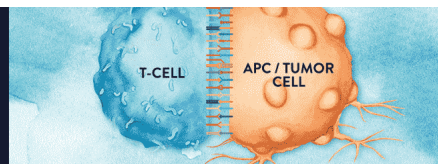


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Mapping Human Monoclonal IgE Epitopes on the Major Dust Mite Allergen Der p 2

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Mapping Human Monoclonal IgE Epitopes on the Major Dust Mite Allergen Der p 2

Geoffrey A. Mueller,* Jill Glesner,[†] Jacob L. Daniel,[‡] Jian Zhang,[‡] Noah Hyduke,[§] Crystal M. Richardson,[†] Eugene F. DeRose,* Martin D. Chapman,[†] R. Stokes Peebles, Jr.,[‡] Scott A. Smith,[‡] and Anna Pomés[†]

IgE Abs drive the symptoms of allergic disease upon cross-linking allergens on mast cells or basophils. If the IgE binding sites on the allergens could be identified, it may be useful for creating new forms of immunotherapy. However, direct knowledge of the human IgE (hIgE) epitopes is limited because of the very low frequency of IgE-producing B cells in blood. A new hybridoma technology using human B cells from house dust mite-allergic patients was used to identify four Der p 2-specific hIgE mAbs. Their relative binding sites were assessed and compared by immunoassays with three previously studied murine IgG mAbs. Immunoassays showed that the recognition of Der p 2 by the first three hIgE was inhibited by a single murine IgG, but the fourth hIgE recognized a different epitope from all the other mAbs. The functional ability of the hIgE that bind different epitopes to cross-link Der p 2 was demonstrated in a mouse model of passive systemic anaphylaxis. Nuclear magnetic resonance analyses of Der p 2 in complex with IgG and IgE Abs were used to identify specific residues in the epitopes. To our knowledge, the combination of immunoassays to distinguish overlapping epitopes and nuclear magnetic resonance analyses to identify specific residues involved in Ab binding provided the first epitope mapping of hIgE mAbs to an allergen. The technologies developed in this study will be useful in high-resolution mapping of human epitopes on other Ags and the design of improved therapeutics. *The Journal of Immunology*, 2020, 205: 1999–2007.

Immunoglobulin E Abs are produced by susceptible individuals upon allergen exposure and are key to the symptoms of allergic disease (1, 2). Information about the epitopes recognized by IgE would be useful for designing new molecules for immunotherapy. However, very little is known about IgE Ab binding sites, their biochemical features, and their distribution on the allergen molecular surface. Abs from different isotypes have the potential to interact with any surface-exposed element of any given Ag (3), but evidence suggests that differences in epitopes exist among isotypes. For example, early epitope mapping studies showed that linear epitopes of synthetic peptides were recognized differently by IgE and IgG (4–9). Recently, different proteins from

grass pollen were also reported to be targeted by IgE versus IgG (10). A possible mechanism for the observation that IgE and IgG epitopes are different has been suggested by next-generation sequencing studies that found fewer mutations in germline IgE Ab sequences from allergic individuals than in IgG (11). The differences in isotype may be due to factors related to the location where the B cell matures (12). It is also possible that the differences in isotype are driven by the molecular or chemical features of epitopes (13, 14). For example, the analysis of a well-characterized epitope recognized by an IgE derived from a combinatorial library suggested that IgE epitopes are potentially more planar than other known epitopes (15). The distribution of IgE epitopes on an allergen may also vary among patients, but some allergens have epitopes clustered in certain areas. For example, a single mAb can block 80% of IgE binding to Phl p 2 (16). All of these studies suggest that a better understanding of the IgE response and localization of epitopes is needed, which would be useful in designing therapies that either seek to block the symptomatic IgE, or modify the allergen for immunotherapy (17–19).

The main reason for the limited knowledge of IgE epitopes is the challenge of cloning IgE Abs because of the extremely rare B cells harboring IgE genetic information. Instead of being able to study human monoclonal IgE, indirect evidence of human IgE (hIgE) epitopes was originally obtained by site-directed mutagenesis (20, 21) or the analysis of IgE binding to synthetic or recombinant peptides (22). Later on, approaches that take into account the conformational nature of most IgE epitopes on aeroallergens were developed; murine IgG (mIgG) mAbs that inhibit IgE Ab binding have been used as surrogates for IgE in x-ray crystallography studies of allergen–Ab complexes (23). Phage display libraries have also been used to simulate human-like IgE constructs that allowed either an indirect identification of epitopes by immunoassays (24) or directly determined the structure of two allergen–IgE Fab complexes (15, 25). These surrogate technologies

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The online version of this article contains supplemental material.

Abbreviations used in this article: hIgE, human IgE; HMQC, heteronuclear multiple-quantum coherence; mIgG, murine IgG; nDer p 2, natural Der p 2; NMR, nuclear magnetic resonance.

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have provided valuable information, but further in vivo studies would be desirable.

