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Isoallergen Variations Contribute to the Overall Complexity of Effector Cell Degranulation: Effect Mediated through Differentiated IgE Affinity

Lars H. Christensen,^{*,†} Erik Riise,[†] Lærke Bang,[†] Chunqing Zhang,[‡] and Kaare Lund*

Most allergens exist in several variants (isoallergens), each of which may be recognized differently by patient IgE. We have previously shown that several properties of the IgE repertoire, including IgE affinity and IgE clonality, are important factors determining degranulation responses of effector cells involved in type I allergic reactions. However, less is known about how the repertoire of naturally occurring isoallergens may affect this response. Thus, in this study, we investigated how individual rIgE Ab clones derived from a human subject are able to distinguish among variants of Der p 2 isoallergens and assessed the impact on basophil degranulation. Biacore analyses showed that individual rIgE clones cloned from an individual allergic to house dust mites recognized Der p 2 with binding affinities varying up to 100-fold between different Der p 2 isoforms. In a well-defined biological system consisting of human basophils sensitized with low rIgE clonality, degranulation responses were directly related to rIgE affinity toward particular rDer p 2 isoallergens. However, basophils sensitized with polyclonal patients' sera showed no differences in degranulation responses toward the different rDer p 2 isoallergens. In conclusion, our study shows that individual IgE Abs are able to bind single allergens with a broad range of affinities due to natural isoallergen variations, contributing further to the overall complexity of IgE–allergen interactions at the effector cell surface, which is, however, blurred by the polyclonal nature of patients' IgE repertoires. *The Journal of Immunology*, 2010, 184: 4966–4972.

House dust mites (HDMs) rank among the most common allergen sources and cause allergic reactions in >50% of all allergic patients (1). Most individuals allergic to the HDM species *Dermatophagoides pteronyssinus* (Der p) are sensitized toward the major allergen, Der p 2 (2) which is a 129-aa, 14-kDa, nonglycosylated protein (3, 4) with unknown biological function. Involvement in lipid transport has, however, been suggested due to its hydrophobic cavity (5). Recently, the allergenicity of Der p 2 was linked to its structural and functional homology with the TLR4 LPS-binding component MD-2 (6).

In both cultured and natural populations of HDM, certain allelic polymorphisms exist that lead to expression of several variants of many HDM genes, including those encoding for allergenic proteins (7). Currently, 15 isoforms of Der p 2 have been described (according to the Allergome allergen database) with individual isoallergens varying between 1 and 7 aa in nine different amino acid positions corresponding to between 95 and 99% identity.

The similarity between Der p 2 and homologous proteins from other HDM species is lower than between the Der p 2 isoforms. Still, there is a considerable level of Ab cross-reactivity between

the closely related group 2 HDM allergens Der p 2, Der f 2, and Eur m 2 (>80% amino acid sequence identity) (4, 8–11). Conversely, Ab cross-reactivity between Der p 2 and the more distant related group 2 storage mite allergens Lep d 2, Tyr p 2, and Gly d 2 is low, consistent with a low (40%) sequence similarity between Der p 2 and these allergens (2, 9).

The ability to distinguish among a limited number of different Der p 2 isoallergens has been shown for IgG mAbs produced in mice (9, 10, 12) and for human IgE Abs at the level of polyclonal patient sera in ELISA (13, 14). Still, the biological mechanism at the level of effector cell activation triggered by interactions between individual IgE Abs and Der p 2 isoallergens is largely unknown.

In this study, we report the cloning and expression of fully human HDM-specific IgE Abs and demonstrate how even small variations in the allergen surface that exist between different isoforms of Der p 2 can affect IgE affinity and how this transfers to effector cell degranulation.

We have previously used rIgE Abs, including the clones described in this study, to show how individual properties of the IgE repertoire affect effector cell degranulation upon allergen challenge (15). In that publication, we stated that IgE clones C–K were cloned by the phage display method, but without a detailed description (only mentioned “as described in detail elsewhere”). Thus, the current study includes this detailed description of full-size, fully human rIgE cloning/production, although part of the method is only included as Supplemental Material.

Materials and Methods

Construction and production of an IgE-Fab phage library

HDM-specific IgE-Fab Abs were cloned by means of the phage display technique from peripheral IgE⁺ B cells of a patient allergic to HDM as schematically outlined in Supplemental Fig. 1.

A total of 100 ml peripheral blood was collected from an individual with a clinical history of atopic rhinitis and dermatitis toward HDMs. Serological samples from this subject showed specific IgE concentrations of 31.5 kU/l toward Der p extract and 50.7 kU/l toward Der p 2. Blood was donated with

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The online version of this article contains supplemental material.

Abbreviations used in this paper: D, aspartic acid; HDM, house dust mite; K, lysine; L, leucine; M, methionine; N, asparagine; T, threonine; V, valine.

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informed consent from the donor and the protocol approved by the regional ethical committee (De Videnskabetiske Komitéer for Region Hovedstaden). Following initial purification of PBMCs, IgE-producing B cells (IgE⁺ B cells) were further purified by removal of B cells producing irrelevant isotypes using Dynabeads (Invitrogen, CA). Dynabeads precoated with anti-CD3 and anti-CD2 for removal of T cells and anti-CD14 for removal of monocytes were also included.

For amplification of IgE-Fd fragments and Ab L chains, a three-step nested RT-PCR strategy was developed as depicted in Supplemental Fig. 2. The third PCR product of the IgE-Fd fragment and L chains were cloned into the pFab60 phagemid vector (16) generating the phagemid library pFab60IgE-Fab. The pFab60IgE-Fab phagemid library was electroporated into ultracompetent TOP10F⁺ bacteria prepared as described (17) in 30 separate electroporations. The transformed TOP10F⁺ bacteria were plated on LB-agar plates with ampicillin and incubated overnight. A total of 400 ml 2XYT medium with ampicillin, tetracycline, and 1% glucose was inoculated with the transformed pFab60IgE-Fab library to a start-OD₆₀₀ of 0.05 and incubated at 37°C to a density of OD₆₀₀ 0.5. At this point, a 100-fold surplus (4×10^{12}) of R408 helper phages (Promega, Madison, WI) were added to the culture followed by infection in 30 min at 37°C. Infected bacteria were then sedimented and resuspended in 400 ml fresh 2XYT medium with ampicillin plus isopropyl β -D-thiogalactoside (inducer). The IgE-Fab phages were expressed overnight at 22°C. The next day, IgE Fab-phage particles were precipitated from the cell supernatant with phage precipitation buffer (20% PEG₆₀₀₀ plus 2.5 M NaCl) and dissolved in PBS. This IgE-Fab phage library was used for the panning procedure described below.

Isolation of HDM-specific IgE-Fab phages

The following panning procedure was applied: Three 4 ml MaxiSorp immunotubes (Nunc, Roskilde, Denmark) were coated with rDer p 2.0101 (produced in *Pichia pastoris*), Der p extract, and Der f extract, respectively. Additional immunotubes were coated with BSA as negative controls. The coated immunotubes were blocked with blocking buffer (2% skim milk in PBS) for 2 h and washed several times in washing buffer (0.05% Tween 20 in PBS). A total of 10^{10} to 10^{11} IgE-Fab phages were added to each precoated Immunotube and incubated at room temperature with slow tilt rotation for 2 h. Immunotubes were washed several times and bound IgE-Fab phages eluted with 10 mM glycine-HCl (pH 2) for 15 min followed by neutralization of the eluates with 0.2 M Tris-base. The eluates were then added to exponentially growing TOP10F⁺ bacterial cultures (OD₆₀₀ ~0.5) to prepare IgE-Fab phages for subsequent panning rounds.

Single IgE-Fab phage clones from third, fourth, and fifth panning rounds were monoclonally produced and tested for their ability to bind HDM allergens in ELISA. IgE-Fab phage clones that showed specific reactivity toward HDM allergens in ELISA were DNA sequenced and converted into full-size rIgE Abs as described below.

Conversion of IgE-Fab phages into full-size, fully human rIgE Abs

Previously, a set of two mammalian expression vectors encoding human IgG3 H chain (pLNOH2) and human κ L chain (pLNOK) was constructed by Norderhaug et al. (18). For expression of the rIgE Abs described in this study, the human constant IgG3 region of pLNOH2 was replaced with the human constant IgE region (CeH1–CeH4), giving the new vector pLNOH2IgE (see details in Supplemental Fig. 4). For optional expression of human λ L chains, the human constant κ L chain insert in pLNOK was replaced with λ C₁, C₂, or C₃ λ L chain subtypes, respectively (Supplemental Fig. 4).

Variable heavy and L chain regions from mite-specific IgE-Fab phage clones B–K were subcloned into these vectors and full-size, fully human rIgE Abs produced in HEK293 cell cultures (Invitrogen) as illustrated in Supplemental Fig. 4.

