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Interplay between Affinity and Valency in Effector Cell Degranulation: A Model System with Polcalcin Allergens and Human Patient-Derived IgE Antibodies

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An allergic reaction is rapidly generated when allergens bind and cross-link IgE bound to its receptor FcεRI on effector cells, resulting in cell degranulation and release of proinflammatory mediators. The extent of effector cell activation is linked to allergen affinity, oligomeric state, valency, and spacing of IgE-binding epitopes on the allergen. Whereas most of these observations come from studies using synthetic allergens, in this study we have used Timothy grass pollen allergen Phl p 7 and birch pollen allergen Bet v 4 to study these effects. Despite the high homology of these polcalcin family allergens, Phl p 7 and Bet v 4 display different binding characteristics toward two human patient-derived polcalcin-specific IgE Abs. We have used native polcalcin dimers and engineered multimeric allergens to test the effects of affinity and oligomeric state on IgE binding and effector cell activation. Our results indicate that polcalcin multimers are required to stimulate high levels of effector cell degranulation when using the humanized RBL-SX38 cell model and that multivalency can overcome the need for high-affinity interactions. *The Journal of Immunology*, 2019, 203: 000–000.

The initiating event of an allergic immune response is the cross-linking by a multivalent allergen of IgE Abs that are bound to their high-affinity receptor (FcεRI) on the surface of effector cells, such as mast cells and basophils (1). Small amounts of multivalent allergen are sufficient to cross-link IgE–FcεRI complexes on the effector cell surface and, on a timescale of seconds to minutes, initiate the early phase of the allergic reaction. This results in the rapid release of inflammatory mediators (e.g., histamines and leukotrienes) (1–4).

Naturally occurring allergens are most commonly complex multivalent proteins. The Timothy grass pollen allergen Phl p 5, despite its small size of 29 kDa, is highly allergenic and has multiple independent IgE epitopes (5). Similarly, the major peanut allergen Ara h 3 contains at least four distinct immunodominant epitopes on its surface (6). Allergens may also exist in multimeric

forms and present identical epitopes. For example, the dimeric form of major birch pollen allergen Bet v 1 is required for effective IgE cross-linking, whereas the monomeric Bet v 1 form does not trigger an allergic response in a murine model (7). Most allergens, such as Ole e 1 from olive trees (8) and house dust mite allergen Der p 2 from *Dermatophagoides pteronyssinus* (9, 10), exist in several variants (isoallergens). These isoallergen variations further contribute to the complexity of allergens, as multiple isoforms may be differentially recognized by the same mAb (10). Similarly, there exists cross-reactivity between structurally homologous allergens from different sources, especially between food allergens and pollen (11, 12).

Because of the complexity of naturally occurring allergens and the polyclonality of IgE Abs generated against these allergens in vivo, researchers often employ simplified model systems using synthetic allergens to study the mechanisms of allergic reactions. One well-characterized system is the hapten/Ab pair DNP/anti-DNP IgE. In these systems, small synthetic allergens are often conjugated to a carrier protein, such as BSA (13), a synthetic scaffold (14–17), or incorporated into liposomes (18). Alternatively, artificial allergens have been generated by grafting IgE epitopes onto a scaffold protein in different numbers and spacings (19). Use of rigid or flexible linkers of different lengths provided information about the optimal spacing of the epitopes for intermolecular and intramolecular cross-linking of IgE–FcεRI complexes (20–25). Some systems incorporate more than one hapten into the model system, such as combining DNP and dansyl, as described in work by Handlogten et al. (16, 17). The use of such experimental model systems has allowed investigations of the importance of allergen valency, affinity, spacing, and synergy in allergen–Ab interactions. However, it is generally understood that simplified model systems using small molecules as allergens may not fully reflect the complexity of naturally occurring allergens.

In this study, we describe an Ab–allergen interaction model system, consisting of polcalcin allergens from pollen and patient-derived, polcalcin-specific human IgE Abs. In this study, termed human anti-Phl p 7 IgE Abs (HAPPIE), these human Abs were

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Abbreviations used in this article: HAPPIE, human anti-Phl p 7 IgE Ab; RBL, rat basophilic leukemia; RBL-SX38, RBL cell line; SA, streptavidin; SPR, surface plasmon resonance.

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identified from single B cell clones and expressed recombinantly (26, 27); they have specificity for the Timothy grass pollen allergen Phl p 7, a small 2-EF hand calcium-binding allergen from polcalcin family (28, 29). Recently, an x-ray crystal structure of the Fab fragment of HAPPIE1 (called 102.1F10 in earlier studies) was solved in complex with the Phl p 7 allergen (30). In this study, we characterized the interactions between recombinantly expressed HAPPIE Abs and polcalcin allergens, and this enabled us to assess the role of allergen affinity, valency, and oligomeric state in effective IgE binding and cross-linking on effector cells.

Polcalcin allergens from different pollen sources, such as alder (Aln g 4), birch (Bet v 4), and olive (Ole e 3), share high sequence and structure similarity with Phl p 7 (31), and, in this study, we show that they display various degrees of cross-reactivity to two HAPPIE Abs. We have used these differences to study the role of allergen affinity in effective IgE-FcεRI cross-linking on effector cells using the humanized rat basophilic leukemia (RBL) cell line (RBL-SX38) (32) expressing human FcεRI, as a basophil cell model. Previously, the effects of number and proximity of IgE binding sites on an allergen have been studied using an artificial allergen generated by grafting IgE-reactive Phl p 1 peptides onto the myoglobin molecule (19). In this study, we have used monomeric polcalcin proteins, naturally occurring polcalcin dimers, and engineered multivalent allergens to study the role of allergen valency, epitope spacing, and flexibility in effective IgE binding and triggering of cell degranulation.

Our results suggest that polcalcin multimers are required to stimulate high levels of basophil degranulation and that basophils respond most effectively to multimeric allergens with high affinity for IgE. Further, a low-affinity IgE Ab can still mediate effector cell activation, using the avidity effects of a polyvalent allergen. Thus, the allergen–Ab model system we have established, and the results presented in this study will contribute to a better understanding of the factors affecting allergen–IgE interactions and enable further elucidation of the mechanisms of the human allergic immune responses to these clinically relevant allergens.

Materials and Methods

Protein expression and purification

Recombinant allergens were expressed and purified as previously described (30). Stable HEK293F cell lines expressing full length HAPPIE1 and HAPPIE2 Abs, as well as HAPPIE2 Fab, were produced using FuGene HD (Promega) as a transfection agent according to established protocols (33). In earlier work, HAPPIE1 and HAPPIE2 Abs were called 1021F10 and CS09G6K, respectively (30). For IgE purification, an anti-IgE affinity column was prepared by conjugating the anti-IgE Ab Xolair (Novartis) onto NHS-activated Sepharose 4 Fast Flow preactivated media (GE Healthcare Life Sciences) according to the manufacturer's instructions. rIgE proteins were then purified using affinity chromatography. His-tagged HAPPIE2 Fab was purified using nickel affinity chromatography methods. All recombinant proteins were further purified using size-exclusion chromatography using Superdex 75 Increase 10/300 GL or Superdex 200 Increase 10/300 GL columns (GE Healthcare Life Sciences).

