# Interplay between affinity and valency in effector cell degranulation: a model system with polcalcin allergens and human patient-derived IgE antibodies

**Running title: An allergen-IgE interaction model system**

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1 Abbreviations used in this article: RBL-SX38, rat basophilic leukemia cells; SA, streptavidin;

2 SPR, surface plasmon resonance.

**Abstract**

An allergic reaction is rapidly generated when allergens bind and crosslink immunoglobulin E bound to its receptor FcεRI on effector cells, resulting in cell degranulation and release of pro-inflammatory mediators. The extent of effector cell activation is linked to allergen affinity, oligomeric state, valency and spacing of IgE binding epitopes on the allergen. While most of these observations come from studies utilising synthetic allergens, here 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 towards two human patient-derived polcalcin-specific IgE antibodies. 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 humanised RBL-SX38 cell model, and that multivalency can overcome the need for high-affinity interactions.

**Keywords: Allergy, Antibodies, IgE, Allergens, Fc Receptors, Mast Cells/Basophils**

## Introduction

The initiating event of an allergic immune response is the cross-linking by a multivalent allergen of IgE antibodies 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 time-scale 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 tree (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 recognised by the same monoclonal antibody (10). Similarly, there exists cross-reactivity between structurally homologous allergens from different sources, especially between food allergens and pollen (11, 12).

Due to the complexity of naturally occurring allergens and the polyclonality of IgE antibodies generated against these allergens *in vivo*, researchers often employ simplified model systems using synthetic allergens to study the mechanisms of allergic reactions. One well-characterised system

is the hapten/antibody pair, dinitrophenyl (DNP)/anti-DNP IgE. In these systems, small synthetic allergens are often conjugated to a carrier protein, such as bovine serum albumin (BSA) (13), or 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-antibody 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.

Here we describe an antibody-allergen interaction model system, consisting of polcalcin allergens from pollen and patient-derived polcalcin-specific human IgE antibodies. Here termed HAPPIE (Human Anti-Phl p 7 Immunoglobulin E antibodies), these human antibodies were 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 characterised the interactions between recombinantly expressed HAPPIE antibodies 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 here we show that they display varying degrees of cross-reactivity to two HAPPIE antibodies. We have utilised 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-SX38) cell line (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). Here, 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 antibody can still mediate effector cell activation, utilising the avidity effects of a polyvalent allergen. The allergen-antibody model system we have established and the results presented here thus 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.

## 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 antibodies, 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 antibodies were called 1021F10 and CS09G6K, respectively (30). For IgE purification, an anti-IgE affinity column was prepared by conjugating the anti-IgE antibody Xolair (Novartis) onto NHS-activated Sepharose 4 Fast Flow pre-activated media (GE Healthcare Life Sciences) according to the manufacturer's instructions. Recombinant IgE 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).

### Production of SA 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. Biotin reagent molecule included a PEG3 linker, serving to reduce steric hindrance and provide orietational flexibility within the streptavidin-allergen complex. Unreacted biotin was removed by size exclusion chromatography and biotinylated Phl p 7 was then incubated with streptavidin (SA) (Sigma) at different molar ratios and then passed through a Superdex 200 Increase column (GE Healthcare) to separate streptavidin-Phl p 7 (SA-Phl p 7) complexes from unbound Phl p 7. Fractions containing SA-Phl p 7 were visualised using non-denaturing SDS PAGE to confirm the

presence of the streptavidin-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'-GGAGTTTACCGACTTTTGTCGTGCAAACCGTGG-3' for forward and 5'-CCACGGTTTGCACGACAAAAGTCGGTAAACTCC-3' for reverse primers.

Distance calculations for epitope spacing in different SA-allergen dimer conformations were a summation of an allergen radius of gyration, PEG3 linker Flori radius (calculated as 0.7 nm) (34) and the distance between selected biotin molecules bound to streptavidin (measured using biotin bound streptavidin (PDB ID 1NDJ) structure as a template (35)). A full list of measurements is available in supplemental Table S3.

