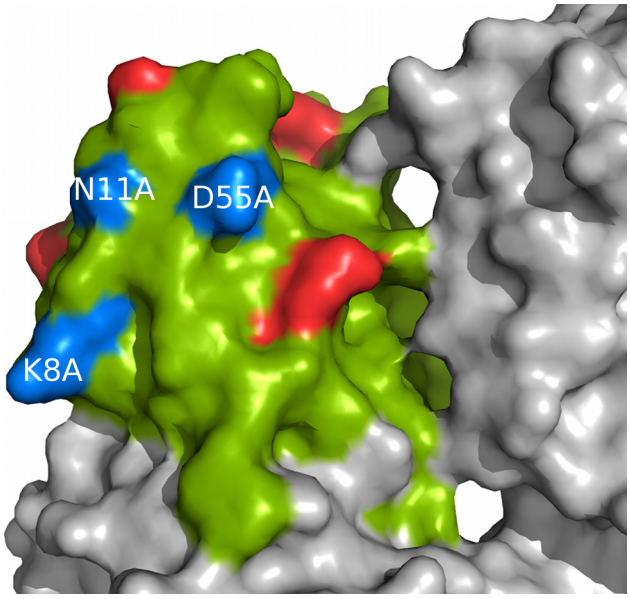

Figure 5

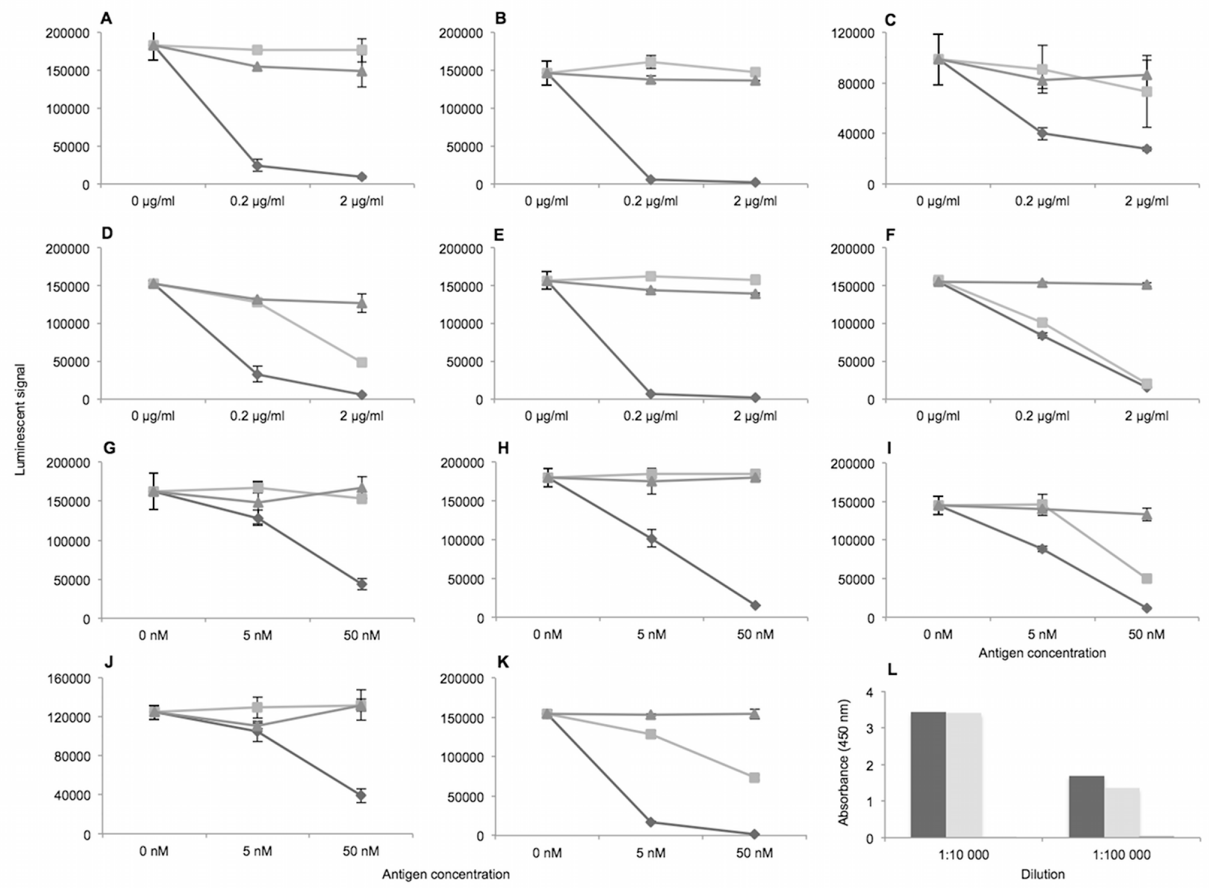
A
B
C
D
E
F
G
H
I
J
K
L
Luminescent signal
0 µg/ml
0.2 µg/ml
2 µg/ml
0 nM
5 nM
50 nM
Antigen concentration
Absorbance (450 nm)
1:10 000
1:100 000
Dilution