Expression of rDer p 2 isoallergens

We were able to obtain cDNA from five different Der p 2 isoforms: Der p 2.0101, 2.0103, 2.0104, and 2.0105 were kindly provided by Prof. Wayne R. Thomas (Telethon Institute for Child Health Research, Subiaco, Australia), whereas Der p 2 Q was a clone previously cloned in-house from cultured HDMs. The Der p 2 isoallergens were subcloned into a mammalian expression vector and expressed in HEK293 cell cultures (Invitrogen) as illustrated in Supplemental Fig. 5. The rDer p 2 isoallergens were normalized to the same concentration (10 mg/ml) in a Biacore assay utilizing an Ab that binds an epitope conserved on all these Der p 2 isoallergens (i.e., binding with equally high affinity to all the Der p 2 isoallergens) (red epitope, Fig. 3).

Binding profiles of rIgE Abs interacting with HDM allergens

Interactions between rIgE Abs and HDM extracts/rDer p 2 isoallergens were analyzed on a Biacore 2000 (GE Healthcare, Piscataway, NJ). A total of 8000

resonance units of a monoclonal anti-human IgE (developed in-house at ALK-Abelló, Hørsholm, Denmark) was immobilized on a CM5-chip (GE Healthcare) using an Amine Coupling Kit (GE Healthcare). Individual rIgE clones were injected for 5 min (corresponding to 800–1000 resonance units) followed by injection of 50 μ g/ μ l Der p extract (made in-house), 50 μ g/ml Der f extract (made in-house), or 10 μ g/ml rDer p 2.0101 for 1.5 min (Fig. 2) or each of the five rDer p 2 isoforms described above (1 μ g/ml) for 3 min (Fig. 4).

Affinity determinations of rIgE Abs interacting with rDer p 2 isoallergens

Surface plasmon resonance (Biacore, GE Healthcare) affinity measurements were carried out using a CM5-chip immobilized with anti-human IgE as described above. Cycles were run as follows: individual rIgE clones were injected for 5 min followed by injection of the rDer p 2 isoallergen for 5 min with a dissociation time of 10 min. Two-fold dilutions of the rDer p 2 isoallergen in a concentration range of 714–0.698 nM were used for a complete coverage from $R_{\max}$ to zero response. The K_D (k_d/k_a) was calculated with BIAevaluation software (Biacore, GE Healthcare).

Basophil degranulation

Basophil degranulation experiments were essentially carried out as previously described (15) and illustrated in Supplemental Fig. 6. Briefly, PBMCs isolated from nonatopic donors whose basophils showed equally high maximal degranulation responses (>80%) were stripped for native IgE followed by passive sensitization with either different combinations of two rIgEs (Fig. 5) or with six different sera (Fig. 6) from individuals with atopic rhinitis toward HDMs collected after informed consent and having the following characteristics: serum 1, 236 kU/l sIgE toward Der p extract and 197 kU/l toward Der p 2; serum 2, 31.5 kU/l sIgE toward Der p extract and 50.7 kU/l toward Der p 2; serum 3, 27.7 kU/l sIgE toward Der p extract and 24.7 kU/l toward Der p 2; serum 4, 24.6 kU/l sIgE toward Der p extract and 24.6 kU/l toward Der p 2; serum 5, 18.5 kU/l sIgE toward Der p extract and 11.4 kU/l toward Der p 2; and serum 6, 4.91 kU/l sIgE toward Der p extract and 4.16 kU/l toward Der p 2. Sensitized basophils were subsequently challenged for 1 h with different rDer p 2 isoallergens diluted in RPMI 1640 medium containing 0.5% HSA plus 2 ng/ml IL-3.

Basophil degranulation was assessed on a FACSAria cell sorter (BD Biosciences, San Jose, CA) subsequent to labeling with a mixture consisting of anti-CD63–FITC, anti-CD123–PE, and anti-HLA-DR–PerCP (catalog number 341068; BD Biosciences) as illustrated in Supplemental Fig. 6.

Results

Cloning of HDM-specific IgE Abs from an HDM-allergic individual

In an attempt to obtain monoclonal mite-specific IgE Abs of human origin, we constructed an IgE-Fab phagemid library from an HDM-allergic patient's peripheral IgE-producing B cells, as illustrated in Supplemental Fig. 1. The library consisted of 1.1×10^7 single clones and was found to be quite diverse, as deduced from DNA sequences of randomly picked clones (Supplemental Fig. 3).

After five rounds of panning on HDM allergens, HDM-specific IgE-Fab phages were enriched 3000-fold on rDer p 2, 1200-fold on Der p extract, and 700-fold on Der f extract compared with the respective background levels by panning on BSA (Fig. 1A).

Forty single IgE-Fab phage clones from third, fourth, and fifth panning rounds were monoclonally produced and their ability to bind HDM allergens confirmed in ELISA (data not shown). Ten different IgE Fab-phage clones were identified and named clones B–K (Fig. 1B). A total of nine different H chains (VH-Seq1–9) and five different L chains (VL-Seq10–14) were found in these 10 clones as shown in Fig. 1C and summarized in Fig. 1B.

Binding profiles of full-size, fully human rIgEs interacting with the same HDM allergen preparations as those used for isolation of the IgE-Fab phages

IgE-Fab phage clones B–K were converted into full-size, fully human IgE Abs, as illustrated in Supplemental Fig. 4. Binding profiles of these full-size rIgEs toward the same mite allergen preparations as those used in the phage panning procedure were

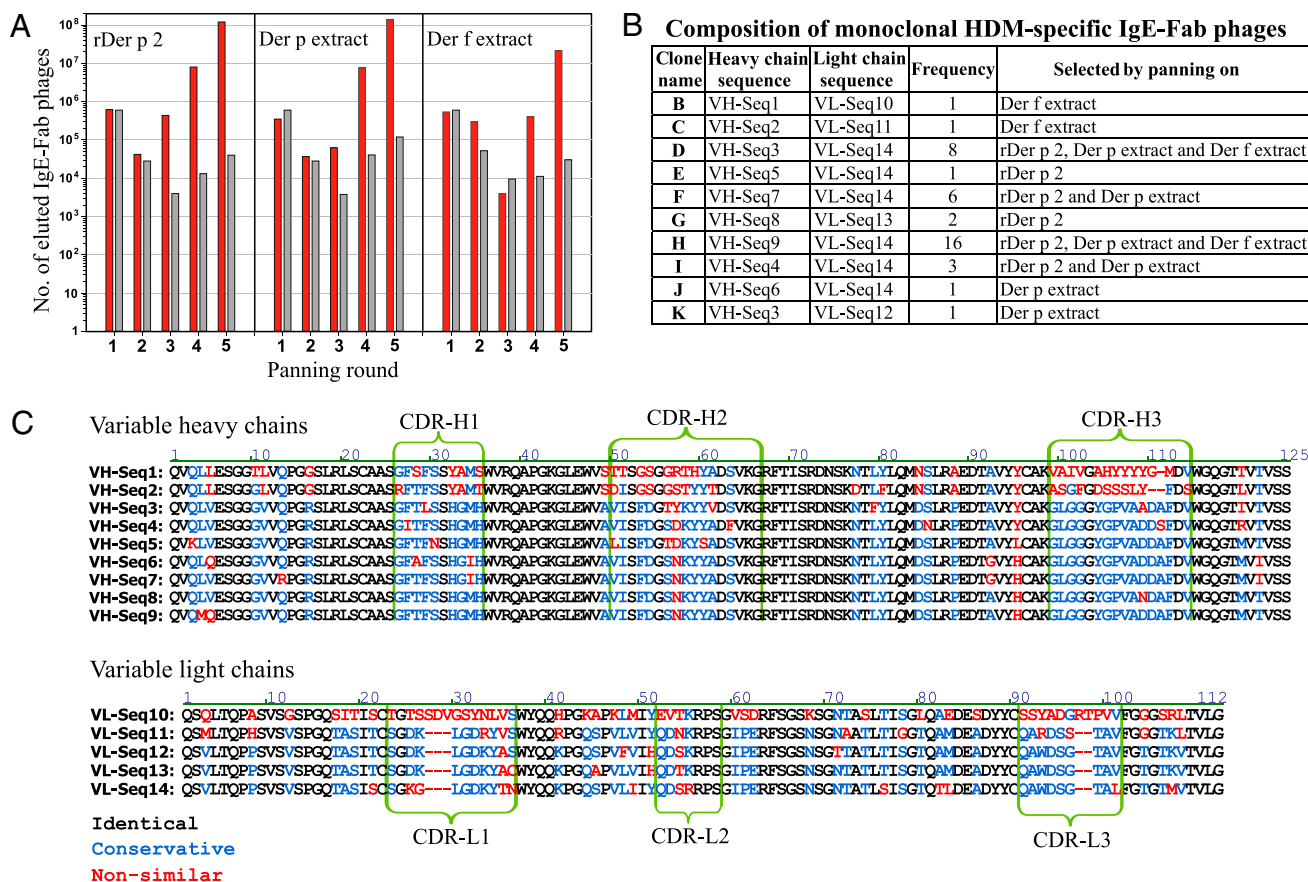


FIGURE 1. Isolation and composition of HDM-specific IgE-Fab phages. **A**, Enrichment of IgE-Fab phages following panning on rDer p 2.0101, Der p extract, or Der f extract. Red columns indicate panning on HDM allergens; gray columns indicate background levels from panning on BSA. **B**, Composition of isolated HDM-specific IgE-Fab phages. **C**, Amino acid sequences of variable IgE H and L chains of isolated IgE-Fab phages.

obtained by means of Biacore analyses (Biacore, GE Healthcare) (Fig. 2). Generally, five different binding patterns represented by rIgE clones B, C, D, E, and F were seen (as rIgE clones D plus K and clones F–J showed mutually similar binding profiles). Except rIgE clone B that selectively bound an allergen exclusively present in Der f extract (which was, however, not one of the major allergens as deduced from crossed radio immunoelectrophoresis; data not shown), all rIgE clones could bind the three allergen preparations (Fig. 2). Most clones (rIgE clone D–K) showed similar binding profiles for Der p and Der f extracts. One exception was clone C, which showed reduced affinity for Der p extract (as deduced from the steeper slope of the dissociation phase; Fig. 2). Unexpectedly, several rIgE clones (C, D, and F–K; Fig. 2) were binding rDer p 2 with markedly lower affinity than Der p extract. We hypothesized that this discrepancy was related to isoform variations (i.e., that rIgEs were able to discriminate between Der p 2 isoallergens with differentiated binding affinities and therefore were binding one or more Der p 2 isoallergens present in the Der p extract with higher affinity than the single rDer p 2.0101 isoallergens tested in this experiment).