More recently, there has been a return to the challenge of cloning human B cells that produce IgE from allergic patients. Anti-peanut IgE Abs were cloned from patients by selecting for anti-Ara h 2 B cells followed by single-cell RNA sequencing (26). The low frequency of B cells in blood is still an important limiting step for this approach. A novel strategy to clone IgE was followed in the current study to isolate hIgE mAbs by hybridoma technology. This approach was first used to isolate IgE specific for *Aspergillus* allergens (27). The advantage is that the isolated IgE clones contain the natural pairing of the H and L chains as it occurs in vivo, which opens a new avenue for the analysis of IgE epitopes. In this study, four IgE human mAbs against Der p 2 were cloned from mite-allergic patients using this new hybridoma technology, the relative positions of their epitopes were analyzed by immunoassays, and residues involved in binding Der p 2 were identified by nuclear magnetic resonance (NMR).

Der p 2 was selected as the model system because of its relevance as a major allergen from the house dust mite *Dermatophagoides pteronyssinus*. A large proportion ($\geq 79\%$) of mite-allergic patients have specific IgE Abs to this allergen (28, 29). In addition to the medical relevance, there are many previous molecular studies of Der p 2 that can provide additional insight. The epitopes of several mIgG Abs to Der p 2 were previously studied by hydrogen-exchange protection NMR, which identified a few residues protected by the Abs in complex with the allergen (30, 31). Recently, the x-ray crystal structure of one of them, mAb 7A1, in complex with Der p 2, was determined, and IgE binding sites were identified by site-directed mutagenesis analysis (23). In this study, we capitalized on previous knowledge and used NMR studies of methyl-labeled Der p 2 in complex with Abs, both mIgG and hIgE, to map epitopes. In combination with immunoassays, the information obtained using this approach gives some of the first site-specific information of the hIgE epitopes that will be used for designing molecules with reduced allergenic activity for immunotherapy.

Materials and Methods

Human hybridoma generation

IgE-secreting human hybridomas were generated using a methodology that was recently described (27) and is detailed below. Dust mite-specific IgE mAbs were created from dust mite-allergic research subjects recruited from within the Vanderbilt University Medical Center. The protocol for recruiting and collecting blood samples from allergic subjects was approved by the Vanderbilt University Medical Center Institutional Review Board (IRB 141330 and 142030). All human study participants provided written informed consent. Diagnosis was based on clinical history and testing serum for the presence and quantity of IgE Ab to dust mite, specifically *Dermatophagoides farinae* and/or *D. pteronyssinus*. Der p 2-specific IgE Ab levels were measured in human sera by using ImmunoCAPs (d203) in a Thermo Fisher Scientific ImmunoCAP system (Phadia 250 immunoassay analyzer; Thermo Fisher Scientific, Portage, MI).

IgE-secreting human hybridomas were generated from previously cryopreserved samples that were thawed, washed, and counted before plating. For every 2 million viable cells, the following were added: 30 ml of prefusion medium (ClonaCell-HY 03801; STEMCELL Technologies), 20 μ l of CpG stock (2.5 mg/ml; ODN 2006), 1 μ l each of mouse anti-human κ (9230-01; SouthernBiotech) and mouse anti-human λ (9180-01; SouthernBiotech), and 1 million gamma-irradiated NIH3T3 cells, a fibroblast line genetically engineered to constitutively express cell surface human CD154 (CD40L), secreted BAFF, and human IL-21 (provided by Dr. D. Bhattacharya; Washington University in St. Louis, St. Louis, MO). The mixture was then plated into 96-well flat-bottom culture plates at 300 μ l/well and incubated at 37°C with 5% CO₂ for 6 d prior to screening for IgE secretion using an ELISA.

Omaliuzumab, which is specific for the unique constant domain of IgE, was used as a capture Ab, coating 384-well black ELISA plates at a

concentration of 10 μ g/ml. After blocking, 100 μ l of supernatant was transferred from each well of the 96-well plates containing B cell lines using a VIAFLO 384 electronic pipetting device (INTEGRA Biosciences). Secondary Ab (mouse anti-hIgE Fc, 9160-05; SouthernBiotech) was applied at a 1:1000 dilution in blocking solution using 25 μ l/well. After 10 washes with PBS, fluorogenic peroxidase substrate solution (QuantaBlu, 15162; Thermo Fisher Scientific) was added at 25 μ l/well, as per manufacturer instructions. Relative fluorescence intensity was measured on a Molecular Devices plate reader. Wells were counted as positive if the relative fluorescence intensity was > 5 times background. IgE B cell frequencies were then expressed as the number of IgE-positive wells per 10 million PBMCs.

HMMA2.5 nonsecreting myeloma cells (provided by M. Posner, Mount Sinai, New York, NY) were counted and suspended in cytofusion medium composed of 300 mM sorbitol, 1.0 mg/ml BSA, 0.1 mM calcium acetate, and 0.5 mM magnesium acetate. Cells from IgE-positive wells were pipetted gently into microcentrifuge tubes containing 1 ml of cytofusion medium. B cells and HMMA2.5 cells were washed three times in cytofusion medium to ensure buffer equilibration. HMMA2.5 cells were then suspended in cytofusion medium to achieve a concentration of 10 million cells/ml. The HMMA2.5 cell suspension was added to each sample tube and the mixture pipetted into cuvettes (450125; BTX). Cytofusion was performed using a BTX cuvette holder (BTX Safety Stand, model 630B) with a BTX ECM 2001 generator, 45-0080; BTX) programed to run with following settings: a prefusion alternating current of 70 V for 40 s, followed by a direct current pulse of 360 V for 0.04 ms, then a postfusion alternating current of 40 V for 9 s. After fusion, the content of each cuvette was added to 20 ml of hypoxanthine-aminopterin-thymidine medium containing ouabain and composed of the following: 500 ml of postfusion medium (03805; STEMCELL Technologies), one vial 50 $\times$ hypoxanthine-aminopterin-thymidine (H0262; Sigma-Aldrich), and 150 μ l of a 1 mg/ml stock of ouabain (013K0750; Sigma-Aldrich). Fusion products then were plated into 384-well plates and incubated for 14 d before screening hybridomas for IgE Ab production by ELISA.