Production of streptavidin oligomeric allergens

Purified recombinant Phl p 7 was conjugated via the sulfhydryl of residue Cys64 to ChromaLink Biotin-Maleimide reagent (Solulink), according to the manufacturer's instructions. The biotin reagent molecule included a PEG3 linker, serving to reduce steric hindrance and provide orientational flexibility within the streptavidin (SA)–allergen complex. Unreacted biotin was removed by size-exclusion chromatography, and biotinylated Phl p 7 was then incubated with SA (Sigma-Aldrich) at different molar ratios and then passed through a Superdex 200 Increase column (GE Healthcare Life Sciences) to separate SA–Phl p 7 complexes from unbound Phl p 7. Fractions containing SA–Phl p 7 were visualized using nondenaturing SDS-PAGE to confirm the presence of the SA–allergen complexes. A similar protocol was followed to produce multivalent Bet v 4 complexes. Site-directed mutagenesis was used to mutate an alanine residue

(Ala71) in Bet v 4 to cysteine in the homologous position to residue cysteine 64 in Phl p 7. Mutagenic primers used were 5'-GGAGTTTACCGACTTTT-GTCGTGCAAACCGTGG-3' for forward and 5'-CCACGGTTTGACGA-CAAAAGTCGGTAAACTCC-3' for reverse.

Distance calculations for epitope spacing in different SA–allergen dimer conformations were a summation of an allergen radius of gyration, PEG3 linker Flory radius (calculated as 0.7 nm) (34) and the distance between selected biotin molecules bound to SA (measured using biotin bound SA [PDB ID 1NDJ] structure as a template (35). A full list of measurements is available in Supplemental Table I.

Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed on a Biacore T200 instrument (GE Healthcare Life Sciences). The running buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 4 mM CaCl₂, and 0.001% [v/v] Surfactant P20) was the same for all experiments. All experiments were performed at 25°C unless stated otherwise. Sensor chip surfaces were prepared by covalently coupling specific proteins using the well-established amine coupling protocol (GE Healthcare Life Sciences). Experiments to study the thermodynamics of HAPPIE2 interaction (Fab was used in these experiments) with Phl p 7 and Bet v 4 were performed at 5, 15, 25, and 35°C. SPR data were analyzed using BIAevaluation Software (GE Healthcare Life Sciences) and Origin 7 (OriginLab). In all cases, standard double-referencing data subtraction methods were applied (36). Data fitting was performed using Origin 7 (OriginLab) or Python scientific computing library SciPy (version 0.18.1) with the scripts for curve fitting, available at https://github.com/gintarebucaite/SPR_data_fitting. Analysis of thermodynamic data was performed using a linear form of the van 't Hoff Equation (37).

Cell culture

The RBL cell line expressing the α-, β-, and γ-chains of human FcεRI (RBL-SX38) was cultured in RBL media (DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml SA, and 500 μg/ml geneticin) in a humidified, 37°C, 5% CO₂ incubator.

Cell degranulation assays

The assay protocol for mast cell degranulation was adapted from Bax et al. (38). RBL-SX38 cells were plated in 96-well plates at 1×10^4 cells per well and incubated overnight at 37°C, 5% CO₂. The following day, cells were sensitized with 200 ng/ml of IgE in RBL media (100 μl/well) for 24 h. Following washes with the assay buffer (HBSS containing 1% BSA), the allergen was added over a concentration range of 0.5 pM–5 μM (100 μl/well, diluted in the assay buffer). A polyclonal rabbit anti-human IgE Ab (A0094; Dako Products) was used as a positive control. Assay buffer with 1% Triton X-100 (100 μl/well) was used to lyse the cells and determine the maximum degranulation. Assay buffer on its own was used (100 μl/well) as a negative control. Cells were incubated for 1 h at 37°C and then 25 μl/well of the supernatant was transferred to a black 96-well plate for a β-hexosaminidase release assay using 4-methylumbelliferyl N-acetyl-β-D-glucosaminide substrate (Sigma-Aldrich) as described previously (38). The percentage of maximum degranulation was calculated, and data were analyzed and plotted using Prism 7 (GraphPad).

Results

Polcalcin allergen binding to human patient-derived IgEs

We recombinantly expressed and purified four polcalcin allergens (Aln g 4, Bet v 4, Ole e 3 and Phl p 7) and determined their binding affinities and kinetics to two monoclonal human patient-derived Phl p 7-specific IgE Abs (HAPPIE1 and HAPPIE2) using SPR (Fig. 1). Consistent with previous studies (30), we found that both anti-Phl p 7 IgE Abs are cross-reactive with homologous polcalcin allergens from different pollen sources. Although there is both high sequence and structure similarities (31) among these four polcalcin allergens (sequence identities are between 67 and 91%), markedly different binding characteristics were observed. Phl p 7 and Ole e 3 bind to HAPPIE1 (Fig. 1A, 1B, these data are summarized in Table I) and HAPPIE2 (Fig. 1E, 1F) with high-affinity (the K_D values for the interactions are as follows, for binding to HAPPIE1 and HAPPIE2, respectively: Phl p 7, 0.9 and 0.11 nM; Ole e 3, 1.0, and 0.8 nM). The allergens Aln g 4 and Bet v 4, although highly homologous to Phl p 7 (the Phl p 7 sequence identity is 68% with Aln g 4 and 67% with Bet v 4),