Epitope characteristics of five rDer p 2 isoallergens

To test whether the different allergen binding patterns of the rIgEs observed in Fig. 2 were due to isoallergen variations, we produced a panel of five Der p 2 isoallergens, of which four were known (described in databases) plus one novel, in this paper called Der p 2 “Q” (Fig. 3).

We have previously mapped and shown that the rIgE clones C–K bind the same epitope-cluster on Der p 2 (15) (Fig. 3B, black circle). As displayed in the figure, this epitope contains two surface-exposed

variable amino acids in the periphery (Der p 2 aa 111 and 114, highlighted in yellow).

The figure also shows the position of two additional epitopes (Fig. 3B, marked by green and red circles) bound by two chimeric rIgE Abs, named H1 and H12:H7, having their Der p 2 binding domains cloned from mice (15). Two surface-exposed variable amino acids (Fig. 3B, Der p 2 aa 15 and 40, highlighted in yellow) are positioned very centrally in the rIgE H1 epitope (marked by a green circle), whereas rIgE H12:H7 binds an epitope (Fig. 3B, marked by a red circle) that is conserved among all the Der p 2 isoallergens (Fig. 3B).

Single rIgE Abs are able to discriminate between different Der p 2 isoallergens through differentiated binding affinity

The Der p 2-specific rIgEs (clones C–K) were further characterized in regard to their Der p 2 isoallergen-binding capabilities. Binding profiles and affinities of four representative rIgE clones are shown in Fig. 4A (the four representative clones, clones C, D, E, and F, were selected on the basis of their unique binding profiles, depicted in Fig. 2). The general tendency of the rAbs was that if they displayed strong binding affinities to one Der p 2 isoform, they also displayed relatively stronger affinities to the other isoforms and vice versa. Still, single rIgEs showed differentiated binding capabilities, with affinities spanning up to 100-fold between different Der p 2 isoallergens. In agreement with a theoretical point of view, each rIgE clone bound Der p 2.0104 and Der p 2 Q with mutually equal affinity, consistent with these two isoforms having identical amino acid composition in the respective epitope (Fig. 3B, epitope marked in black). Exchange of leucine (L) in Der p 2 position 111 with a methionine (M), as is the case with Der p

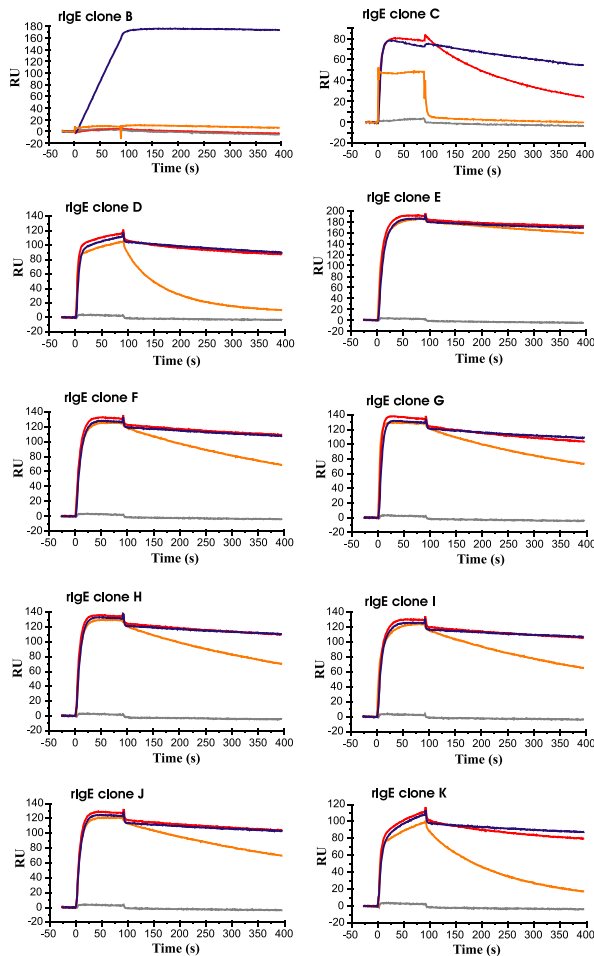


FIGURE 2. Binding profiles of full-size rIgE Abs interacting with the same HDM allergen preparations as those used for selection in the phage-display procedure. On a Biacore chip, rIgE clones B–K were individually bound by covalently immobilized anti-IgE (not shown). At time 0 s to 90 s, Der f extract (blue), Der p extract (red), rDer p 2.0101 (orange), or buffer control (gray) was injected (association phase). At time 90 s, the injection was stopped and allergen dissociated from the rIgE (dissociation phase) until the time of 400 s, when the analysis was stopped.

2.0103 (Fig. 3A), conferred slightly lower binding affinity (higher K_D values) (Fig. 4). Additional exchange of asparagine (N) with aspartic acid (D) (Fig. 3B) in position 114 as in Der p 2.0101 and Der p 2.0105 conferred markedly reduced binding affinity. The two latter isoforms have identical amino acid composition in this epitope (Fig. 3B, black circle) but differ in the non-surface-exposed amino acid, position 47. Still, this amino acid may contribute to the overall rigidity of the molecule, which is a plausible explanation why these two isoforms were bound with slightly different affinities (Fig. 4A, blue and orange curves).

Fig. 4B shows the binding profiles of chimeric rIgE clones H1 and H12:H7. As expected, clone H12:H7, which binds a fully conserved epitope (red circle, Fig. 3B), bound all Der p 2 isoallergens with equally high affinity (Fig. 4B). Similarly, clone H1 bound isoforms 2.0103, 2.0104, and 2.0105 with equal affinity consistent with these three isoallergens having identical amino acid composition in the epitope bound by this Ab (Fig. 3, green circle). However, exchange of lysine (K) to threonine (T) in position 15 as in Der p 2 isoform Q markedly decreased the binding affinity, whereas exchange of leucine (L) to valine (V) in position 40 as in Der p 2.0101 markedly increased the affinity (Fig. 4B, clone H1).

Isoallergen variations affect effector cell degranulation through differentiated IgE affinity

To test whether the observed isoallergen-dependent IgE affinity manifests into differentiated effector cell degranulation, human basophils were passively sensitized with combinations of two rIgEs followed by challenge with individual Der p 2 isoallergens as illustrated in Fig. 5. In one experiment (Fig. 5A), each human-derived rIgE (clones C–F) was combined with chimeric rIgE H12:H7 (clone binding all Der p 2 isoallergens with equally high affinity). The largest differences seen were basophils sensitized with rIgE clone C plus H12:H7. In this case, the extremely low-affinity interaction of rIgE clone C with Der p 2.0101 and 2.0105 led to basophil degranulation responses of >1000-fold lower sensitivity (observed as the shift toward lower rDer p 2 concentration eliciting a given effector cell response) compared with the higher-affinity interactions with isoform 2.0103 and 2.0104. In agreement with previous findings (15), high- and midrange affinity interactions between rIgEs and Der p 2 were generally triggering more equal effector cell responses, as shown for basophils sensitized with rIgE clones D–F instead of clone C (Fig. 5A). As expected, basophil degranulation responses were even more complex when both sensitizing rIgEs were directed toward nonconserved epitopes (Fig. 5B).

The effect of isoallergen variations on effector cell degranulation is blurred by patient's polyclonal IgE repertoire

Finally, to test whether the observed isoallergen dependency on individual rIgE affinity would further manifest into complex patient sera, basophils were sensitized with sera from HDM-allergic individuals followed by challenge with individual Der p 2 isoallergens. Contrary to what was observed with basophils sensitized with pairs of rIgEs, basophils sensitized with polyclonal patients' IgE showed highly similar degranulation responses disregarding which isoform was used for challenge (Fig. 6).