Surface plasmon resonance

SPR experiments were performed on a Biacore T200 instrument (GE Healthcare). The running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 4 mM $CaCl_2$, and 0.001% (v/v) Surfactant P-20) 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). Experiments to study the thermodynamics of HAPPIE2's interaction (Fab was used in these experiments) with Phl p 7 and Bet v 4 were performed at 5 °C, 15 °C, 25 °C and 35 °C. SPR data were analysed using BIAevalution 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 rat basophilic leukaemia (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 units/mL penicillin, 100 μg/mL streptomycin, and 500 μg/mL geneticin) in a humidified, 37 °C, 5% $CO_2$ 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 $10^4$ cells/well and incubated overnight at 37 °C, 5% $CO_2$. The following day cells were sensitised with 200 ng/mL of IgE in RBL media (100 μL/well) for 24 h. Following washes with the assay buffer (Hank's Balanced Salt Solution (HBSS) containing 1% bovine serum albumin), the allergen was added over a concentration range of 0.5 pM to 5 μM (100 μL/well, diluted in the assay buffer). A polyclonal rabbit anti-human IgE antibody (Dako, A0094) 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 analysed 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 antibodies (HAPPIE1 and HAPPIE2) using surface plasmon resonance (Fig. 1). Consistent with previous studies (30), we found that both anti-Phl p 7 IgE antibodies 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. 1 A and B; these data are summarised in Table 1) and HAPPIE2 (Fig. 1 E and F) 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 nM and 0.11 nM; Ole e 3, 1.0 nM 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), cross-reacted less strongly with both HAPPIE1 (Fig 1 C and D) and HAPPIE2 (Fig. 1 G and H) (the $K_D$ values for Aln g 4 are 2320 nM and 390 nM; Bet v 4 are 4430 nM 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 $^{o}$C to 35 $^{o}$C, we measured binding kinetics and affinities of the allergen to the covalently immobilised HAPPIE2 Fab fragment. For each temperature, a concentration binding series was performed (Fig. S1) and interaction 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 (Fig. S1). 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 favourable interactions for both enthalpy and entropy ($\Delta H$ = -36.4 kJ mol$^{-1}$, -T $\Delta S$ = -19.7 kJ mol$^{-1}$), while the (weaker) interaction with Bet v 4 was dominated by a favourable entropy term ($\Delta H$ = -7.1 kJ mol$^{-1}$, -T $\Delta S$ = -23.8 kJ mol$^{-1}$). It is not surprising that the cross-reactivity seen with Bet v 4 is less enthalpically favourable than the Phl p 7 interaction; similarly to HAPPIE1, the HAPPIE2 antibody was derived from a patient with grass pollen allergies, and this antibody likely underwent affinity maturation against the Phl p 7 antigen, optimising for non-covalent interactions with this target (30). Cross-reactivity towards 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 non-specific interactions; specific interactions tend to be enthalpically driven, while non-specific 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 (Fig. S2). 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 sensitised 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 stabilised by an internal disulfide bridge, which stabilises the monomer conformation, as described by Magler *et al.* (41). When RBL-SX38 cells were sensitised with either HAPPIE1 or HAPPIE2, no significant degranulation was detected when stimulated either wild-type monomer or disulfide stabilised monomeric allergens (Fig. 3). In previous experiments, monomeric Phl p 7 has been reported to activate HAPPIE1-sensitised basophils, and a superantigen-like mechanisms was proposed for this activity (30). However, in the current experiments the disulfide-stabilised monomeric Phl p 7 showed no capacity to crosslink HAPPIE1 and trigger basophil degranulation (Fig. 3B).

Low concentrations of native Phl p 7 dimer (Fig. 3 B and D), but not dimeric Bet v 4 (Fig. 3 A and C), triggered basophil degranulation when cells were sensitised 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 streptavidin-biotin interaction was utilised 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 streptavidin (SA) to form SA-allergen complexes (resulting complexes had highest possible valency of four biotinylated allergens bound per tetrameric SA (Fig. 4 B)) and the reaction was assessed using SDS PAGE (Fig. 4 A). The unbound biotinylated allergen was separated from the SA-polcalcin complexes using size-exclusion chromatography (Fig. 4 C). Fig. 4 summarises the approach used to produce SA-Bet v 4 complexes; an identical protocol was followed to generate SA-Phl p 7 multimers. Streptavidin-polcalcin complexes with different polcalcin/streptavidin ratios were also produced (Fig. 4 D and E), enabling study of the role of allergen valency in IgE binding and basophil degranulation. Streptavidin-polcalcin dimers and trimers, while distinct peaks in size-exclusion chromatography (Fig. 4 C and D), are heterogenous species due to the intrinsic asymmetry of the streptavidin tetramer (Fig. 4E), while monomers and tetramers are homogeneous.