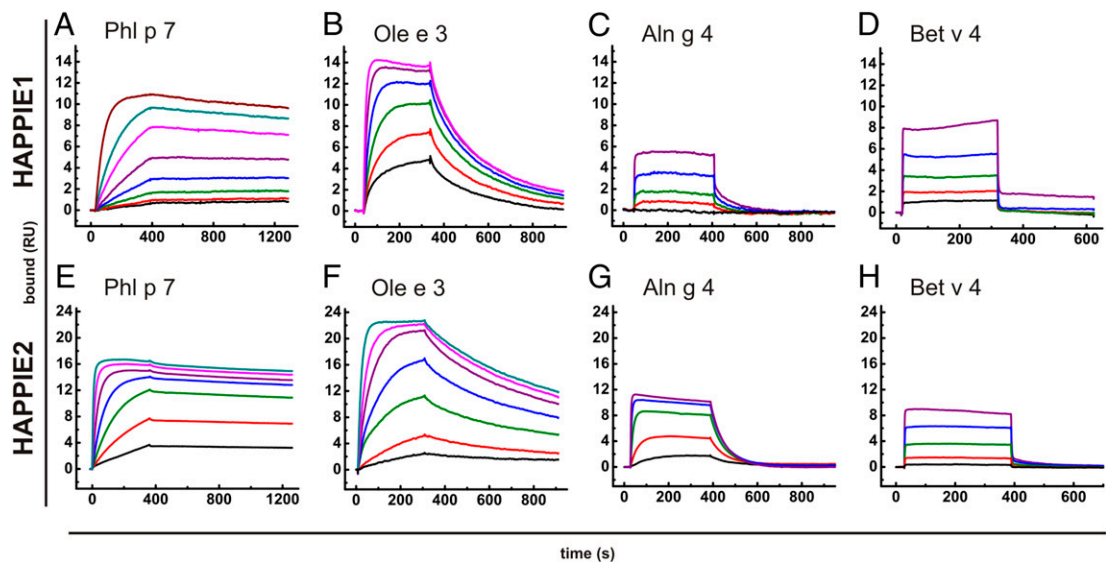


FIGURE 1. Polcalcin allergen binding to HAPPIE1 (A–D) and HAPPIE2 (E–H). Fusion protein of IgG4 Fc and the soluble form of FcεRI receptor (sFcεRIα) (56) was immobilized on an SPR sensor chip, and IgE molecules were captured on the chip via the immobilized sFcεRIα; allergens were then flowed over the IgE surface over a range of concentrations. Allergens were injected using a 2-fold dilution series, with the highest concentrations as follows: 2000 nM for Aln g 4 binding to HAPPIE1, 2000 nM for Aln g 4 binding to HAPPIE2, 2000 nM of Bet v 4 for binding to HAPPIE1, 4000 nM of Bet v 4 for binding to HAPPIE2, 62.5 nM for Ole e 3 binding to both HAPPIE1 and HAPPIE2, and 100 nM for Phl p 7 for binding to both HAPPIE1 and HAPPIE2.

cross-reacted less strongly with both HAPPIE1 (Fig. 1C, 1D) and HAPPIE2 (Fig. 1G, 1H) (the K_D values for Aln g 4 are 2320 and 390 nM; the K_D values for Bet v 4 are 4430 and 3430 nM for binding to HAPPIE1 and HAPPIE2, respectively). Based on these observations, we performed further experiments using Bet v 4 and Phl p 7 as representative low-affinity and high-affinity allergens, respectively, allowing us to explore the role of allergen affinity in IgE binding and effector cell degranulation.

Thermodynamics of Bet v 4 and Phl p 7 binding to HAPPIE2

Additional SPR experiments were performed to derive the thermodynamic parameters of Bet v 4 and Phl p 7 binding to HAPPIE2. Over a range of temperatures, from 5 to 35°C, we measured binding kinetics and affinities of the allergen to the covalently immobilized HAPPIE2 Fab fragment. For each temperature, a concentration binding series was performed (Supplemental Fig. 1), and the interaction of kinetic parameters and affinities were extracted. The affinity of Phl p 7 for the HAPPIE2 Fab decreased with increasing temperature, and the Bet v 4 interaction with the HAPPIE2 Fab was largely temperature independent (Supplemental Fig. 1). A van 't Hoff analysis of the temperature dependence of binding was performed (Fig. 2), which allows the extraction of enthalpic and entropic contributions to binding. HAPPIE2 Fab's interaction with Phl p 7 showed favorable interactions for both enthalpy and entropy ($\Delta H = -36.4 \text{ kJ mol}^{-1}$, $-\Delta S = -19.7 \text{ kJ mol}^{-1}$), whereas the (weaker) interaction with Bet v 4 was dominated by a favorable entropy term ($\Delta H = -7.1 \text{ kJ mol}^{-1}$, $-\Delta S = -23.8 \text{ kJ mol}^{-1}$). It is not surprising that the cross-reactivity seen with Bet v 4 is less enthalpically favorable than the Phl p 7 interaction; similar to HAPPIE1, the HAPPIE2 Ab was derived from a patient with grass pollen allergies, and this Ab likely underwent affinity maturation against the Phl p 7 Ag, optimizing for noncovalent interactions with this target (30). Cross-reactivity toward Bet v 4 would be expected to have imperfect complementarity with the conserved but not identical Bet v 4 protein. The Bet v 4 interaction would, therefore, be expected to be comprised of fewer hydrogen bonds and ionic interactions and less optimal van der Waals interactions but would

still be capable of solvent exclusion of a similar protein surface, resulting in a similar entropy term. Different thermodynamic signatures can be used to distinguish specific versus nonspecific interactions; specific interactions tend to be enthalpically driven, whereas nonspecific interactions tend to be entropically driven (39, 40).

Native polcalcin dimers trigger RBL-SX38 cell degranulation

The family of polcalcin proteins have been shown to exist both as monomers and domain-swapped homodimers (41). Recombinantly expressed polcalcins can form monomers and intertwined dimers, and these oligomeric states can be separated using size-exclusion chromatography during the purification process (Supplemental Fig. 2). To determine the capacity of naturally forming polcalcin dimers to cross-link IgE–FcεRI complexes on the effector cell surface, β-hexosaminidase release was measured for RBL-SX38 cells sensitized with monoclonal Phl p 7-specific IgE and then stimulated with allergens in either monomeric or dimeric form (separated by size-exclusion chromatography) at concentrations ranging from 0.5 pM to 5 μM (Fig. 3). We have also produced monomeric polcalcin mutants stabilized by an internal disulfide bridge, which stabilizes the monomer conformation, as described by Magler et al. (41). When RBL-SX38 cells were sensitized with either HAPPIE1 or HAPPIE2, no significant degranulation was

Table I. Polcalcin binding to IgE data

Allergen	Ab	$K_D \pm \text{SD}$ (nM)
Aln g 4	HAPPIE1	2320 ± 50
	HAPPIE2	390 ± 110
Bet v 4	HAPPIE1	4430 ± 1400
	HAPPIE2	3430 ± 770
Ole e 3	HAPPIE1	1.0 ± 0.45
	HAPPIE2	0.8 ± 0.14
Phl p 7	HAPPIE1	0.9 ± 0.1
	HAPPIE2	0.11 ± 0.01

Summary of selected polcalcin binding affinity values to HAPPIE1 and HAPPIE2 Abs. Data given as ± SD; experiments were run in duplicate.

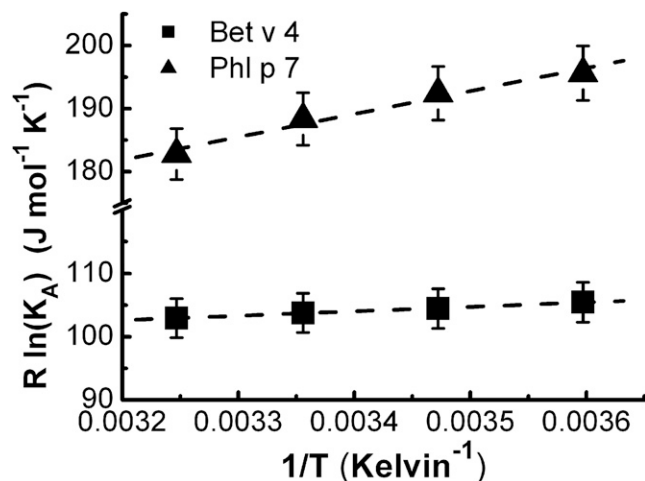


FIGURE 2. The thermodynamic parameters of Phl p 7 (triangles) and Bet v 4 (squares) interaction with HAPPIE2 Fab. SPR binding experiments were performed at temperatures of 5, 15, 25, and 35°C. Data are shown as van 't Hoff plots, and the enthalpic contributions to binding were extracted using a linear van 't Hoff analysis (57) (dashed lines show linear fit of the data). HAPPIE2 Fab/Phl p 7 interaction: $\Delta H = -36.4 \text{ kJ mol}^{-1}$, $-T\Delta S = -19.7 \text{ kJ mol}^{-1}$, $\Delta G = -56.1 \text{ kJ mol}^{-1}$; HAPPIE2 Fab/Bet v 4 interaction: $\Delta H = -7.1 \text{ kJ mol}^{-1}$, $-T\Delta S = -23.8 \text{ kJ mol}^{-1}$, $\Delta G = -30.9 \text{ kJ mol}^{-1}$.