Figure 6

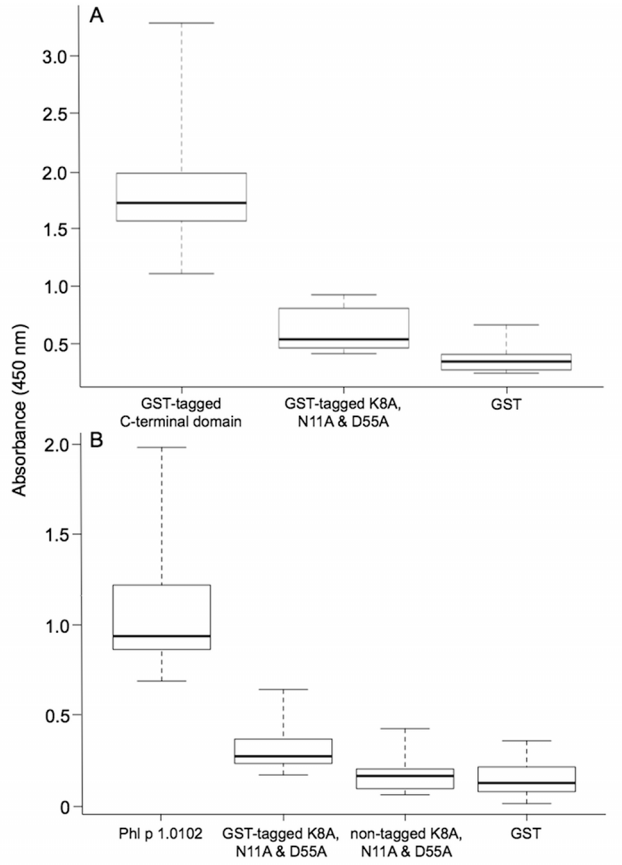
A
3.0
2.5
2.0
1.5
1.0
0.5
Absorbance (450 nm)
GST-tagged
C-terminal domain
GST-tagged K8A,
N11A & D55A
GST
B
2.0
1.5
1.0
0.5
0
Phl p 1.0102
GST-tagged K8A,
N11A & D55A
non-tagged K8A,
N11A & D55A
GST

Figure 7

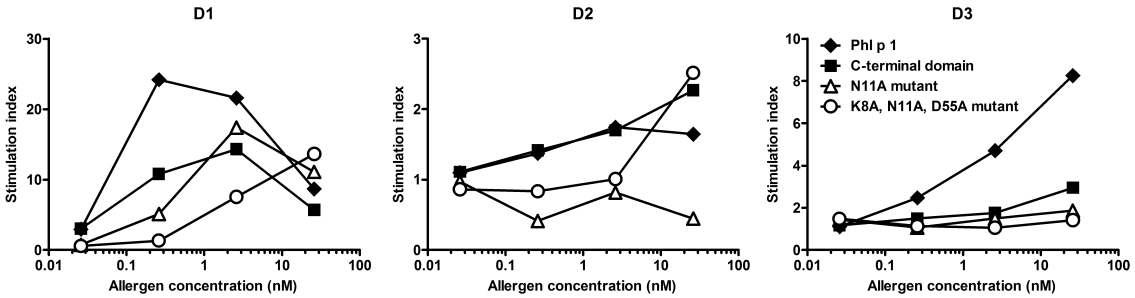
D1
D2
D3
Stimulation index
Allergen concentration (nM)
Phl p 1
C-terminal domain
N11A mutant
K8A, N11A, D55A mutant

Figure 8