Based on the radii of gyration calculated for the proteins used here (full details about estimated distances are available in supplemental Table S3), 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 therefore be anticipated that engineered multimeric allergens would display improved IgE cross-linking when compared to native dimers. Additionally, the spacing between the IgE-binding epitopes on streptavidin-polcalcin multimers would not be great enough to cross-link both Fabs on a single IgE antibody (Fabs may be cross-linked by ligands of around 11 to 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 RU, 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 antibody surface (Fig. 5 A). Native Bet v 4 dimers showed moderate avidity effects (Fig. 5 B), characterised 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 due to avidity effects (Fig. 5 C and D). 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 due to 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). $EC_{50}$ values are summarised in Table 2.

Similar levels of degranulation were triggered by bivalent ($EC_{50}$ = 0.5 nM and 0.7 nM for cells sensitised with HAPPIE1 and HAPPIE2, respectively), trivalent (HAPPIE1 $EC_{50}$ = 0.4 nM; HAPPIE1 $EC_{50}$ = 0.7 nM (HAPPIE2)) or tetravalent Phl p 7 (HAPPIE1 $EC_{50}$ = 0.7 nM; HAPPIE1 $EC_{50}$ = 0.3 nM) constructs (Fig 6 B and D). For all of these constructs there were no statistically significant differences in $EC_{50}$ 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 antibodies 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 6 A and C). ($EC_{50}$ = 0.3 nM (tetramer) vs $EC_{50}$ = 1.8 nM (dimer), **p ≤ 0.01; $EC_{50}$ = 0.6 nM (trimer) vs $EC_{50}$ = 1.8 nM (dimer), **p ≤ 0.01; $EC_{50}$ = 0.3 nM (tetramer) vs $EC_{50}$ = 0.6 nM (trimer) when cells were sensitised with HAPPIE1, n.s.; $EC_{50}$ = 0.2 nM (tetramer) vs $EC_{50}$ = 1.5 nM (dimer), ***p ≤ 0.001; $EC_{50}$ = 0.3 nM (trimer) vs $EC_{50}$ = 1.5 nM (dimer), **p ≤ 0.01; $EC_{50}$ = 0.2 nM (tetramer) vs $EC_{50}$ = 0.3 nM (trimer) when cells were sensitised with HAPPIE2 and stimulated with Bet v 4 complexes, n. s.). This suggests that allergen valency can, under some circumstances, play an important role in effective IgE-receptor cross-linking, and that an antibody's low affinity for allergen can be overcome by avidity effects.

These degranulation experiments using streptavidin-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. 6 A and C) were more potent in degranulation assays than native Bet v 4 dimers (Fig. 3 A and C), which is clearly illustrated when the potency of these allergens is compared directly (Fig. S4). RBL-SX38 cells were activated by native Bet v 4 dimers only at high concentrations (**p ≤ 0.01, HBSS vs 2.5 μM native Bet v 4 dimer, estimated $EC_{50}$ = 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 thousand-fold lower concentrations when compared to native dimers and stimulated RBL-SX38 cells sensitised with either HAPPIE1 or HAPPIE2 (estimated $EC_{50}$ = 1.8 nM and 1.5 nM for cells sensitised 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.

In the polcalcin-streptavidin dimer, distances between polcalcin epitopes are greater, and there is potentially greater orientational flexibility for these molecules, due to 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 modelling server (47–49) and models of monomeric Bet v 4 (50) bound to tetrameric streptavidin (35), we can estimate the average distances between polcalcin epitopes. Engineered polcalcin-streptavidin 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 recognised by an antibody on a native polcalcin would be about 3.0-3.2 nm. In the engineered polcalcin dimer, those epitopes would be on average separated by about 4.1 nm in cis-conformation, and by > 6 nm in either of the trans- conformations.

## Discussion

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

Most of the currently used allergen-antibody model systems utilise small haptens, such as DNP and dansyl, as allergens, where 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 to control valency and allergen spacing. In model systems where haptens are conjugated to a chemically synthesised scaffold (as described by Handlogten *et al.* (15)), these problems can be overcome. But such models still use small synthetic molecules as haptens, while most naturally occurring allergens are complex proteins. Further, the IgE antibodies used in most experimental models are usually of non-human origin (51). The HAPPIE antibodies used in this study are recombinantly-expressed, monoclonal human patient-derived antibodies with matched heavy and light chains (26). Finally, the human origin of the antibodies 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 modelling the human allergic response is RBL-2H3, which expresses rat FcεRI, and mouse antibodies 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 (54) 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 antibody 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 favour bicyclic IgE-FcεRI complex dimers to form, result in very inefficient mast cell activation (20, 21, 55). And, as we show in this work, bivalent allergens with low affinities and fast off-rates may result in only transient IgE crosslinking, 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). Here 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-sensitised cells were stimulated with multivalent Bet v 4 constructs.