detected when stimulated either wild-type monomer or disulfide-stabilized monomeric allergens (Fig. 3). In previous experiments, monomeric Phl p 7 has been reported to activate HAPPIE1-sensitized basophils, and a superantigen-like mechanism was proposed for this activity (30). However, in the current experiments, the disulfide-stabilized monomeric Phl p 7 showed no capacity to cross-link HAPPIE1 and trigger basophil degranulation (Fig. 3B).

Low concentrations of native Phl p 7 dimer (Fig. 3B, 3D) but not dimeric Bet v 4 (Fig. 3A, 3C) triggered basophil degranulation when cells were sensitized with either HAPPIE1 or HAPPIE2. The native Bet v 4 dimer could not effectively cross-link receptor-bound HAPPIE1 (Fig. 3A), and only a very small amount of degranulation was observed for receptor-bound HAPPIE2 at the highest concentrations of Bet v 4 tested (Fig. 3C). We, therefore, designed further experiments to test how effector cell sensitivity

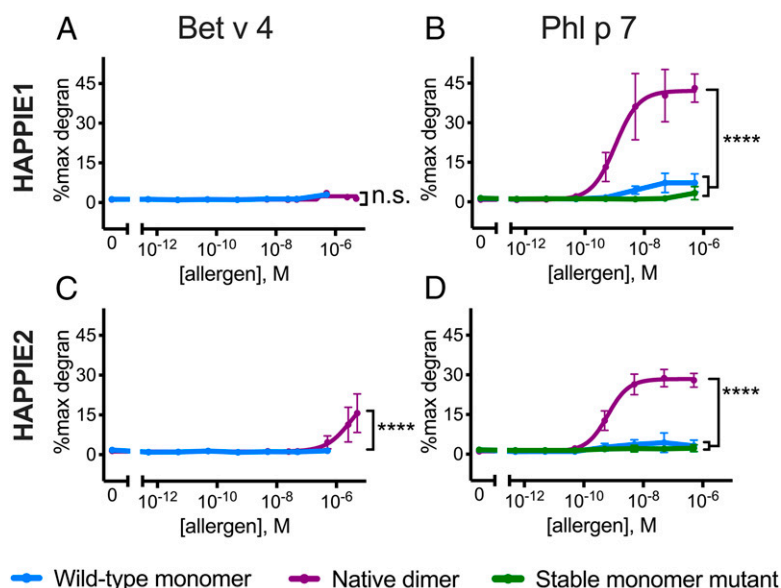
would change when stimulated with higher oligomeric state allergens.

Multimeric allergen design and production

Multivalent Bet v 4 and Phl p 7 allergens were engineered and produced to study the interplay between allergen valency and affinity in IgE binding and effector cell activation. The ultrahigh-affinity SA–biotin interaction was used to generate multimeric allergens. Biotin was site specifically conjugated to the two allergens via the single free sulfhydryl in each protein (at Cys64 in Phl p 7, and a cysteine residue introduced at the homologous position in Bet v 4 using site-directed mutagenesis) using a maleimide-biotin reagent. The biotin-maleimide reagent used in the reaction contained a flexible PEG3 linker and a UV-traceable chromophore, allowing rapid confirmation of the degree of biotinylation. Biotinylated allergens were then incubated at various concentrations with tetrameric SA to form SA–allergen complexes [the resulting complexes had the highest possible valency of four biotinylated allergens bound per tetrameric SA (Fig. 4B)], and the reaction was assessed using SDS-PAGE (Fig. 4A). The unbound biotinylated allergen was separated from the SA–polcalcin complexes using size-exclusion chromatography (Fig. 4C). Fig. 4 summarizes the approach used to produce SA–Bet v 4 complexes; an identical protocol was followed to generate SA–Phl p 7 multimers. SA–polcalcin complexes with different polcalcin/SA ratios were also produced (Fig. 4D, 4E), enabling study of the role of allergen valency in IgE binding and basophil degranulation. SA–polcalcin dimers and trimers, although distinct peaks in size-exclusion chromatography (Fig. 4C, 4D), are heterogeneous species because of the intrinsic asymmetry of the SA tetramer (Fig. 4E), whereas monomers and tetramers are homogeneous.

Based on the radii of gyration calculated for the proteins used in this study (full details about estimated distances are available in Supplemental Table I), we estimate that the distances between the epitopes in engineered Bet v 4 multimers would range between 2.4 and 7.3 nm, which is close to the distances between epitopes observed in published Fab–allergen–allergen–Fab crystal structures, such as 4 nm in the *Bos d 5* dimer structure (42) and 6.3 nm in the *Bla g 2* dimer (43). Native polcalcin dimers would have a maximum diameter of around 3.0–3.2 nm, which is less than the suggested optimal distance of ~6 nm between epitopes as determined in synthetic allergen model systems (24, 25). It would,

FIGURE 3. RBL-SX38 cell degranulation assays with recombinant Bet v 4 and Phl p 7 monomeric and dimeric proteins. RBL-SX38 were sensitized with HAPPIE1 (A and B) or HAPPIE2 (C and D) and then stimulated with wild-type monomer or native dimer Bet v 4 (A and C), or they were stimulated with disulfide stabilized monomer, wild-type monomer, or native dimeric versions of Phl p 7 (B and D). Data were normalized as the percentage of maximum degranulation (%max degran; determined by cell lysis with 1% Triton X-100) and fitted to determine the EC_{50} values. Data shown are the averages $\pm$ SD of at least three experiments with three biological replicates per experiment. One-way ANOVA, followed by Tukey multiple comparison tests were used to determine the statistical significance between the levels of β -hexosaminidase release when stimulated with wild-type monomers, disulfide stabilized monomers, or native dimeric allergens when compared with background levels of degranulation (HBSS only); statistical tests are based on the highest concentrations of allergen tested. **** $p < 0.0001$.