Wells containing hybridomas producing IgE Abs were cloned biologically by indexed single-cell flow cytometric sorting into 384-well culture plates. Once clonality was achieved, each hybridoma was expanded in postfusion medium in 75-cm² flasks. The mAb was expressed by large-scale growth of the hybridoma in serum-free medium (Life Technologies Hybridoma-SFM, 12045084; Invitrogen) in 225-cm² flasks. IgE Ab was then purified by immunoaffinity chromatography (omalizumab covalently coupled to GE Healthcare HiTrap NHS-Activated column; 17-0717-01) and visualized by SDS-PAGE for purity.

The mAbs used are annotated in Table I.

Immunoassays to assess the relative positions of mAb epitopes on Der p 2

For the two-site immunoassays, a microplate was coated overnight with the mIgG mAbs [7A1, 1D8, or α DpX (32) (Fig. 1)] or IgE mAbs [2G1, 1B8, or 2F10 (Fig. 3)] at 10 μ g/ml in 50 mM carbonate/bicarbonate buffer (pH 9.6). The plate was washed and blocked with PBS (pH 7.4) containing 0.05% Tween 20 and 1% BSA. To the wells coated with murine mAbs (Fig. 1), natural Der p 2 (nDer p 2) was added at a concentration of 100 ng/ml, followed by a 1-h incubation and addition of biotinylated hIgE mAbs (1:1000 and diluted 1:2 across the plate). To the plates coated with IgE mAbs (Fig. 3), nDer p 2 was added at the concentration of 100 ng/ml and diluted 1:2 across the plate, followed by a 1-h incubation and addition of biotinylated hIgE mAbs (1:1000) (same protocol for Supplemental Fig. 2, regardless of the IgG or IgE mAb used for coating). For both immunoassays, addition of streptavidin peroxidase (1:1000) (Sigma, St. Louis, MO) for 30 min followed, and the plates were then developed using ABTS in 70 mM citrate phosphate buffer (pH 4.2) and 1:1000 dilution of H₂O₂. Regarding the allergen, nDer p 2 was affinity purified from mite culture by affinity chromatography using 7A1; the purity was $> 95\%$ by SDS-PAGE.

For dose-response inhibition experiments of IgE mAb binding to Der p 2, microplates were coated overnight with nDer p 2.0103 at 10 μ g/ml expressed in *Pichia pastoris* as previously described (23). The IgG mAbs were then added at 0.1, 1, 10, and 100 μ g/ml to the plate wells and incubated 1 h. The plate was washed, and IgE mAb (1:500–1:50,000) was added to wells and incubated 3 h. A 1-h incubation of mouse anti-hIgE Fc-HRP (SouthernBiotech, Birmingham, AL) (1:1000) followed, and the plate was developed as above.

For additional inhibition experiments using sera or plasma from mite-allergic patients, the maximum concentration of inhibitor (100 μ g/ml of IgG) was selected. Microplates were coated overnight with nDer p 2 at 10 μ g/ml. The IgG mAbs were then added at 100 μ g/ml to the plate wells in duplicate and incubated with plasma from allergic patients ($n = 8$) at 1:2 dilution for 3 h. A 1-h incubation of mouse anti-hIgE Fc-HRP (SouthernBiotech) (1:1000) followed, and the plate was developed, and absorbance was read as above.

NMR samples and data acquisition

Der p 2 and Der f 2 have been studied previously by NMR (31, 33–36). Allergen expression and purification followed the optimized protocol of Nakamura (37), with a few adjustments. Benzoylase was added prior to sonication, and the insoluble fraction was incubated with 6 M guanidium chloride for 1 h at room temperature with rapid stirring prior to dialysis and refolding. Assignments were determined in PBS buffer using standard NMR techniques, with a [^{13}C , ^{15}N] Der p 2 sample (29). For maximum sensitivity in a high m.w. complex with Abs, a Der p 2 [^{13}C , ^{15}N , ILV ^{13}H , ^{13}C -methyl] sample was made according to Goto et al. (38) with the exception that ^2H -glycerol was used instead of ^2H -glucose as the primary carbon source. This expression protocol labels the Ile 81 methyl, both methyls of Leu and Val, and otherwise perdeuterates the rest of the protein. For simplicity, we refer to this molecule as the “methyl-labeled Der p 2.”