While the native Bet v 4 dimer triggered low levels of degranulation and showed only a small amount of enhanced binding due to avidity in the SPR binding experiments (Fig. 5), dimeric streptavidin-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 signalling cascade leading to cell

degranulation (52, 56). While a previous study using artificial allergens has concluded that placing IgE epitopes in close proximity increases allergen potency (19), using streptavidin 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 due to the incorporation of a flexible linker between the streptavidin scaffold and the allergen itself, allowing some movement and repositioning of multiple epitopes in respect to each other.

In summary, here we describe a model allergen-antibody system utilising patient-derived IgE antibodies and the protein allergens to which patients react, and demonstrate the importance of allergen affinity, oligomeric state and epitope spacing in IgE binding and mast cell degranulation.

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## Tables

| Allergen | Antibody | $K_D$ ± 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 |

**Table 1. Summary of selected polcalcin binding affinity values to HAPPIE1 and HAPPIE2 antibodies**. Data given as ±SD, experiments were run in duplicate.

| $EC_{50}$ ± SD (nM) | **Bet v 4** | | **Phl p 7** | |
|---|---|---|---|---|
| | **HAPPIE1** | **HAPPIE2** | **HAPPIE1** | **HAPPIE2** |
| **Dimer** | 1.8 ± 0.1 | 1.5 ± 0.1 | 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 |

**Table 2**. **Estimated $EC_{50}$ values for dimeric, trimeric and tetrameric polcalcin potency to trigger RBL-SX38 degranulation.** Data shown are the averages ±SD of at least three experiments with three biological replicates per experiment.

## Figures

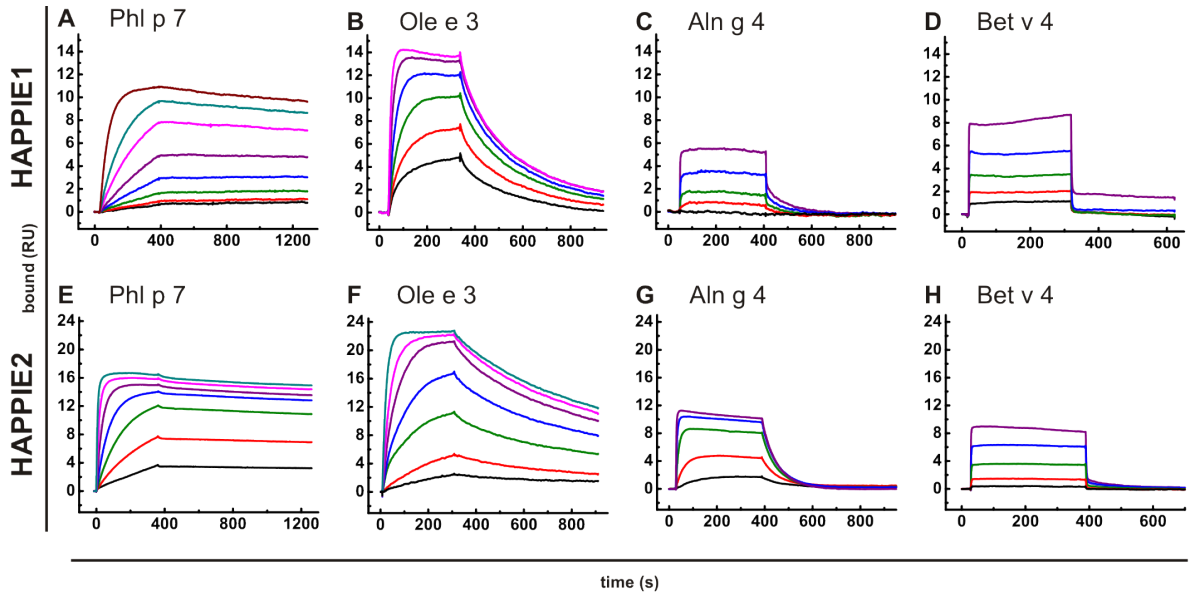


Fig.1 **Polcalcin allergen binding to HAPPIE1 (A-D) and HAPPIE2 (E-H).** Fusion protein of $IgG_4$ Fc and the soluble form of FcεRI receptor (sFcεRIα) (57) was immobilized on an SPR sensor chip and IgE molecules were captured on the chip via the immobilized sFcεRIα; allergens were then flowed over the IgE surface over a range of concentrations. Allergens were injected using a two-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 HAPPPIE1 and HAPPIE2; 100 nM for Phl p 7 for binding to both HAPPIE1 and HAPPIE2.