Discussion

We have previously reported how individual factors, such as concentration, affinity, and clonality of the IgE repertoire, are crucial determinants of the outcome of IgE-allergen complex-mediated effector cell degranulation (15). In this study, we demonstrate that individual IgE Abs derived from an HDM-allergic patient's IgE repertoire are able to bind recombinant constructs of naturally occurring Der p 2 isoforms with a broad range of affinities. Thus, as established for the IgE-repertoire, the complexity of the isoallergen repertoire in, for example, inhaled dust is adding up to the highly complex interplay between patients' IgE repertoire and encountered allergen repertoire shaping the outcome of effector cell degranulation.

At the clinical level, this effect of isoallergen variation may be less significant, as we found that effector cells sensitized with sera from HDM-allergic individuals show equal responses upon challenge with different isoallergens. The results, however, indicate that allergic patients' IgE repertoires are of relatively high clonality, even toward single allergens. Although the affinity effect of individual isoallergens may be of minor importance in individuals having a highly polyclonal IgE repertoire, the IgE affinity may play an important role in the context of allergenic cross-reactivity, in which recognition of allergens from species distantly related to the sensitizing allergen source will be based on a reduced subset of the IgE repertoire.

An inherent feature of phage-displayed combinatorial Ab libraries is that H and L chains are randomly paired, which means that it is never known for sure whether Abs obtained by this technique are authentically composed of their original H and L

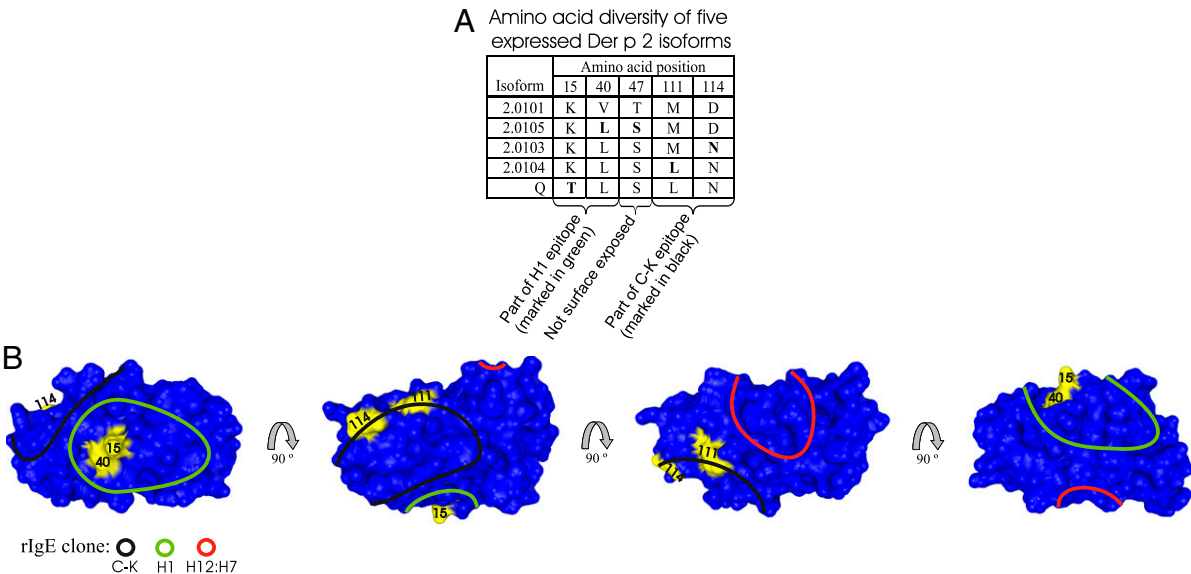


FIGURE 3. Diversity of five expressed Der p 2 isoallergens and map of IgE epitopes. **A**, Table showing amino acid variations of five expressed Der p 2 isoallergens. **B**, Crystal structure (5) of Der p 2 with variable amino acids marked in yellow. IgE epitopes bound by fully human rIgE clones C-K (black circle), chimeric rIgE clone H1 (green circle), and chimeric rIgE clone H12:H7 (red circle) were previously mapped (15). D, aspartic acid; K, lysine; L, leucine; M, methionine; N, asparagine; T, threonine; V, valine.

chains. Still, even though the IgE H chain library and, in particular, the L chain libraries of the unselected randomly picked IgE-Fab phages were relatively diverse, only a very narrow repertoire of both H and L chains were used by the isolated HDM-specific IgE-Fab phages, even after independent panning on different HDM

allergen preparations. Further, all isolated IgE-Fab phage clones were binding to at least one of the tested allergen preparations with very high affinity (Fig. 2). Together, these observations strongly indicate that the Ab chains of the selected IgE-Fab phages have in fact evolved from encountering HDM allergens.

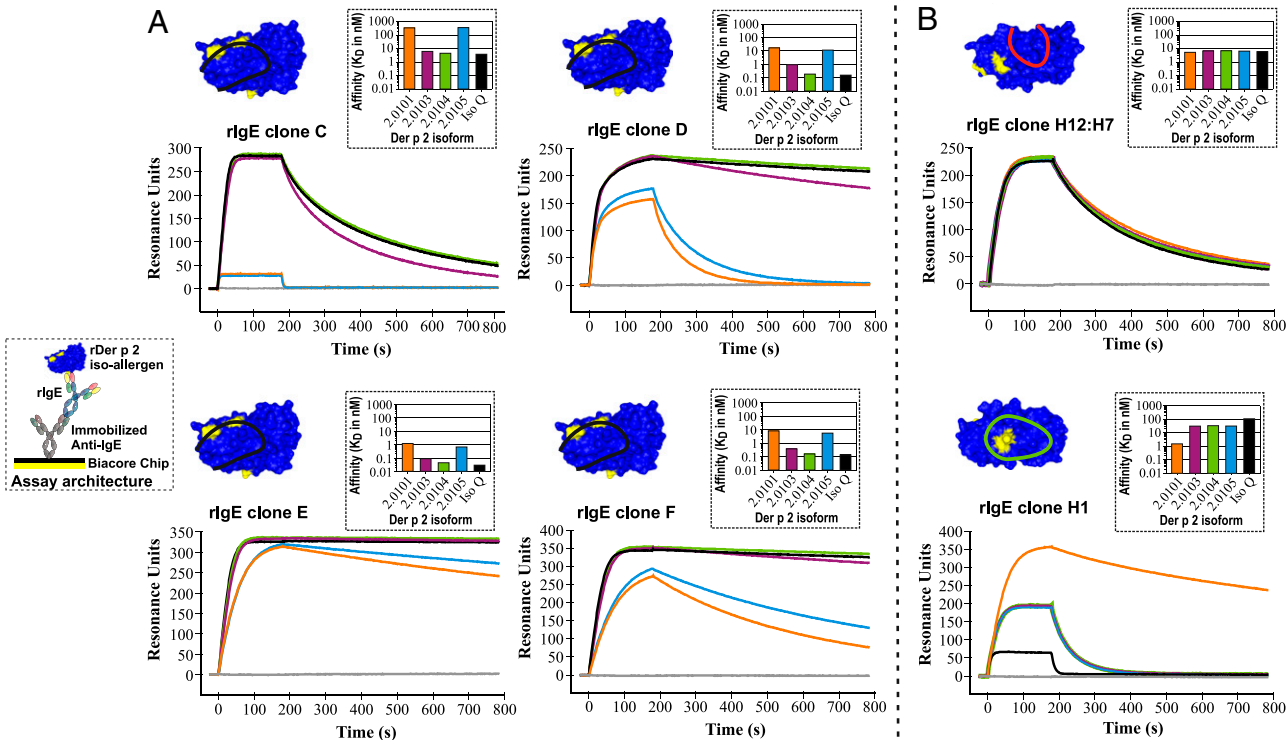


FIGURE 4. Binding profiles and affinities of rIgEs interacting with five different Der p 2 isoallergens. On a Biacore chip, fully human rIgE clones C-F (**A**) or chimeric rIgE clone H1 and H12:H7 (**B**) were bound by covalently immobilized anti-IgE (shown in the illustration to the left, but not included on the graphs). Graphs: at time 0 s to 180 s, 1 μ g/ml rDer p 2.0101 (orange), rDer p 2.0103 (purple), rDer p 2.0104 (green), rDer p 2.0105 (blue), rDer p 2 Q (black), or buffer control (gray) was injected (association phase). At time 180 s, the injection was stopped and rDer p 2 dissociated from the rIgE (dissociation phase) until time $\sim$ 800 s, at which the analysis was stopped. Exact affinities (K_D values) are shown in the upper right corner of the graphs. A lower K_D value means a higher affinity (stronger binding strength).