Complexes were made by mixing 5–8 mg of Ab with an excess of methyl-labeled Der p 2 and incubated for at least 30 min at room temperature before applying the sample to a 26–600 Superdex S200 column flowing at 0.3 ml/min at 4°C with PBS. Three example purifications are overlaid in Supplemental Fig. 1 with an example gel separation of the components. The Der p 2–mAb fractions (region 2 in Supplemental Fig. 1) were exchanged into ^2H -PBS and concentrated to 400 μl . Data were acquired in a 4-mm tube on a Varian 800 MHz spectrometer with a cryogenically cooled probe using the heteronuclear multiple-quantum coherence (HMQC) pulse sequence, typically for 8–16 h at 37°C. The structure of Der p 2 in all figures uses Protein Data Bank file 1KTJ (39).

Human transgenic mouse anaphylaxis experiments

Human Fc ϵ RI transgenic mice (B6.Cg-Fc ϵ RI ^{tm1Knt} Tg[FCER1A]) 1Bhk/J were purchased from The Jackson Laboratory (stock number 010506), brought out of cryogenic storage, bred, and genotyped. Mice were maintained under specific pathogen-free conditions and used in compliance with the revised 2011 *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. These double mutant mice express the human Fc fragment of IgE, high-affinity I α receptor for α polypeptide, (FCER1A), under the control of the human FCER1A promoter and carry the Fc ϵ RI ^{tm1Knt} targeted mutation (40). Mice that are hemizygous for the transgene and homozygous for the targeted deletion of the mouse Fc ϵ RI respond to experimental induction of anaphylaxis with hIgE. Transgenic mice were sensitized by i.p. injection with 100 μg of total purified IgE in PBS and challenged 3 d later by i.p. injection with 50 μg of purified nDer p 2 protein in PBS. Temperature was monitored using implanted temperature probes. Data were combined from two separate experiments containing 12 mice each. The first experiment contained the following groups: 1B8 alone, 1B8 plus 2G1, 2F10 plus 1B8, and 2F10 plus 2G1. The second experiment contained the following groups: 2F10 alone, 1B8 plus 2G1, 2F10 plus 1B8, and 2F10 plus 2G1. The protocols for animal care and use for these experiments have been approved by the Institutional Animal Care and Use Committee (IACUC M1700156-00). All personnel are trained on the topic of responsible conduct of research and certified to conduct animal experiments by the Institutional Animal Care and Use Committee and a veterinarian. Temperatures of sensitized mice and 2F10 alone-sensitized mice were compared independently at each time point following Der p 2 challenge using ANOVA (MATLAB; MathWorks). Error bars for the mouse temperature measures represent SEM.

Results

Isolation of hIgE mAbs

Two research subjects were recruited from Vanderbilt University Medical Center allergy clinic during a routine evaluation (Table II). The first subject (P1) had eczema and rhinitis, whereas the second subject (P2) had asthma. Skin prick testing for both subjects demonstrated sensitization to dust mite, having 10–15 mm wheal reactions. The Der p 2-specific IgE Ab levels were 289.2 kU/L for subject P1 and 51.8 kU/L for subject P2. Further patient characteristics are given in Table II. PBMCs were isolated, and cells were grown as described previously (27). IgE encoding B cells were identified by screening culture supernatants for the presence of IgE Ab. IgE B cell frequencies and the number of IgE-producing B cells per 10 million PBMCs grown were calculated. Both subjects had nearly identical IgE B cell frequencies; subject P1 had 10 and P2

Table I. Origin and isotype of Abs

Name	Origin	Isotype	Patient Number
2G1	Human	IgE	P1
5D10	Human	IgE	P2
1B8	Human	IgE	P2
2F10	Human	IgE	P2
7A1	Murine	IgG	—
1D8	Murine	IgG	—
αDpX	Murine	IgG	—

showed 12 IgE B cells for every 10 million PBMCs cultured. IgE-containing B cell cultures were then immortalized, and human hybridomas were created without secondary screening for IgE specificity. Once hybridomas were generated, they were screened for binding to dust mite allergens. One hybridoma from subject P1 and three from P2 were found to be Der p 2 specific. Additional hybridomas with specificity to allergens of peanut and tree nuts were identified from P2 (data not shown).

Relative epitope mapping on Der p 2 by immunoassays

Three mIgG mAbs 7A1, 1D8 and αDpX , were used as reference Abs for epitope mapping of IgE mAbs because they have been reported as follows: 1) they bind to nonoverlapping epitopes (31, 32), and 2) they are able to inhibit polyclonal IgE from mite-allergic patients (31), which indicates that they overlap with IgE Abs. We confirmed that these three IgG mAbs, 7A1, 1D8, and αDpX inhibited binding of IgE Abs from sera/plasma to Der p 2 up to 10.3, 23.2, and 36.8%, respectively (with high variability in the percentage of inhibition depending on the subject; average $\pm$ SD was 2.4 ± 4.6 , 5.7 ± 11.2 , and $16.7 \pm 13.4\%$, respectively, for $n = 8$).