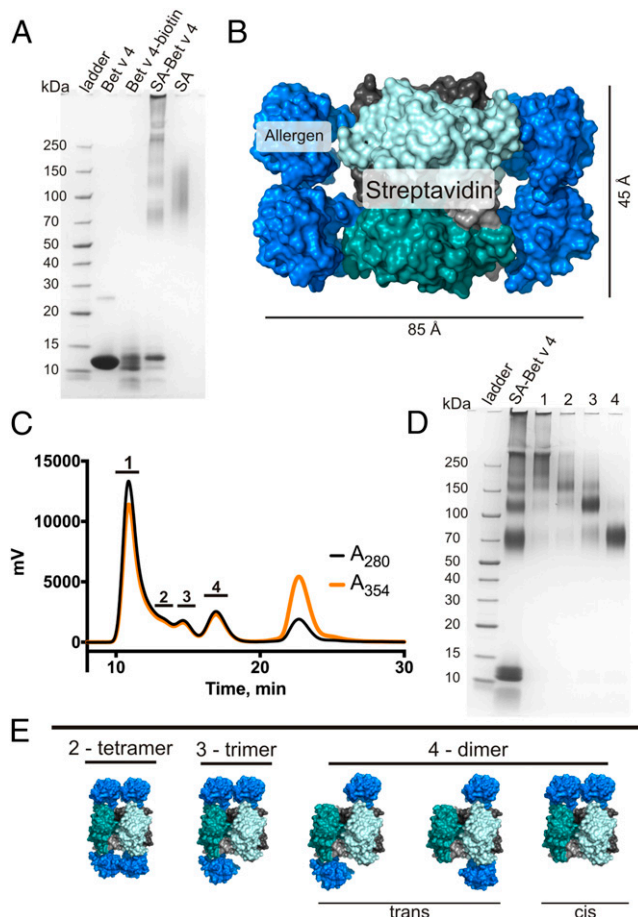


FIGURE 4. Multimeric allergen production and purification using SA as a scaffold. SA was incubated with biotinylated allergen to yield multivalent allergen complexes (**A**); a model of the tetrameric SA–allergen complex is shown in (**B**) allergen is in dark blue, and SA monomers are in black, gray, teal, and light cyan. The SA–allergen mixture was purified using size-exclusion chromatography, and the eluted analyte was detected by measuring the absorbance at 280 and 354 nm (**C**). Individual peaks were visualized using SDS-PAGE (**D**), and possible multimeric configurations (tetramer, trimer, *cis* and *trans* dimers) are shown in (**E**).

therefore, be anticipated that engineered multimeric allergens would display improved IgE cross-linking when compared with native dimers. Additionally, the spacing between the IgE-binding epitopes on SA–polcalcin multimers would not be great enough to cross-link both Fabs on a single IgE Ab (Fabs may be cross-linked by ligands of around 11–15 nm (20, 44, 45)).

Multimeric allergens demonstrate avidity in SPR

We used SPR to determine whether increasing allergen valency in engineered polcalcin constructs results in avidity and, consequently, more efficient binding by measuring how Bet v 4 binding to HAPPIE1 changed with increased valency. For this, a high-density SPR surface was prepared. HAPPIE1 IgE was captured on a sensor surface at ~1000 resonance units, a density sufficiently high to allow observation of multivalent effects. Monomeric Bet v 4 binding to HAPPIE1 reached equilibrium rapidly, and the allergen dissociated rapidly from the Ab surface (Fig. 5A). Native Bet v 4 dimers showed moderate avidity effects (Fig. 5B), characterized by slightly slower off-rates. Engineered Bet v 4 dimers and trimers, however, dissociated from the IgE surface markedly more slowly, indicating that increased valency resulted in tighter binding because of avidity effects (Fig. 5C, 5D). Similar experiments were performed with Bet v 4 constructs binding to HAPPIE2 and Phl p 7 constructs binding

to HAPPIE1 and HAPPIE2 captured on the sensor surface. In all experiments, we observed slower dissociation rates at increased allergen valency because of avidity effects. In higher oligomeric state complexes, dissociation at one of the binding sites is more likely to be followed by reassociation at another binding site rather than complete dissociation of the complex (46). We next performed effector cell degranulation assays using multivalent SA–allergens to test whether the increase in valency would have an effect on the binding and cross-linking of IgE–receptor complexes on effector cells.

RBL-SX38 degranulation by multimeric polcalcin allergens

We used the multivalent polcalcin allergens in RBL-SX38 degranulation assays to investigate the role of allergen valency in effective cross-linking of IgE–FcεRI complexes on the surface of the effector cells. When RBL-SX38 cells were saturated with either HAPPIE1 or HAPPIE2, high levels of cellular degranulation, as measured by β-hexosaminidase release, were observed when stimulated with either Bet v 4 or Phl p 7 multimers (Fig. 6). EC₅₀ values are summarized in Table II.

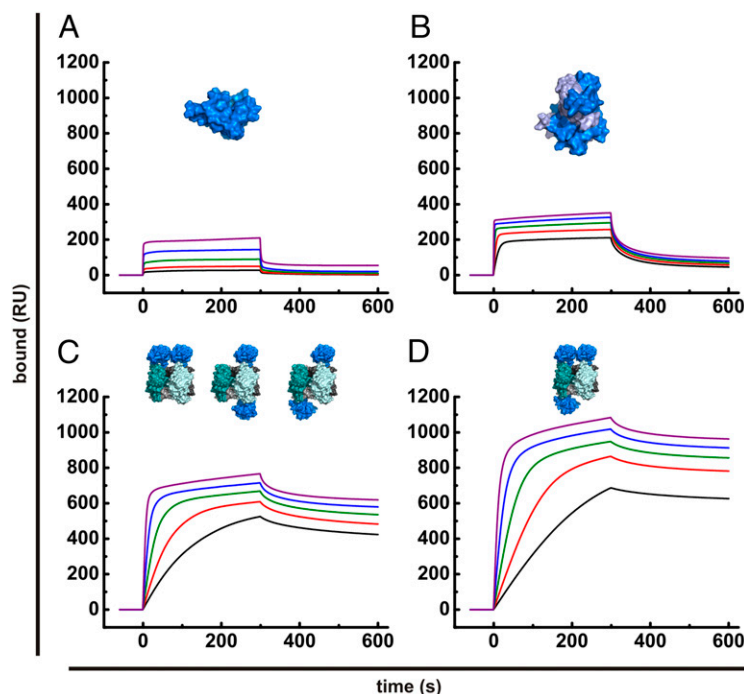
Similar levels of degranulation were triggered by bivalent (EC₅₀ = 0.5 and 0.7 nM for cells sensitized with HAPPIE1 and HAPPIE2, respectively), trivalent (HAPPIE1 EC₅₀ = 0.4 nM; HAPPIE2 EC₅₀ = 0.7 nM), or tetravalent Phl p 7 (HAPPIE1 EC₅₀ = 0.7 nM; HAPPIE2 EC₅₀ = 0.3 nM) constructs (Fig. 6B, 6D). For all of these constructs, there were no statistically significant differences in EC₅₀ values; all the different Phl p 7 multimers cross-linked and activated RBL cells at subnanomolar concentrations.