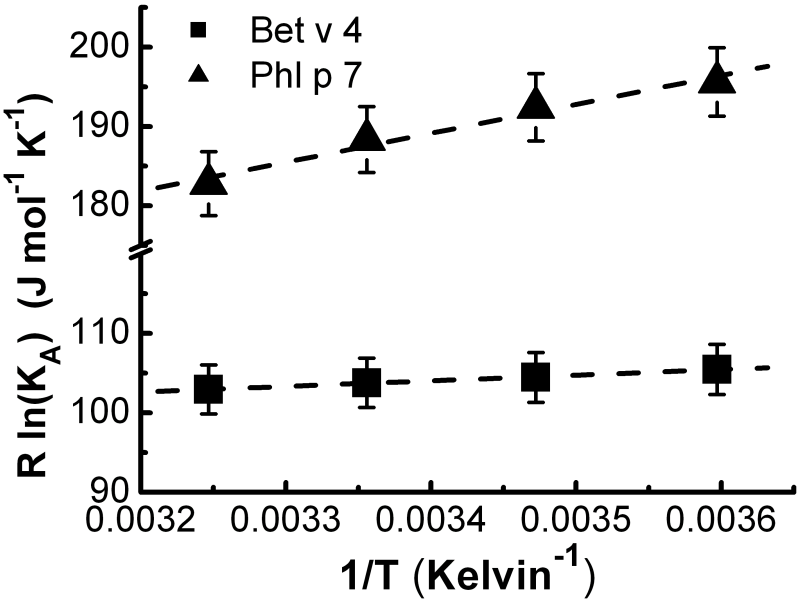


Fig. 2 **The thermodynamic parameters of Phl p 7 (triangles) and Bet v 4 (squares) interaction with HAPPIE2 Fab.** SPR binding experiments were performed at temperatures of 5, 15, 25 and 35 $^{o}$C. Data are shown as van't Hoff plots and the enthalpic contributions to binding were extracted using a linear van't Hoff analysis (58) (dashed lines show linear fit of the data). HAPPIE2 Fab/Phl p 7 interaction: $\Delta H$ = -36.4 kJ mol$^{-1}$, -T$\Delta S$ = -19.7 kJ mol$^{-1}$, $\Delta G$ = -56.1 kJ mol$^{-1}$; HAPPIE2 Fab/Bet v 4 interaction: $\Delta H$ = -7.1 kJ mol$^{-1}$, -T$\Delta S$ = -23.8 kJ mol$^{-1}$, $\Delta G$ = -30.9 kJ mol$^{-1}$.