To perform a relative epitope mapping of the four identified hIgE mAbs, we investigated whether these IgE mAbs overlapped with any of these three murine mAbs by two-site ELISA and inhibition assays. For the two-site ELISA, each of the murine mAbs was used as coating Ab to capture Der p 2, followed by incubation with each of the IgE mAbs. The two-site ELISAs showed that three hIgE mAbs (2G1, 5D10, and 1B8) bound Der p 2 presented by mAb 1D8 and 7A1 but not αDpX (Fig. 1, Supplemental Fig. 2A), which indicates that αDpX and the three IgE mAbs 2G1, 5D10, and 1B8 bind to overlapping epitopes. However, the IgE mAb 2F10 bound Der p 2 presented by each of the three coating mIgG mAbs (1D8, 7A1, and αDpX) (Fig. 1). This result shows that the epitope for mAb 2F10 differs from the mAb IgG epitopes. The differences in binding level of the biotinylated IgE mAbs are most likely due to differences in the concentration of Ab used (concentrations of purified IgE mAbs before biotinylation ranged from 2.8 to 5.4 mg/ml) in combination with possible differences in Ab affinity (which was not measured).

To confirm the epitope overlap between the mIgG mAb αDpX and the three hIgEs observed in the two-site ELISAs, inhibition assays were performed (Fig. 2). αDpX at 100 $\mu\text{g}/\text{ml}$ inhibited 50–71% of the binding of IgE mAbs 2G1, 5D10, and 1B8 but not 2F10 to Der p 2. The murine mAbs 7A1 and 1D8 did not inhibit binding of these IgE mAbs in a similar way as the negative control the anti-Der p 1 mAb 4C1. None of the IgG mAbs showed a dose-response inhibition of IgE mAb 2F10 binding to Der p 2 (Fig. 2D). Overall, these results showed the interference of mIgG mAb αDpX with binding of hIgE mAbs 1B8, 2G1, and 5D10 to Der p 2 and confirmed the unique epitope of 2F10.

In addition, to assess relative epitope binding, hIgE mAbs were tested in pairs in two-site ELISAs, using combinations of a non-biotinylated IgE for coating with a biotinylated IgE for detection

Table II. Subject allergy history, B cell frequency, and hybridoma yield

Subject Code	Age	Sex	Allergic Disease	Total Serum IgE (kU IgE/L)	Dust Mite, <i>D. pteronyssinus</i> SPT (mm Wheal)	Dust Mite, <i>D. farinae</i> SPT (mm Wheal)	IgE B Cell Frequency (per 1×10^7 PBMCs)	Dust Mite-Specific IgE Hybridomas Generated ^a
P1 ^b	8	M	FA, eczema, rhinitis	ND	10 × 10	10 × 10	10	1
P2 ^c	18	M	FA, asthma	677	10 × 8	15 × 10	12	3

Subject age, total serum IgE, and dust mite skin prick testing results are shown. IgE B cell frequencies are expressed as the number of IgE-positive cells per 10 million PBMCs. Subjects' allergy histories are listed in descending order from most to the least reactive.

^aThe total of dust mite-specific IgE expressing human hybridomas generated for each subject is listed.

^bP1: dust mite, fish, peanut, cashew, egg.

^cP2: peanut, walnut, dust mite, cockroach.

FA, food allergy; M, male; SPT, skin prick test.

(Fig. 3, Supplemental Fig. 2B). The three biotinylated IgE mAbs 2G1, 5D10, and 1B8 detected the Der p 2 presented by coating IgE mAb 2F10 (Fig. 3A); and vice versa, biotinylated 2F10 detected Der p 2 presented by either the IgE mAb 2G1 or 1B8 (Fig. 3B). Note that IgE mAbs 5D10 and 1B8 are derived from the same patient and likely represent somatic variants. No reactivity was observed for any mAb pair combination of 2G1, 5D10, and 1B8, which indicates that these three IgE mAbs bind to overlapping epitopes (Fig. 3B, Supplemental Fig. 2B).

In summary, recognition of Der p 2 by three hIgE (5D10, 1B8, and 2G1) was inhibited by α DpX, but IgE mAb 2F10 binds to a different site than the epitopes for all the other six mAbs.

NMR analyses of complexes and mapping epitopes to the structure

Previously, an analysis of the crystal structure and NMR data from Der p 2 in complex with a single chain Fv of 7A1 demonstrated that surface-exposed methyl groups would provide the best signal-to-noise and most reliable epitope information (23). These probe residues were identified as follows: I97, I28, I121, V40, V63, V81, V105, V116, L17, and L61. The analysis showed that the Der p 2 methyls responded to both remote conformational changes because of Ab complexation and that resonances from residues immediately in the epitope disappeared. In more technical terms, the close proximity of methyls in the epitope to the large protonated Ab caused the resonances to broaden through a mechanism called cross-relaxation, thus making them hard to observe (41). Fig. 4A and 4B show both of these effects when Der p 2 is bound to hIgE 2F10. The methyls of residues L61, V63, and V105 have broadened below detection, whereas I97 has shifted. Fig. 4C shows that these residues are in close proximity on Der p 2. Using the knowledge that I97 is buried in the 7A1 epitope (23), and that 7A1 does not inhibit 2F10 binding to Der p 2 (Fig. 2), the shift of I97 is suggested to be a distal conformational change because of the complex formation. Therefore, the 2F10 epitope is proposed to center around V105, as shown in Fig. 4D. Fig. 4G shows a combination Venn diagram and color legend for Fig. 4E and 4F, demonstrating that a few residues may overlap between the proposed 2F10 and 7A1 epitopes, although this overlap is not functionally important.