In contrast, we observed that the oligomeric state does affect effector cell sensitivity when cells are primed with IgE Abs with low affinity for the allergen. Tetravalent and trimeric Bet v 4 triggered significant levels of basophil degranulation at lower concentrations than dimeric complexes (Fig. 6A, 6C) (EC₅₀ = 0.3 nM [tetramer] versus EC₅₀ = 1.8 nM [dimer], $^{**}p \leq 0.01$; EC₅₀ = 0.6 nM [trimer] versus EC₅₀ = 1.8 nM [dimer], $^{**}p \leq 0.01$; EC₅₀ = 0.3 nM [tetramer] versus EC₅₀ = 0.6 nM [trimer] when cells were sensitized with HAPPIE1, NS; EC₅₀ = 0.2 nM [tetramer] versus EC₅₀ = 1.5 nM [dimer], $^{***}p \leq 0.001$; EC₅₀ = 0.3 nM [trimer] versus EC₅₀ = 1.5 nM [dimer], $^{**}p \leq 0.01$; EC₅₀ = 0.2 nM [tetramer] versus EC₅₀ = 0.3 nM [trimer] when cells were sensitized with HAPPIE2 and stimulated with Bet v 4 complexes, NS). This suggests that allergen valency and avidity effects can, under some circumstances, play an important role in effective IgE–receptor cross-linking, even when the Ab's affinity for the allergen is low.

These degranulation experiments using SA–polcalcin complexes have also suggested the role epitope spacing may play in the effective receptor cross-linking on the cell surface. Engineered Bet v 4 dimers (Fig. 6A, 6C) were more potent in degranulation assays than native Bet v 4 dimers (Fig. 3A, 3C), which is clearly illustrated when the potency of these allergens is compared directly (Supplemental Fig. 3). RBL-SX38 cells were activated by native Bet v 4 dimers only at high concentrations ($^{***}p \leq 0.01$, HBSS versus 2.5 μM native Bet v 4 dimer, estimated EC₅₀ = 2.9 μM) and when primed with HAPPIE2 but not HAPPIE1. SA–Bet v 4 dimers, however, triggered high levels of degranulation at more than a 1000-fold lower concentration when compared with native dimers, and stimulated RBL-SX38 cells sensitized with either HAPPIE1 or HAPPIE2 (estimated EC₅₀ = 1.8 and 1.5 nM for cells sensitized with HAPPIE1 or HAPPIE2, respectively).

The number of available epitopes is the same in the engineered Bet v 4 dimer as in the native dimeric Bet v 4; therefore, it is likely that it is the difference in spacing between epitopes that results in different effector cell activation profiles for these two different dimeric structures.

FIGURE 5. SPR binding analysis of wild-type Bet v 4 (A), native Bet v 4 dimer (B), dimeric SA-Bet v 4 (C), and trimeric SA-Bet v 4 complexes (D) to HAPPIE1 captured on the sensor surface. Wild-type (A) and native dimeric (B) Bet v 4 was injected over HAPPIE1 sensor surface in a 2-fold dilution series, with a highest concentration of 2000 nM. (C and D) Engineered Bet v 4 dimers (C) and trimers (D) were added to HAPPIE1 over a range of 2-fold dilution series concentrations, starting with 200 nM as the highest concentration.



In the polcalcine-SA dimer, distances between polcalcine epitopes are greater, and there is potentially greater orientational flexibility for these molecules because of the biotin-maleimide-PEG3 linker (with a calculated Flory radius of ~0.7 nm (34)). Based on measurements of the dimeric Bet v 4 model generated by SWISS-MODEL homology modeling server (47–49) and models of monomeric Bet v 4 (50) bound to tetrameric SA (35), we can estimate the average distances between polcalcine epitopes. Engineered polcalcine-SA dimers are heterogenous and can exist in both *cis* or *trans* conformations (Fig. 4E), but all possible conformations would likely have larger distances between epitopes than native Bet v 4 dimer. For example, the longest distance between two epitopes recognized by an Ab on a native polcalcine would be ~3.0–3.2 nm. In the engineered polcalcine dimer, those epitopes would be on average separated by ~4.1 nm in *cis* conformation and by >6 nm in either of the *trans* conformations.

Discussion

The results presented in this study describe an Ab-allergen interaction model system that can be used to study how certain allergen properties, such as affinity toward the IgE Ab or the allergen's oligomeric state, affect the extent of effector cell activation as a result of IgE-FcεRI complex cross-linking. In this study we used monoclonal human patient-derived IgE Abs against pollen allergens as model Abs, and we have used recombinant Phl p 7 from Timothy grass pollen and its homologs from different pollen sources, such as alder, birch, and olive tree, as representative allergens.

Most of the currently used allergen-Ab model systems use small haptens, such as DNP and dansyl, as allergens, in which the hapten is usually conjugated to a carrier protein, such as BSA, or a scaffold (13–18). Haptens are most commonly conjugated to BSA through reactive amine groups; consequently, it is not always straightforward as to controlling valency and allergen spacing. In

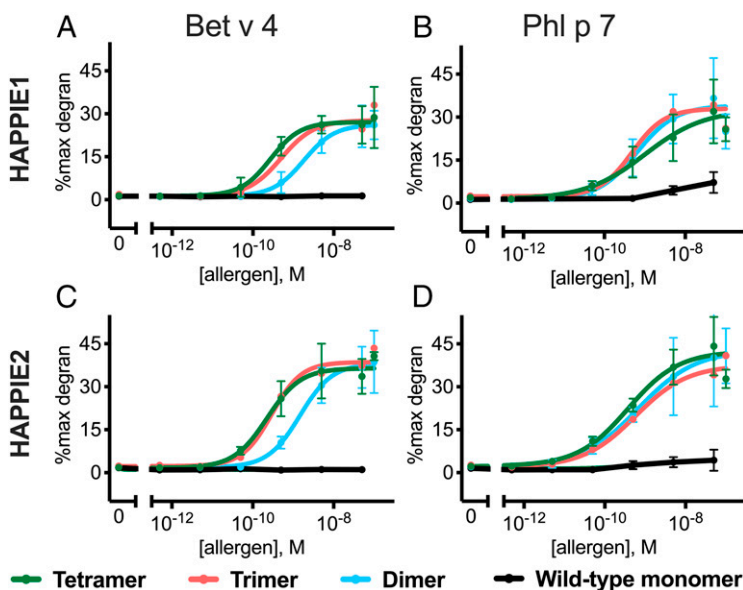


FIGURE 6. Degranulation of RBL-SX38 cells sensitized with HAPPIE1 (A and B) or HAPPIE2 (C and D) and stimulated with multimeric variants of Bet v 4 (A and C) or Phl p 7 (B and D). Responses for cells stimulated with wild-type monomeric proteins is replicated from Fig. 3 and included for comparison purposes. Data were normalized as the percentage of maximum degranulation (cell lysis with 1% Triton X-100). Data shown are the averages $\pm$ SD of at least three experiments with three biological replicates per experiment.