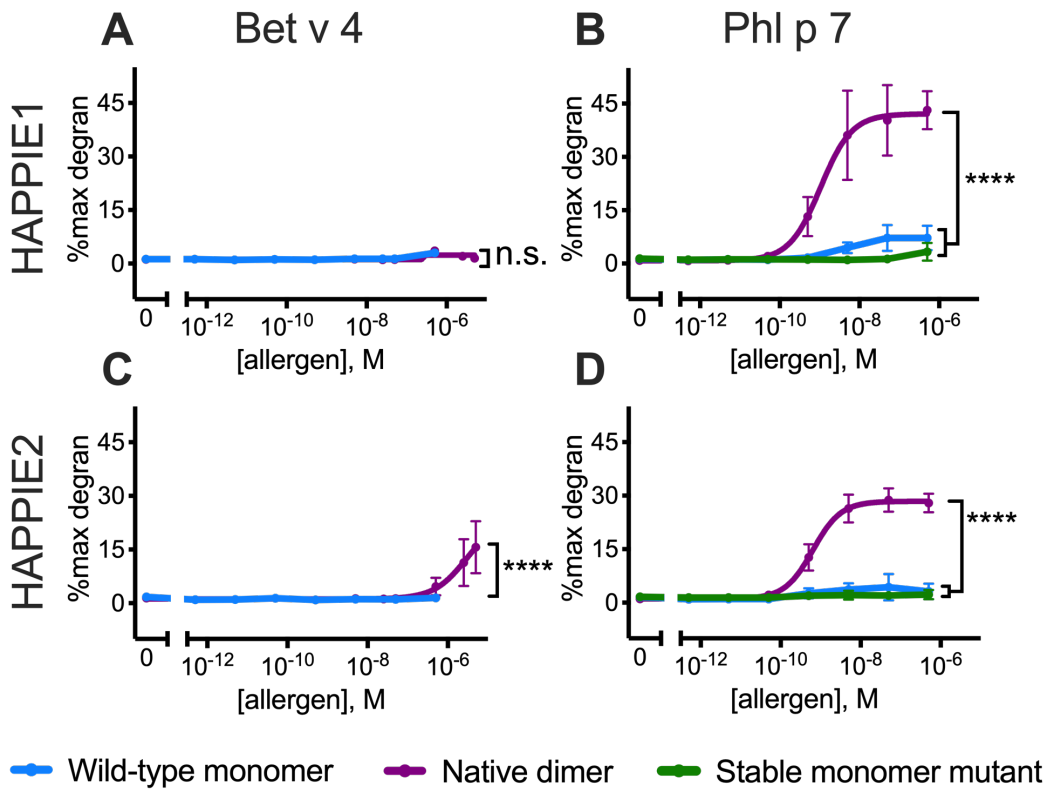


Fig. 3. **RBL-SX38 cell degranulation assays with recombinant Bet v 4 and Phl p 7 monomeric and dimeric proteins.** RBL-SX38 were sensitised with HAPPIE1 (A and B) or HAPPIE2 (C and D) and then stimulated with wild-type monomer or native dimer Bet v 4 (A and C), or disulfide stabilised monomer, wild-type monomer or native dimeric versions of Phl p 7 (B and D). Data were normalised as the percentage of maximum degranulation (%max degran; determined by cell lysis with 1% Triton-X100) and fitted to determine the $EC_{50}$ values. One-way ANOVA, followed by Tukey's multiple comparison tests were used to determine the statistical significance between the levels of β-hexosaminidase release when stimulated with wild-type monomers, disulfide stabilised monomers or native dimeric allergens when compared to background levels of degranulation (HBSS only); statistical tests are based on the highest concentrations of allergen tested (****$p < 0.0001$). Data shown are the averages ±SD of at least three experiments with three biological replicates per experiment.