Next, we analyzed the data for the complex of Der p 2 and α DpX, shown in Supplemental Fig. 3A and 3B. V81 showed the largest chemical shift changes, with a small change to V40, and possibly a new shift for I88, although this is speculative. Supplemental Fig. 3C shows how these residues are in close proximity on Der p 2. Combining this information with previous knowledge that disruption of the disulfide bond between C73 and C78 affects α DpX binding (20, 32, 42), and that residues A72, C73, and Y75 were protected by α DpX (as suggested by previous hydrogen-exchange experiments; these residues were not detected in the current study because only Ile, Leu and Val were labeled) (31), Supplemental Fig. 3D shows the proposed α DpX epitope with the residues annotated in Supplemental Fig. 3E.

Der p 2 in complex with each of the hIgE mAbs 2G1, 5D10, or 1B8 all showed nearly identical chemical shift changes (Supplemental Fig. 4). There were small changes in the Ile residues,

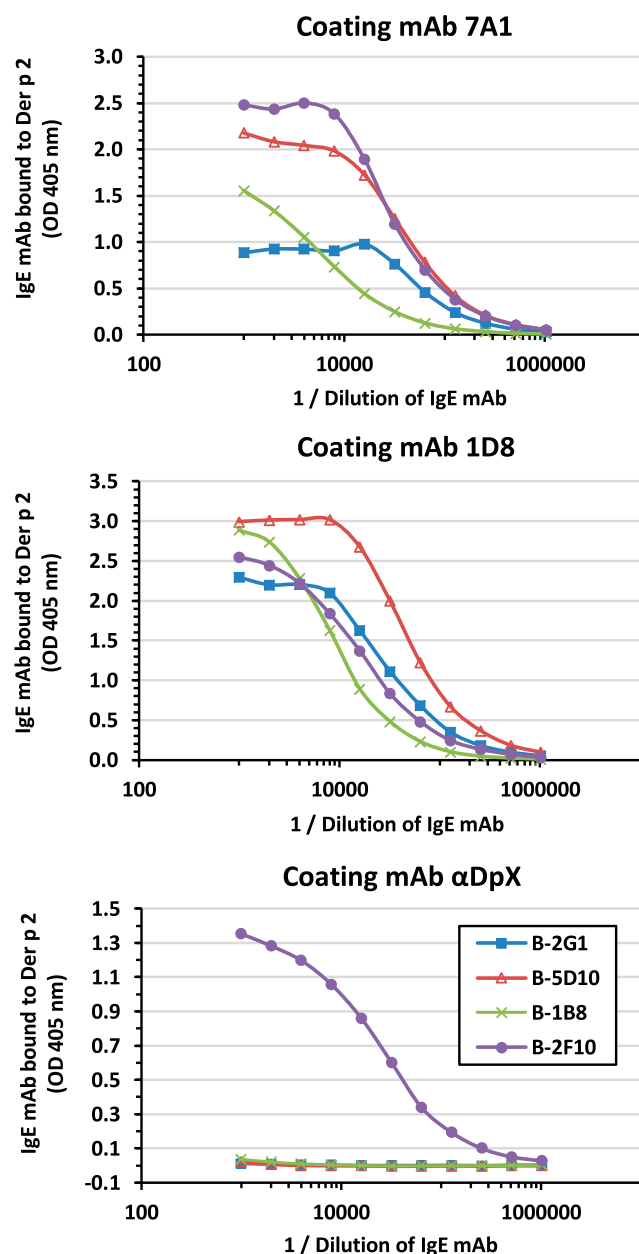


FIGURE 1. Dose-response curves of hIgE mAb binding to Der p 2 (rDer p 2.0103) presented by mIgG mAbs coating the plate (7A1, 1D8, and α DpX, from top to bottom). The two-site ELISA with all the different Ab combinations were done in duplicate with single data points, and a representative experiment is shown (see also Supplemental Fig. 2).