Table II. Estimated EC₅₀ values for dimeric, trimeric, and tetrameric polcalcin potency to trigger RBL-SX38 degranulation

EC ₅₀ ± SD (nM)	Bet v 4		Phl p 7	
	HAPPIE1	HAPPIE2	HAPPIE1	HAPPIE2
Dimer	1.8 ± 0.1 (** <i>p</i> versus trimer, ** <i>p</i> versus tetramer)	1.5 ± 0.1 (** <i>p</i> versus trimer, *** <i>p</i> versus tetramer)	0.5 ± 0.2	0.7 ± 0.1
Trimer	0.6 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.7 ± 0.3
Tetramer	0.3 ± 0.04	0.2 ± 0.06	0.7 ± 0.3	0.3 ± 0.1

Data shown are the averages ± SD of at least three experiments with three biological replicates per experiment. Statistical significance between the estimated EC₅₀ values was calculated using multiple comparison one-way ANOVA followed by Tukey multiple comparison test.
p* ≤ 0.01, *p* ≤ 0.001.

model systems in which haptens are conjugated to a chemically synthesized scaffold [as described by Handlogten et al. (15)], these problems can be overcome. But such models still use small synthetic molecules as haptens, whereas most naturally occurring allergens are complex proteins. Further, the IgE Abs used in most experimental models are usually of nonhuman origin (51). The HAPPIE Abs used in this study are recombinantly expressed, monoclonal human patient-derived Abs with matched heavy and light chains (26). Finally, the human origin of the Abs enabled us to perform functional assays with a cell line expressing a human FcεRI on the effector cell surface, whereas the cell line of choice in most similar experiments modeling the human allergic response is RBL-2H3, which expresses rat FcεRI, and mouse Abs are used. The HAPPIE/polcalcin allergen system offers, we believe, a model system that has the potential to more closely mimic human allergic reactions than many existing model systems.

Some previous studies have concluded that bivalent allergens trigger low levels of mast cell degranulation even in the instances when the allergen affinity is high (17). In contrast, synthetic trivalent ligands have been shown to be highly potent in cross-linking IgE–FcεRI complexes (23, 52). However, from our studies, as well as others [e.g., the dimeric Bet v 1 (7) and Hev b 8 (53) allergens], it is clear that dimeric allergens are fully capable of triggering a robust degranulation response. Dimeric allergens, after all, can theoretically generate large aggregates of cell surface IgE molecules via intermolecular interactions of the bivalent Ab molecules (19), but not all dimeric allergens will do this efficiently. Dimeric allergens, whose structures permit bivalent intramolecular interactions (i.e., with the two Fabs within an IgE molecule), or allergens that favor formation of the bicyclic IgE–FcεRI complex dimers result in very inefficient mast cell activation (20, 21, 54). And, as we show in this work, bivalent allergens with low affinities and fast off-rates may result in only transient IgE cross-linking, which is insufficient for cellular activation.

Our study demonstrates that under some circumstances low-affinity allergens can trigger cell degranulation without the presence of high-affinity binding sites. Previous studies support the importance of low-affinity epitopes when presented together with high-affinity epitopes in effector cell activation (10, 17, 51). In this study, we show that IgE–FcεRI complexes can be efficiently cross-linked with low-affinity allergens alone. High levels of effector cell degranulation were observed when HAPPIE-sensitized cells were stimulated with multivalent Bet v 4 constructs.

Whereas the native Bet v 4 dimer triggered low levels of degranulation and showed only a small amount of enhanced binding because of avidity in the SPR binding experiments (Fig. 5), dimeric SA–Bet v 4 complexes were potent allergens and displayed marked increased avidity in the SPR experiments. This confirms that the spacing and flexibility of IgE-binding epitopes also play a role in efficient IgE–FcεRI cross-linking and initiation of signaling cascade, leading to cell degranulation (52, 55). Although a

previous study using artificial allergens has concluded that placing IgE epitopes in close proximity increases allergen potency (19), using SA as a scaffold and a flexible PEG3 linker to increase the spacing between IgE epitopes resulted in higher allergen potency in our model system. This may have resulted from enhanced availability of IgE binding sites because of the incorporation of a flexible linker between the SA scaffold and the allergen itself, allowing some movement and repositioning of multiple epitopes in respect to each other.

In summary, in this study, we describe a model allergen–Ab system using patient-derived IgE Abs and the protein allergens to which patients react, and we demonstrate the importance of allergen affinity, oligomeric, state and epitope spacing in IgE binding and mast cell degranulation.

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Disclosures

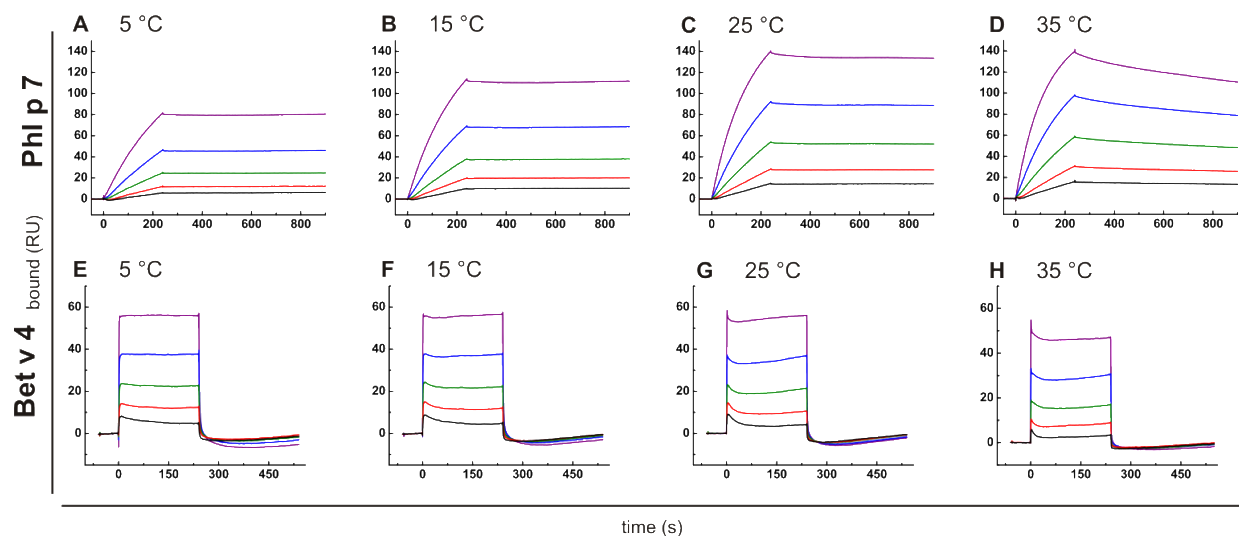
The authors have no financial conflicts of interest.