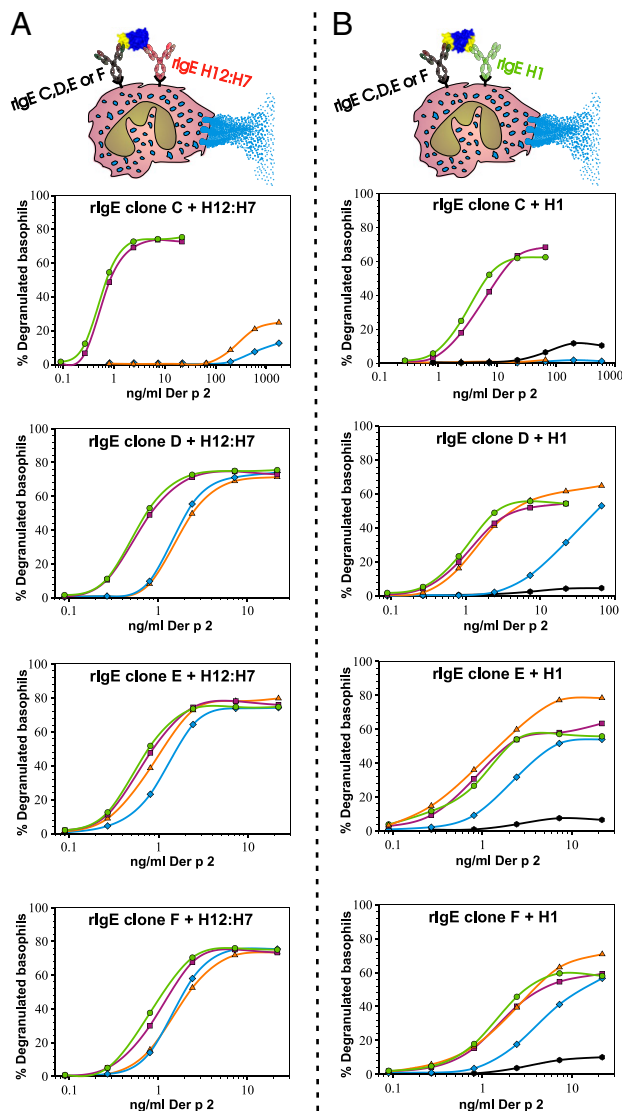


FIGURE 5. Degranulation of human basophils sensitized with different combinations of two rIgEs followed by challenge with individual Der p 2 isoallergens. *A*, Basophils sensitized with chimeric rIgE clone H12:H7 (specific for an epitope conserved on all Der p 2 isoallergens) plus one of each of the human-derived rIgE clones C–F were challenged with four different Der p 2 isoforms (isoform Q was not included in this experiment). *B*, Basophils sensitized with chimeric rIgE clone H1 (specific for a nonconserved epitope) plus one of each of the human-derived rIgE clones C–F (Der p 2 isoform Q was included in this experiment, as it has a profound effect on IgE affinity of clone H1; see Fig. 4). Orange curves indicate rDer p 2.0101, purple curves indicate rDer p 2.0103, green curves indicate rDer p 2.0104, blue curves indicate rDer p 2.0105, and black curves indicate rDer p 2 Q. Data shown in *A* and *B* are from two different experiments.

Most of the isolated Der p 2-specific IgE clones (D–K) show very high amino acid conservation in the allergen binding regions (CDRs), indicating that these clones have probably evolved from the same B cell ancestor through clonal expansion/affinity maturation. Consequently, these IgE clones have the same Der p 2 epitope specificity but bind identical Der p 2 isoforms with the observed altered affinities. Still, the very narrow epitope specificity of the isolated IgE clones underscores that we have not successfully been able to isolate all mite-specific IgE Abs from the HDM-allergic individual, as productive allergen-mediated IgE

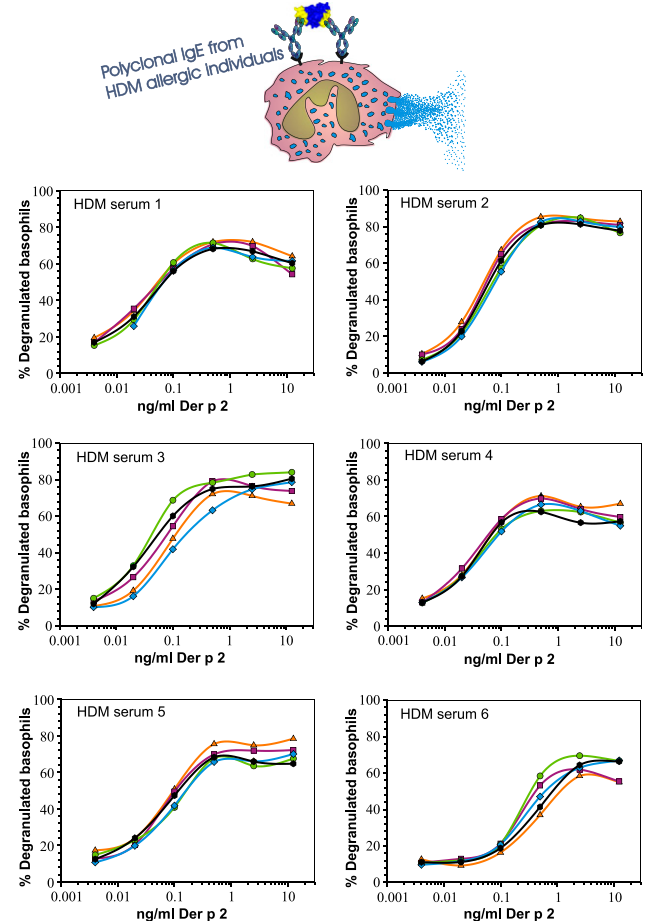


FIGURE 6. Degranulation of human basophils sensitized with sera from six HDM-allergic individuals followed by challenge with five different Der p 2 isoallergens. Orange curves indicate rDer p 2.0101, purple curves indicate rDer p 2.0103, green curves indicate rDer p 2.0104, blue curves indicate rDer p 2.0105, and black curves indicate rDer p 2 Q.

cross-linking triggering an effector cell response requires an absolute minimum of at least two different IgE clones binding two nonoverlapping allergen epitopes. In the fairly limited number of papers (19–23), including this one, in which human allergen-specific IgE Ab fragments have been isolated, combinatorial libraries have always been cloned from peripheral blood samples. However, an essential aspect of the evolving IgE response is the accumulating evidence that both class-switch recombination (24–27) and somatic hypermutation (25) followed by clonal expansion and IgE production (28, 29) occur in local airway mucosa under the influence of the inflamed tissue (reviewed in Ref. 30). Thus, although allergen-specific IgEs may be present in the circulation, B cells producing the IgE are apparently largely confined to the airway mucosa. Hence, peripheral blood may not be a proper source of IgE-producing B cells/plasma cells in relation to IgE repertoire studies.

Finally, single allergens are often quantified or even standardized in complex allergen extracts by use of assays that are based on mAbs. In agreement with previous findings (14), our results have the practical implication that such Ab-based assays should preferably be validated regarding the ability of the mAbs to recognize all present isoallergens. Ideally, the mAbs should recognize an epitope fully conserved among all of the isoallergens to assure equally high binding affinity for correct measurements of the entire isoallergen repertoire present in the particular extract.

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In memory of Prof. Jan Engberg, who was a great contributor to and intended coauthor of this paper. We thank Lars Norderhaug for kindly providing vector pLNOK and pLNOH2 and Prof. Wayne R. Thomas for kindly providing DNA encoding several different Der p 2 isoallergens.

Disclosures

L.H.C. and K.L. are both employees of ALK-Abellø. K.L. owns stocks in ALK-Abello.