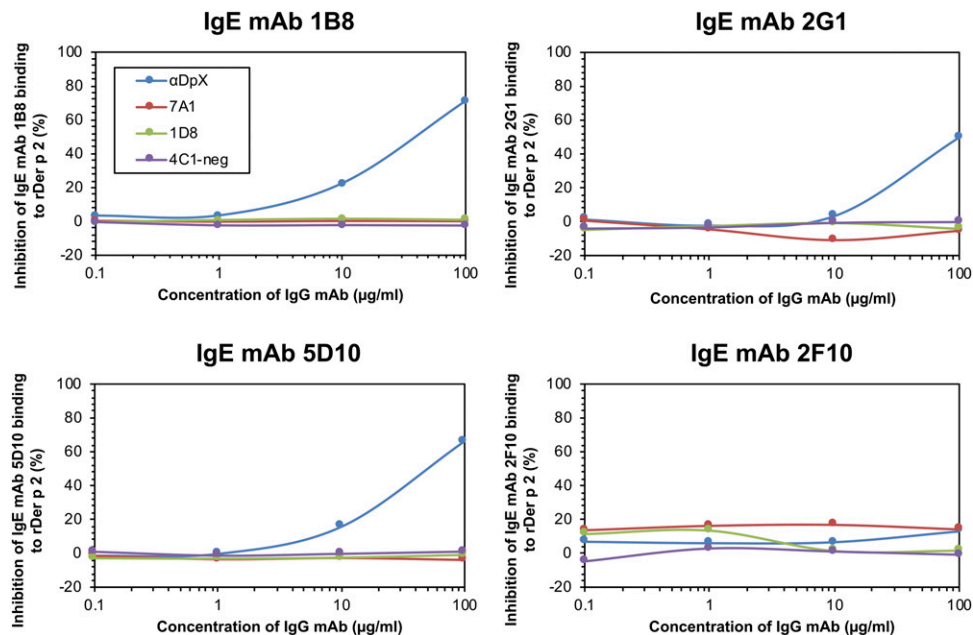


FIGURE 2. Immunoassays showing inhibition of hIgE mAb binding to Der p 2 by mIgG mAbs (α DpX in blue, 7A1 in red, and 1D8 in green). The Der p 1 mAb 4C1 (purple) served as a negative control. Each plot is representative of two to three experiments, in which data were obtained in single (shown) or duplicate.

but a few notable changes are evident in the Val, Leu region seen in Fig. 5A. Similar to the α DpX complex, V81 and V40 showed shift changes, but they were smaller and in different directions. The shifts of V116 were so far novel to the 5D10 family of Abs. There are possibly new shifts for the adjacent L117 that are subtly different for these three Abs. Because α DpX inhibits these hIgE there is likely significant overlap in the epitopes; but because the shift of 116 is different from the α DpX NMR data, the IgE epitope is proposed to be further from V40 and closer to V116 (see Fig. 5C). The epitope overlap is shown in Fig. 5D and 5E, whereas Fig. 5F again provides an explicit Venn diagram of the residues in the epitopes and is a color-coded legend for Fig. 5.

The final Ab complex explored was Der p 2 with the mIgG 1D8. There were numerous chemical shift changes scattered throughout Der p 2 (Supplemental Fig. 5), so additional data are necessary to help interpret the NMR shifts. 1D8 belongs to a family of Abs, including 6D6 and 2B12 that have overlapping epitopes. 1D8 can differentiate Der p 2 isoforms via residue differences at position

114 (33, 43, 44). Hydrogen-exchange protection also suggested residue 111 and 116 were in the 6D6 epitope, which is a related Ab to 1D8. Similarly, another Ab in this family, 2B12, has a reduced affinity for mutations at residues 45–48 (20, 32). Therefore, we suggest the chemical shift changes and broadening of 116 represent changes explicitly in the epitope region. Next, 1D8 partially inhibited the 5D10 family of IgE, likely indicating some epitope overlap reflected in the shift changes to V81, which are, however, at different chemical shifts from the previous Ab complexes. These differences in the V81 complex shifts likely indicate that 1D8 is not binding to the same epitope as 5D10/2G1/1B8 or α DpX. Finally, the shift changes to I121 are unique to 1D8 versus all the other Abs studied. Looking at the available data, it is likely that the 1D8 epitope spreads out from V116 to I121 and partially overlaps with the 5D10 family of epitopes near N44 (Supplemental Fig. 4C, 4D). This suggests that 1D8 might partially overlap with the 2F10 epitope, although functionally, the two Abs do not inhibit each other.

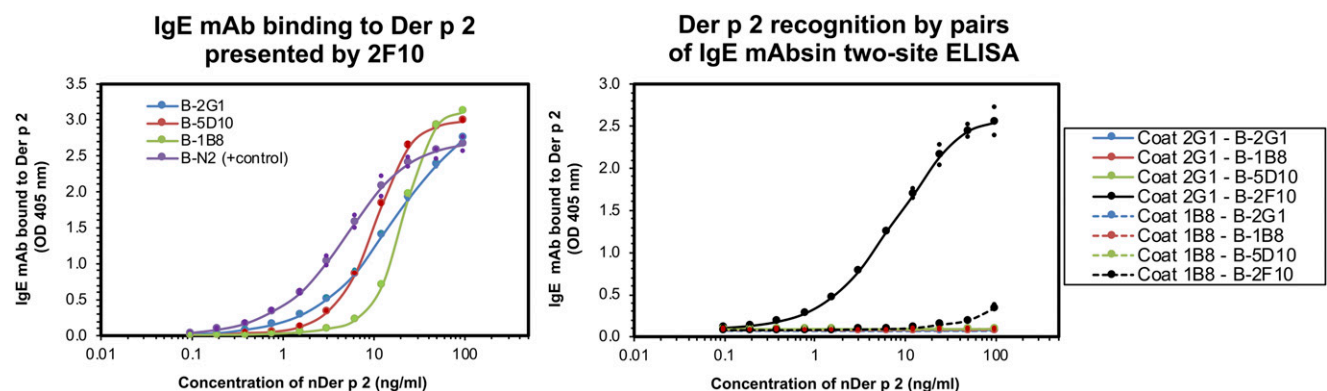


FIGURE 3. Dose-response curves of IgE mAb pairs: Left, Three biotinylated IgE mAbs (2G1, 5D10, and 1B8) detected Der p 2 presented by coating IgE mAb 2F10. Plot is representative of two experiments. Data are average (in the curve) of two duplicates that are shown as smaller symbols around the average. Right, Dose-response curves of each of the four biotinylated IgE mAbs binding to Der p 2 presented by either coating 2G1 or 1B8 IgE mAbs. Epitopes of three IgE mAbs (2G1, 5D10, and 1B8) overlapped, but mAb 2F10 bound to a different site. Plot is representative of two to three experiments, and data are average (in the curve) of two duplicates that are shown as smaller symbols around the average.