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1 **Supplemental data**



2

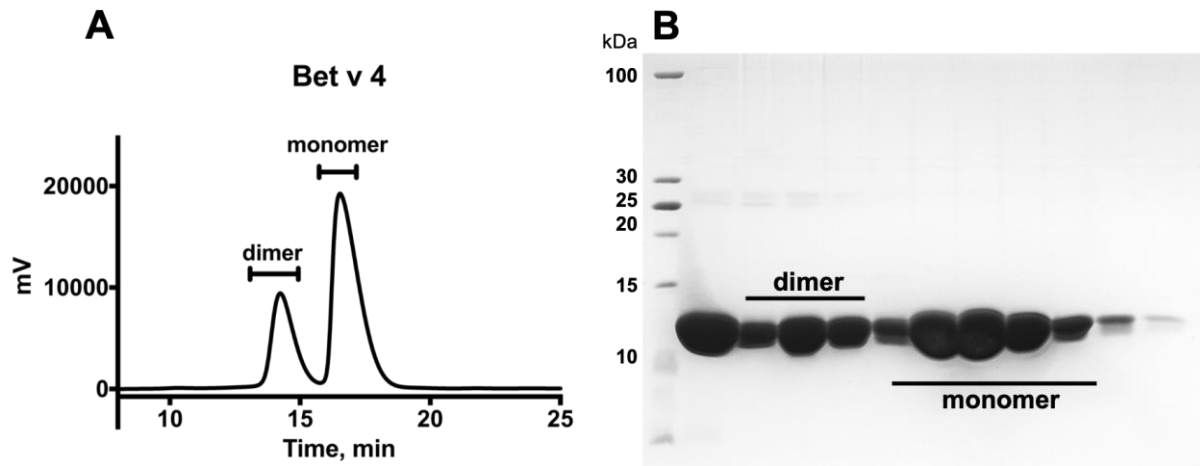
3 Suppl. Figure 1. Phl p 7 and Bet v 4 binding to HAPPIE2-Fab over a range of temperatures.

4 Allergens were flowed over covalently immobilised HAPPIE2-Fab surface over a range of

5 concentrations in a two-fold dilution series. Highest concentration used was 20 nM for Phl p 7

6 and 4000 nM for Bet v 4.

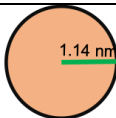
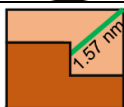
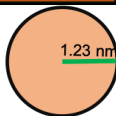
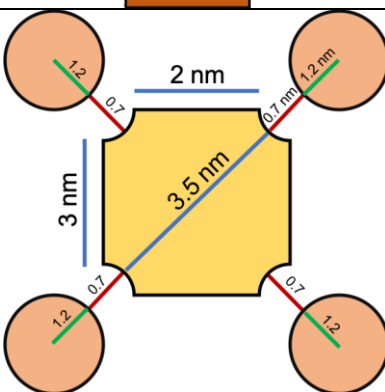
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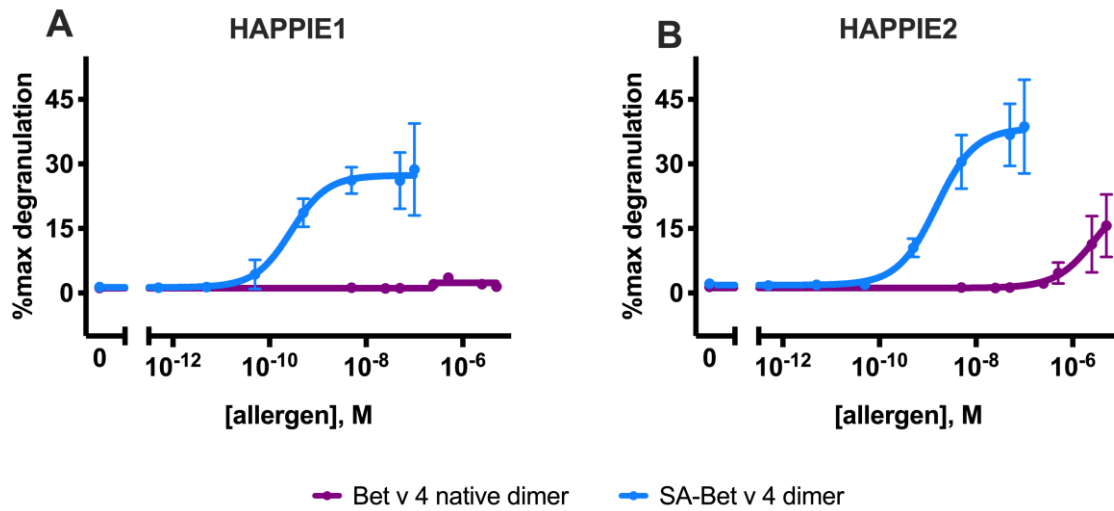
3 Suppl. Figure 2. Separation of monomeric and dimeric recombinantly expressed polcalcin forms
4 by size-exclusion chromatography, Bet v 4 is shown as a representative allergen. Dimeric
5 allergen elutes before monomeric form due to larger mass (A). Fractions containing dimer and
6 monomer allergen (boiled and diluted in reducing loading buffer) were visualised using SDS
7 PAGE (B).

8

Molecule	Measurement (nm)	Distance between epitopes (nm)	Representation
Chromalink® biotin maleimide reagent	0.7 [*]	n/a	
Phl p 7 monomer (2VLK)	1.14 [†]	n/a	
Phl p 7 dimer (1K9U)	1.57 [†]	2 x 1.57 [†]	
Bet v 4 monomer (1H4B)	1.23 [†]	n/a	
Bet v 4 dimer (model)	1.58 [†]	2 x 1.58 [†]	
SA biotins cis (1NDJ)	2 [‡]	n/a	
SA biotins trans (1)(1NDJ)	3 [‡]	n/a	
SA biotins trans (2)(1NDJ)	3.5 [‡]	n/a	
Cis-dimer	See representation	2.4 – 5.8	
Trans-dimer (1)		4.4-6.8	
Trans-dimer (2)		7.3	

2 Suppl. Table 1. Summary of estimated measurements of molecules used to construct multimeric
3 SA-allergens. Measurements denoted with (*) were estimated using an equation to calculate
4 Flori radius (1, 2); those denoted with (†) report molecule's radius of gyration (calculated in
5 YASARA (3)) and (§) measurements between individual biotin molecules bound to streptavidin
6 homotetramer were performed in MacPyMOL. Distances between epitopes in different SA-
7 allergen complexes were a summation of appropriately selected estimated distances of molecules
8 used to construct the complex, namely, radii of gyration of allergen molecules, Flori radius
9 calculated for flexible PEG3 linker and the measured distance between selected biotin molecules
10 in tetravalent streptavidin molecule.

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3 Suppl. Figure 4. Comparison between native Bet v 4 dimer and streptavidin-Bet v 4 dimer ability
 4 to stimulate RBL-SX38 cell degranulation when cells were sensitised with either HAPPIE1 (A)
 5 or HAPPIE2 (B). Data were taken from Fig. 3 for native dimer and Fig. 6 for engineered dimer.
 6 Data were normalised as the percentage of maximum degranulation (cell lysis with 1% Triton-
 7 X100). Data are shown as $\pm$ SD.

1 **References**

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