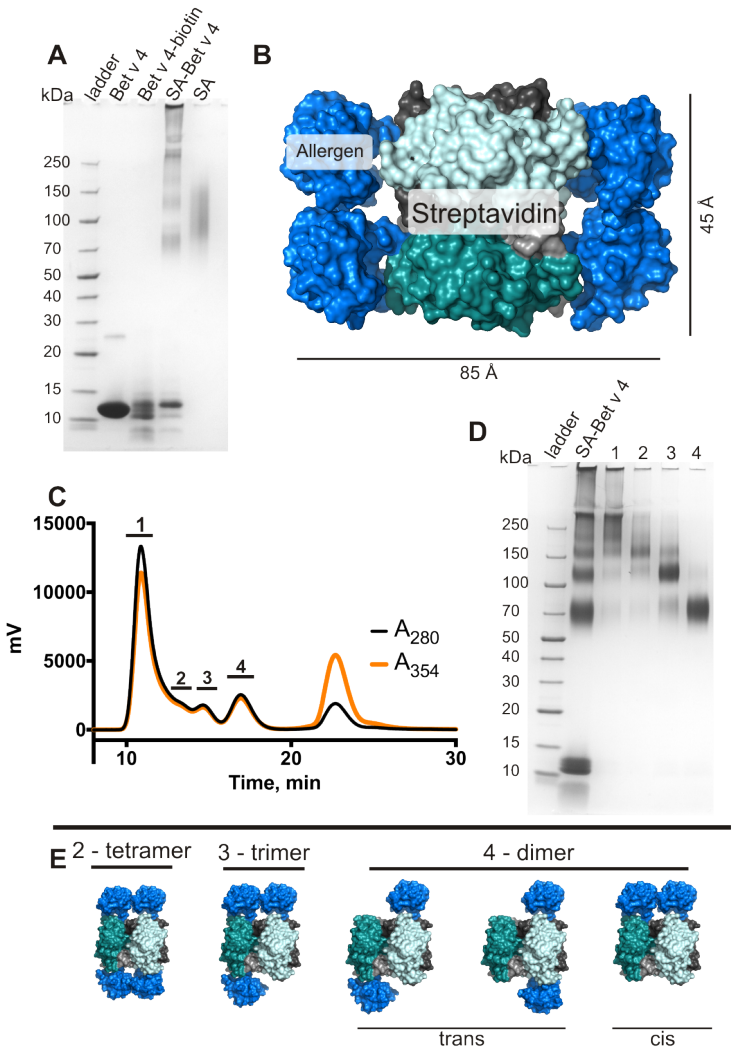


Fig. 4. **Multimeric allergen production and purification using streptavidin as a scaffold.** Streptavidin was incubated with biotinylated allergen to yield multivalent allergen complexes (A); a model of the tetrameric streptavidin-allergen complex is shown in (B) allergen is in dark blue, and streptavidin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