References

- Boulet, L. P., H. Turcotte, C. Laprise, C. Lavertu, P. M. Bédard, A. Lavoie, and J. Hébert. 1997. Comparative degree and type of sensitization to common indoor and outdoor allergens in subjects with allergic rhinitis and/or asthma. *Clin. Exp. Allergy* 27: 52–59.
- Weghofer, M., W. R. Thomas, M. Kronqvist, A. Mari, A. Purohit, G. Pauli, F. Horak, H. Grönlund, M. van Hage, R. Valenta, and S. Vrtala. 2008. Variability of IgE reactivity profiles among European mite allergic patients. *Eur. J. Clin. Invest.* 38: 959–965.
- Chua, K. Y., C. R. Doyle, R. J. Simpson, K. J. Turner, G. A. Stewart, and W. R. Thomas. 1990. Isolation of cDNA coding for the major mite allergen Der p II by IgE plaque immunoassay. *Int. Arch. Allergy Appl. Immunol.* 91: 118–123.
- Heymann, P. W., M. D. Chapman, R. C. Aalberse, J. W. Fox, and T. A. Platts-Mills. 1989. Antigenic and structural analysis of group II allergens (Der f II and Der p II) from house dust mites (*Dermatophagoides* spp.). *J. Allergy Clin. Immunol.* 83: 1055–1067.
- Derewenda, U., J. Li, Z. Derewenda, Z. Dauter, G. A. Mueller, G. S. Rule, and D. C. Benjamin. 2002. The crystal structure of a major dust mite allergen Der p 2, and its biological implications. *J. Mol. Biol.* 318: 189–197.
- Trompette, A., S. Divanovic, A. Visintin, C. Blanchard, R. S. Hegde, R. Madan, P. S. Thorne, M. Wills-Karp, T. L. Gioannini, J. P. Weiss, and C. L. Karp. 2009. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457: 585–588.
- Piboonpocanun, S., N. Malainual, O. Jirapongsananuruk, P. Vichyanond, and W. R. Thomas. 2006. Genetic polymorphisms of major house dust mite allergens. *Clin. Exp. Allergy* 36: 510–516.
- Ovsyannikova, I. G., L. D. Vailles, Y. Li, P. W. Heymann, and M. D. Chapman. 1994. Monoclonal antibodies to group II *Dermatophagoides* spp. allergens: murine immune response, epitope analysis, and development of a two-site ELISA. *J. Allergy Clin. Immunol.* 94: 537–546.
- Smith, A. M., D. C. Benjamin, N. Hozic, U. Derewenda, W. A. Smith, W. R. Thomas, G. Gafvelin, M. van Hage-Hamsten, and M. D. Chapman. 2001. The molecular basis of antigenic cross-reactivity between the group 2 mite allergens. *J. Allergy Clin. Immunol.* 107: 977–984.
- Smith, A. M., D. C. Benjamin, U. Derewenda, W. A. Smith, W. R. Thomas, and M. D. Chapman. 2001. Sequence polymorphisms and antibody binding to the group 2 dust mite allergens. *Int. Arch. Allergy Immunol.* 124: 61–63.
- Yasueda, H., H. Mita, Y. Yui, and T. Shida. 1989. Comparative analysis of physicochemical and immunochemical properties of the two major allergens from *Dermatophagoides pteronyssinus* and the corresponding allergens from *Dermatophagoides farinae*. *Int. Arch. Allergy Appl. Immunol.* 88: 402–407.
- Hakkaert, G. A., M. D. Chapman, R. C. Aalberse, and R. van Ree. 1998. Immune-reactivity of recombinant isoforms of the major house dust mite allergen Der p 2. *Clin. Exp. Allergy* 28: 169–174.
- Hales, B. J., L. A. Hazell, W. Smith, and W. R. Thomas. 2002. Genetic variation of Der p 2 allergens: effects on T cell responses and immunoglobulin E binding. *Clin. Exp. Allergy* 32: 1461–1467.
- Park, J. W., K. S. Kim, H. S. Jin, C. W. Kim, D. B. Kang, S. Y. Choi, T. S. Yong, S. H. Oh, and C. S. Hong. 2002. Der p 2 isoallergens have different allergenicity, and quantification with 2-site ELISA using monoclonal antibodies is influenced by the isoallergens. *Clin. Exp. Allergy* 32: 1042–1047.
- 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.
- Johansen, L. K., B. Albrechtsen, H. W. Andersen, and J. Engberg. 1995. pFab60: a new, efficient vector for expression of antibody Fab fragments displayed on phage. *Protein Eng.* 8: 1063–1067.
- Engberg, J., P. S. Andersen, L. K. Nielsen, M. Dziegiel, L. K. Johansen, and B. Albrechtsen. 1996. Phage-display libraries of murine and human antibody Fab fragments. *Mol. Biotechnol.* 6: 287–310.
- Norderhaug, L., T. Olafsen, T. E. Michaelsen, and I. Sandlie. 1997. Versatile vectors for transient and stable expression of recombinant antibody molecules in mammalian cells. *J. Immunol. Methods* 204: 77–87.
- 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.
- Edwards, M. R., W. Brouwer, C. H. Choi, J. Ruhno, R. L. Ward, and A. M. Collins. 2002. Analysis of IgE antibodies from a patient with atopic dermatitis: biased V gene usage and evidence for polyreactive IgE heavy chain complementarity-determining region 3. *J. Immunol.* 168: 6305–6313.
- Jakobsen, C. G., U. Bodtger, P. Kristensen, L. K. Poulsen, and E. L. Roggen. 2004. Isolation of high-affinity human IgE and IgG antibodies recognising Bet v 1 and Humicola lanuginosa lipase from combinatorial phage libraries. *Mol. Immunol.* 41: 941–953.
- Laukkanen, M. L., S. Mäkinen-Kiljunen, K. Isoherranen, T. Haahela, H. Söderlund, and K. Takkinen. 2003. Hevein-specific recombinant IgE antibodies from human single-chain antibody phage display libraries. *J. Immunol. Methods* 278: 271–281.
- 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.
- Cameron, L., A. S. Gounni, S. Frenkiel, F. Lavigne, D. Vercelli, and Q. Hamid. 2003. S epsilon S mu and S epsilon S gamma switch circles in human nasal mucosa following ex vivo allergen challenge: evidence for direct as well as sequential class switch recombination. *J. Immunol.* 171: 3816–3822.
- Coker, H. A., S. R. Durham, and H. J. Gould. 2003. Local somatic hypermutation and class switch recombination in the nasal mucosa of allergic rhinitis patients. *J. Immunol.* 171: 5602–5610.
- Takhar, P., L. Smurthwaite, H. A. Coker, D. J. Fear, G. K. Banfield, V. A. Carr, S. R. Durham, and H. J. Gould. 2005. Allergen drives class switching to IgE in the nasal mucosa in allergic rhinitis. *J. Immunol.* 174: 5024–5032.
- Takhar, P., C. J. Corrigan, L. Smurthwaite, B. J. O'Connor, S. R. Durham, T. H. Lee, and H. J. Gould. 2007. Class switch recombination to IgE in the bronchial mucosa of atopic and nonatopic patients with asthma. *J. Allergy Clin. Immunol.* 119: 213–218.
- KleinJan, A., J. G. Vinke, L. W. Severijnen, and W. J. Fokkens. 2000. Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients. *Eur. Respir. J.* 15: 491–497.
- Smurthwaite, L., S. N. Walker, D. R. Wilson, D. S. Birch, T. G. Merrett, S. R. Durham, and H. J. Gould. 2001. Persistent IgE synthesis in the nasal mucosa of hay fever patients. *Eur. J. Immunol.* 31: 3422–3431.
- Gould, H. J., and B. J. Sutton. 2008. IgE in allergy and asthma today. *Nat. Rev. Immunol.* 8: 205–217.

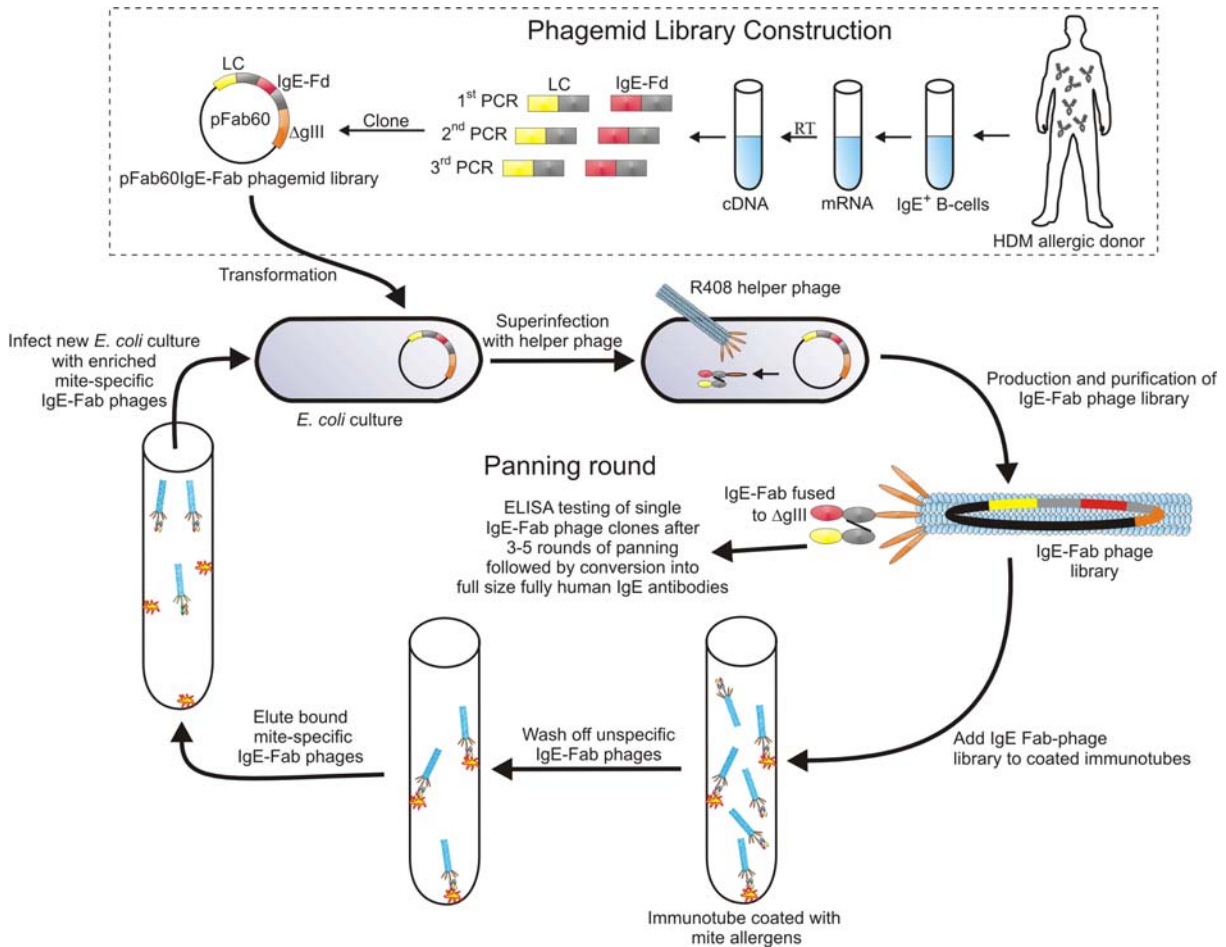


Figure S1. Overview of phage display method used to isolate human HDM-specific IgE antibodies.