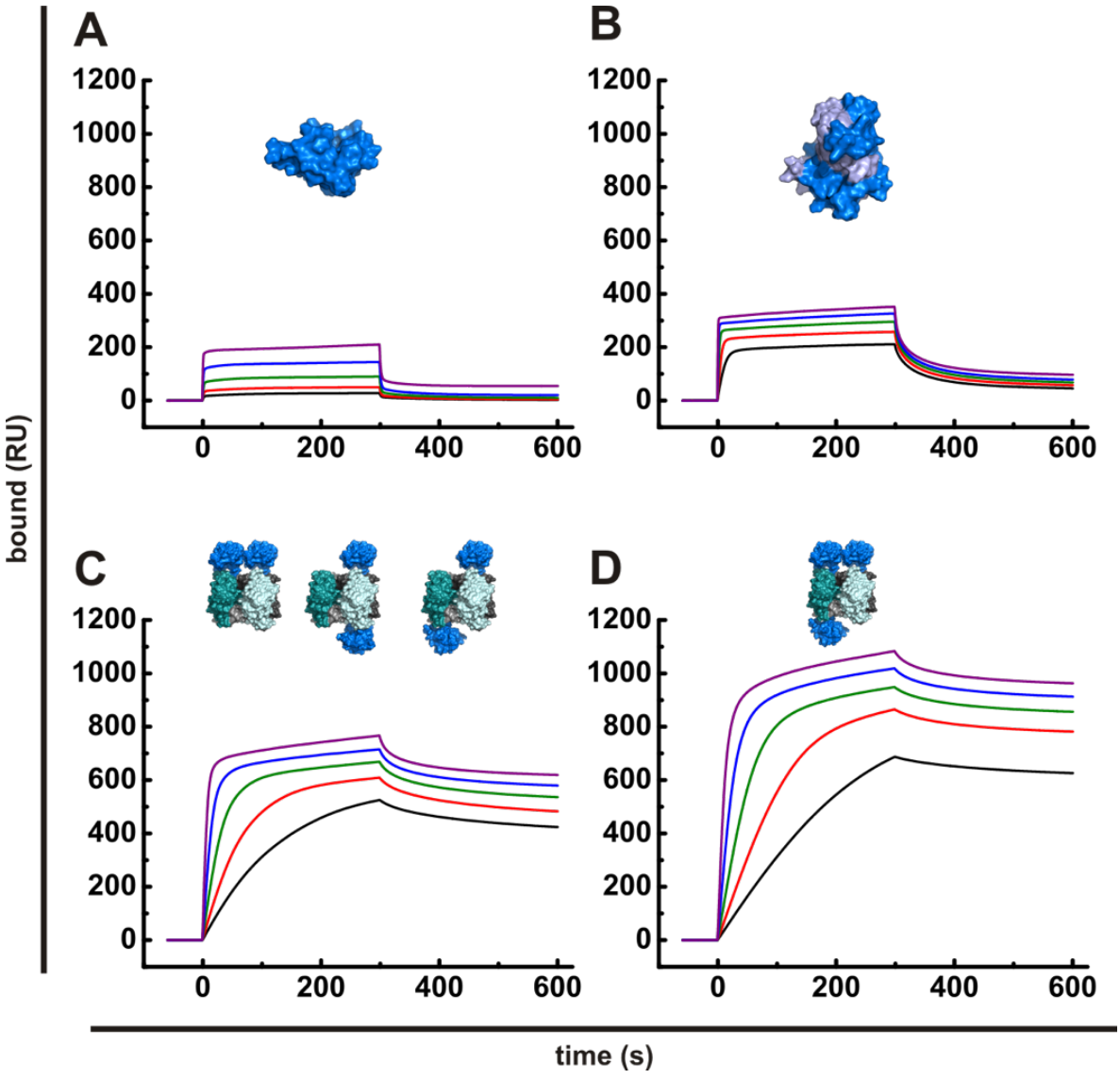


Fig. 5. **SPR binding analysis of wild-type Bet v 4 (A), native Bet v 4 dimer (B), dimeric streptavidin-Bet v 4 (C) and trimeric streptavidin-Bet v 4 complexes (D) to HAPPIE1 captured on the sensor surface.** Wild-type (A) and native dimeric (B) Bet v 4 was injected over HAPPIE1 sensor surface in a two-fold 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 two-fold dilution series concentrations, starting with 200 nM as the highest concentration.

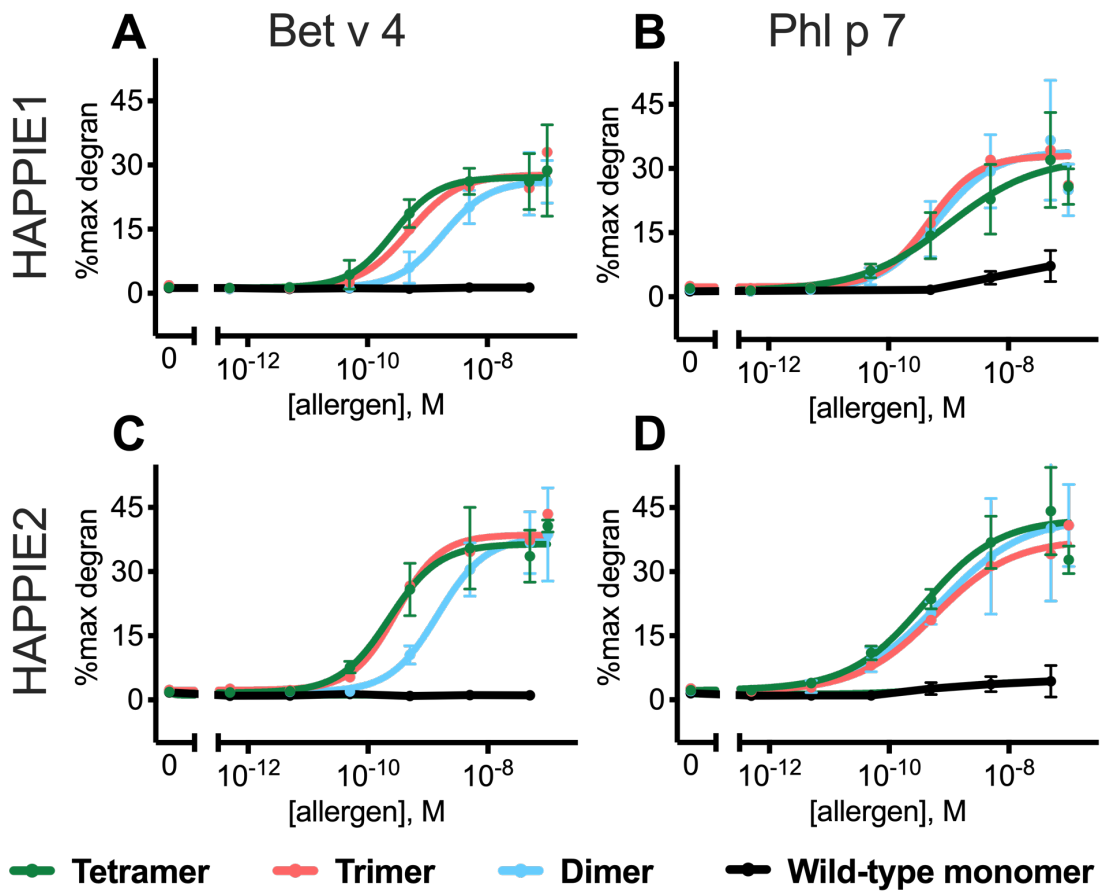


Fig. 6. **Degranulation of RBL-SX38 cells sensitised with HAPPIE1 (A and B) or HAPPIE2 (C and D) and stimulated with multimeric variants of Bet v 4 (A and C) or Phl p 7 (B and D).** Responses for cells stimulated with wild-type monomeric proteins is replicated from Figure 3 and included for comparison purposes. Data were normalised as the percentage of maximum degranulation (cell lysis with 1% Triton-X100). Data shown are the averages ±SD of at least three experiments with three biological replicates per experiment.