Upper part: Schematic representation of phagemid-library construction: IgE producing B-cells were isolated from a HDM allergic donor and mRNA reverse transcribed (RT) into cDNA. IgE-Fd fragments and antibody light chains were subsequently PCR amplified and cloned into a phagemid vector generating the phagemid library, pFab60IgE-Fab.

Lower part: Schematic representation of the panning procedure. A culture of *E. coli* was transformed with the phagemid library followed by super infection with a helper phage providing all the necessary proteins for phage assembly. The resulting library of IgE-Fab phages was purified and added to immunotubes precoated with HDM allergens. Non-HDM specific IgE-Fab phages were washed off followed by elution of bound HDM-specific IgE-Fab phages. A new culture of *E. coli* was subsequently infected with the eluted HDM-specific IgE-Fab phages for further enrichment of HDM-specific IgE-Fab phages. After 3-5 panning rounds, single IgE-Fab phage clones were ELISA tested to confirm binding to HDM allergens.

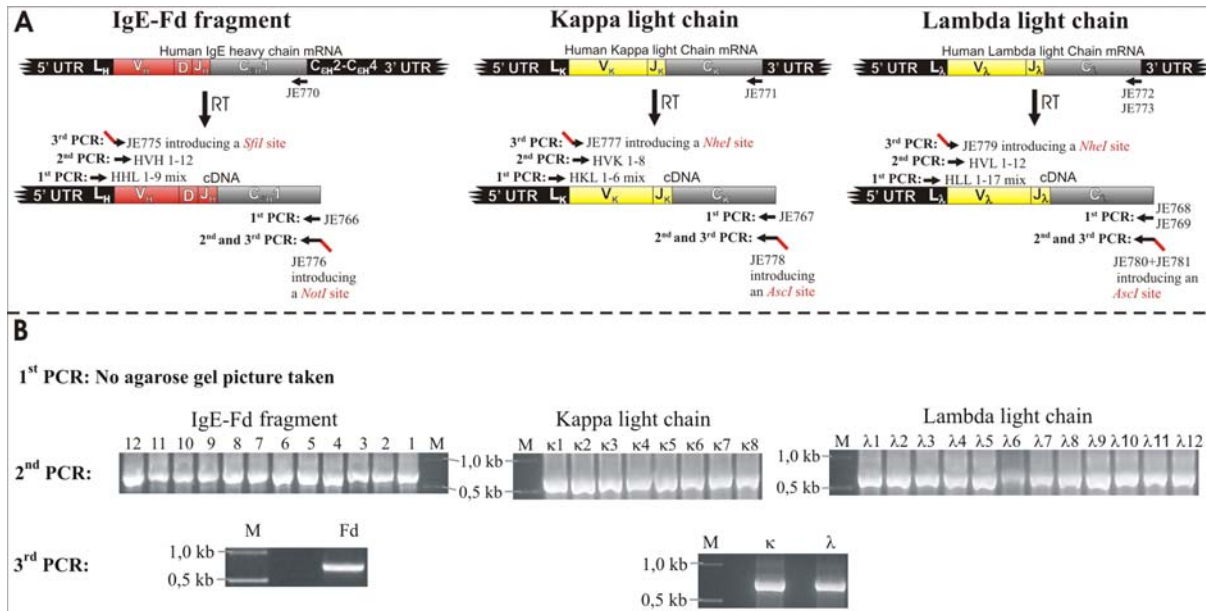


Figure S2. RT-PCR strategy used for amplification of IgE-Fd and light chains. A, schematic illustration:

cDNA synthesis: Initially, m-RNA isolated from the IgE⁺ B-cells was reverse transcribed using gene specific primers (JE770-JE773) annealing the 3'-end of human C_{H1}, C_K, C_λ1-3 and C_λ7 constant antibody genes.

1st PCR: For amplification of the IgE Fd-fragment, degenerate primers (HHL 1-9 mix) annealing the relatively conserved leader sequence of Ig heavy chains in combination with a primer (JE766) annealing the 3'-end of C_{H1} was used. JE766 was 3 bp longer than the JE770 primer used for cDNA synthesis to minimize PCR artefacts. Kappa and Lambda light chains were amplified in a similar way, using a proofreading DNA polymerase, AccuPrime (Invitrogen, CA) for 25 cycles.

2nd PCR: For further amplification, purified PCR products of the first PCR reaction were divided into 12, 8 and 12 individual reactions for amplification of the Fd-fragment, the Kappa and the Lambda light chain, respectively and PCR-amplified for 25 cycles. In this step, primers (HVH1-12, HVK1-8 and HVL1-12) annealing the relatively conserved 5'-end of V_H, V_K and V_L, respectively in combination with primers (JE776, JE778, JE780+JE781) annealing the 3'-end of C_{H1}, C_K and C_L, respectively were used. The latter primers were 2-3 bp longer than the corresponding primers used in the 1st PCR-step, again to minimize PCR artefacts.

3rd PCR: The third PCR-step served to incorporate restriction sites (*SfiI* / *NotI* and *NheI* / *AscI*) for cloning of the IgE-Fd fragment and light chains into phagemid vector, pFab60. Equal amounts of the 12 Fd-fragment reactions, the 8 kappa chain reactions and the 12 lambda chain reactions from the 2nd PCR were pooled into a Fd-pool, a Kappa-pool and a Lambda-pool, respectively and amplified for 15 cycles.

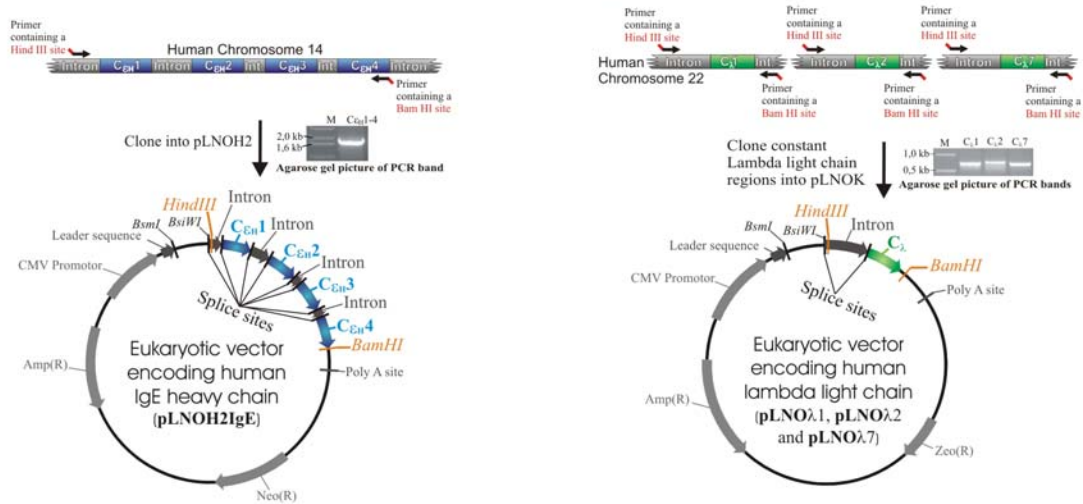
UTR=Untranslated region, RT=reverse transcription, L=leader sequence, V=variable, D=diversity, J=joining.

B, Pictures of PCR bands after agarose gel electrophoresis.

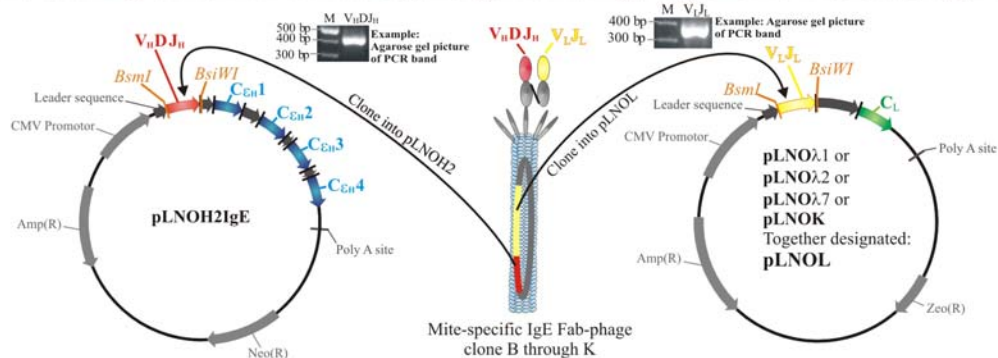
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Figure S3. Amino acid sequences of randomly picked IgE-Fab phagemid clones, sequenced from the unselected IgE-Fab phagemid library. To validate the integrity and diversity of the unselected phagemid library, 45 randomly picked IgE-Fab phagemid clones (21 with a Kappa and 24 with a Lambda light chain) were DNA sequenced. Generally, the diversity of the IgE-heavy chain and in particular the light chain libraries seemed high as most clones were unique. However, one mentionable exception was a variable IgE heavy chain clone that was found in 18 of the 45 sequenced clones. Still, the diversity of the IgE repertoire is expected to be quite limited as only a minor fraction of the peripheral B-cells undergoes isotype class switch recombination to IgE. There were also several clones having heavy chains with highly similar CDR regions indicating that these clones were clonally related and might have arisen by the process of somatic hyper mutation/affinity maturation. We found that most heavy chain clones belong to the VH3 gene family. This heavy chain family is, however, also by far the largest one and hence, no bias towards this gene family can be concluded.

Step 1: Cloning of human constant (FcεRI binding) IgE antibody regions



Step 2: Cloning of human variable (allergen binding) regions from selected IgE-Fab phages



Step 3: Expression of full size, fully human recombinant IgE antibodies in a human cell line

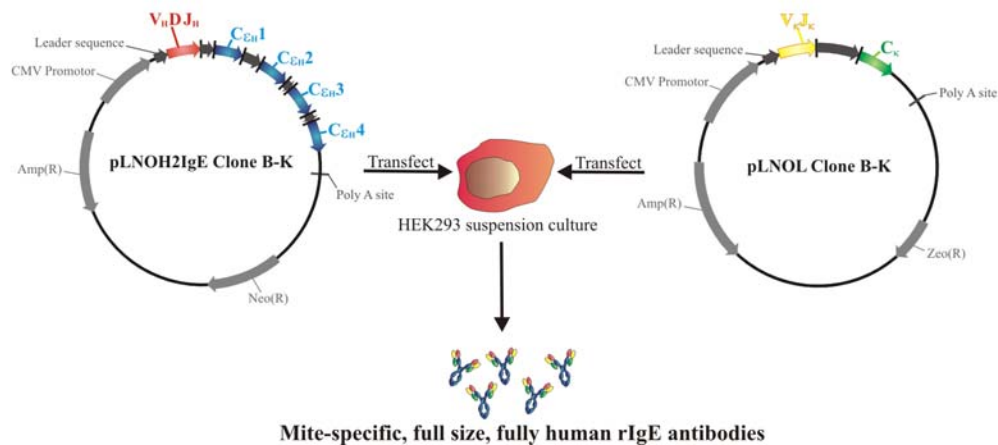


Figure S4. Conversion of IgE-Fab phages into full size, fully human recombinant IgE antibodies.

Mammalian expression vectors encoding the constant domains of human IgE heavy chain and different subtypes of Lambda light chains were constructed. A preexisting vector (pLNOK) encoding the human constant Kappa light chain was also available.

The variable heavy and light chain regions from HDM-specific IgE-Fab phages were sub-cloned into these vectors and HDM-specific, full size, fully human recombinant IgE antibodies produced by transfection of human cell cultures (HEK293).

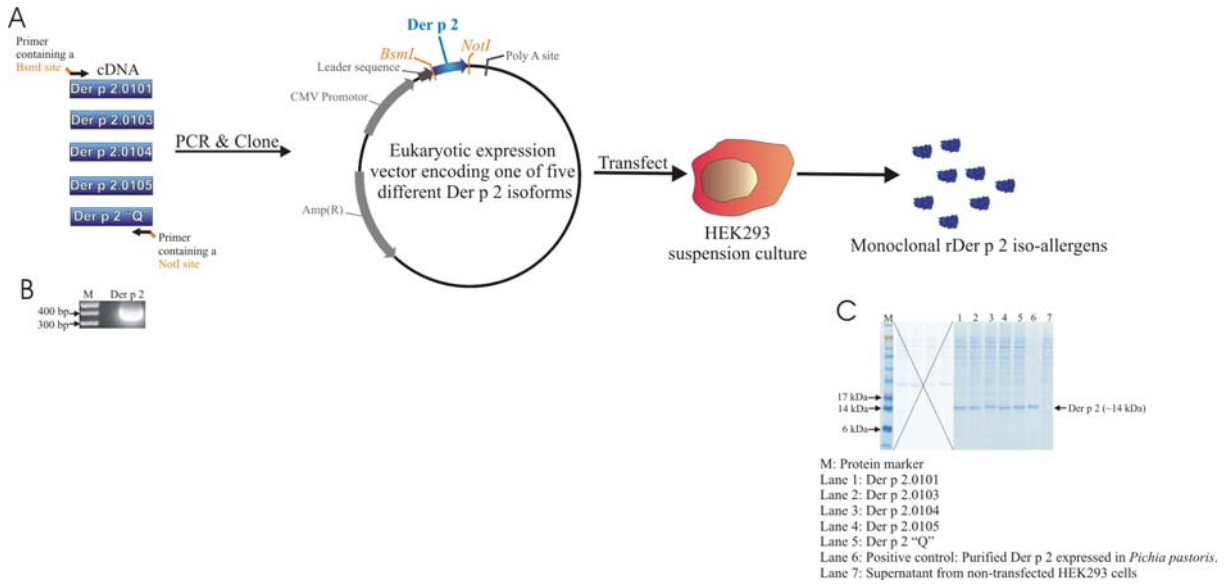


Figure S5. Cloning and expression of five naturally occurring Der p 2 iso-allergens. **A**, cDNA of five Der p 2 iso-allergens were PCR amplified and cloned into a mammalian expression vector. Recombinant allergens were produced by transfection of human cell cultures (HEK293) with these vectors. **B**, Example of a PCR amplified Der p 2 fragment visualized after agarose gel electrophoresis. **C**, SDS-PAGE picture showing expressed Der p 2 iso-allergens of correct size (approximately 14 kDa).

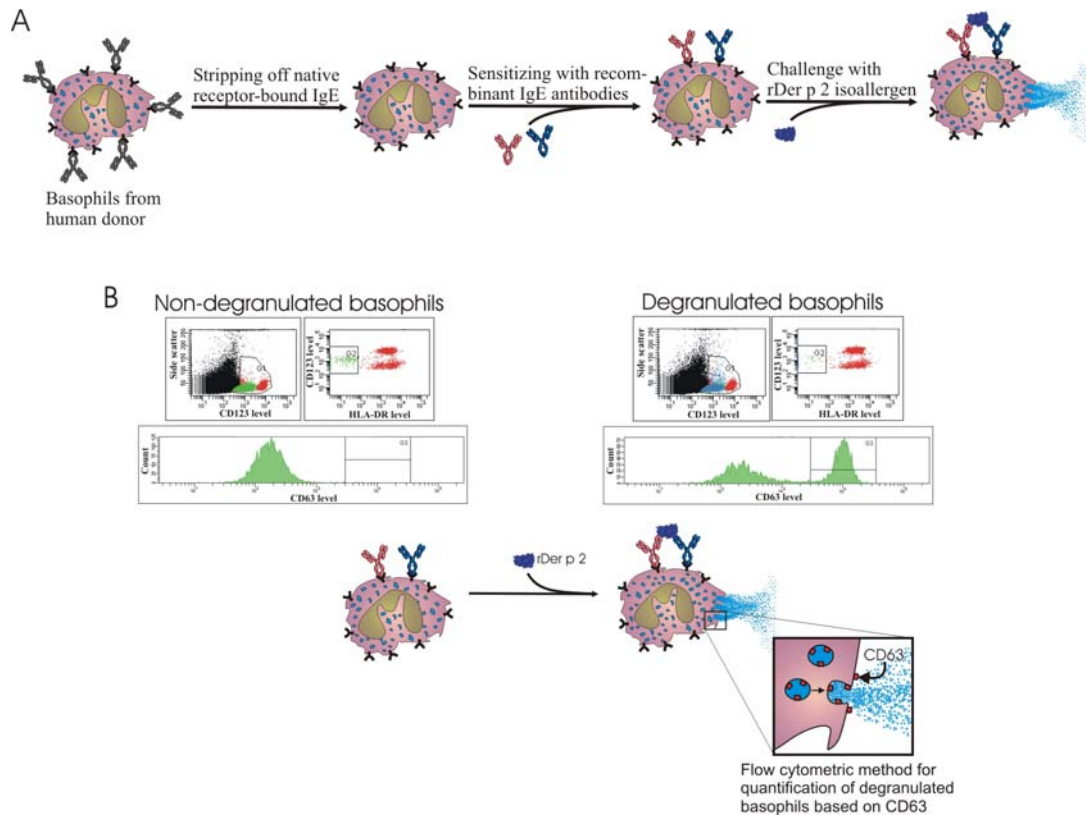


Figure S6. Schematic illustration of basophil degranulation experiments. **A**, Basophils were isolated from human donors and native receptor-bound IgE antibodies stripped off. The stripped basophils were passively sensitized with Der p 2-specific recombinant IgE antibodies followed by challenge with different rDer p 2 iso-allergens.

B, Degranulated basophils were quantified by the degranulation marker, CD63, using flow cytometry a method now widely used alternatively to histamine release measurements. Up on degranulation, CD63, a 53 kDa glycoprotein anchored in the granule membrane, fuses with and becomes exposed on the outside of the basophil plasma membrane which perfectly correlates with histamine release. Initially, basophils and monocytes were separated from other PBMCs by gating on CD123 positive cells. Basophils were then separated from monocytes by further gating on HLA-DR negative cells (basophils). Degranulated (CD63 positive) and non-degranulated (CD63 negative) basophils were quantified.