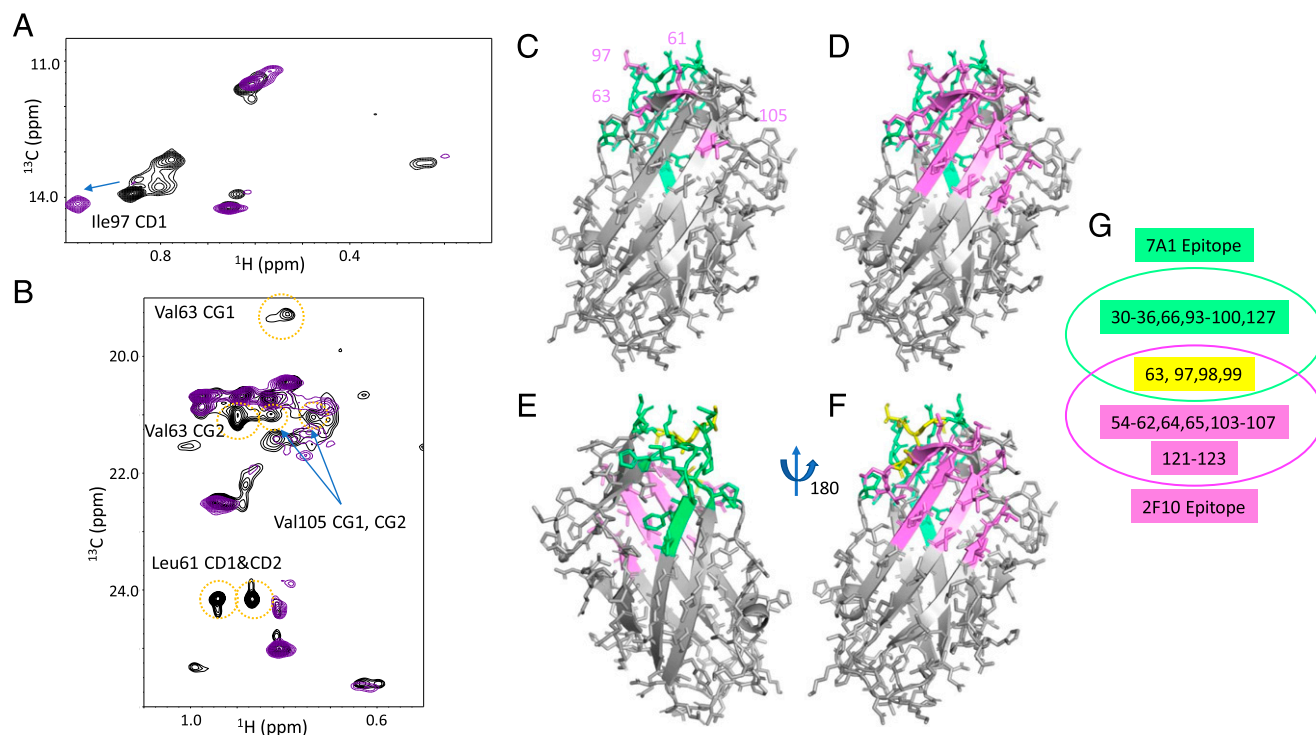


FIGURE 4. Interactions of Der p 2 with hIgE 2F10 and mIgG 7A1. (A) and (B) show NMR $^1\text{H}^{13}\text{C}$ HMQC data of methyl-labeled Der p 2 alone (black contours) and Der p 2 in complex with 2F10 (purple contours). (A) is the Ile region and (B) is Val and Leu region. Arrows indicate shifts, and dotted circles indicate resonances absent in the complex. (C) Der p 2. The 7A1 epitope is colored pale green, as previously identified (23). Residues identified in the 2F10 interactions are indicated in violet. (D) Adjacent residues proposed to be in the 2F10 epitope are colored violet. (E) and (F) show the structure of Der p 2 rotated 180° on the y-axis and colored as shown in (G) to indicate the epitopes of 7A1 and 2F10.

Fig. 6 summarizes all the proposed epitopes in this study, with a Venn diagram indicating the overlap of residues involved in each. The combination of immunoassays and NMR data identified specific residues of Der p 2 that were influenced by Ab binding and confirmed the overlap of hIgE mAbs 5D10, 1B8, and 2G1 and the mIgG mAb αDpX . NMR data also identified different residues influenced by binding of mIgG mAbs 1D8 and 7A1 and the hIgE mAb 2F10, consistent with three functionally non-overlapping epitopes. Dashed lines highlight the proposed hIgE epitopes for 5D10/1B8/2G1 and 2F10 in Fig. 6A and 6B. The methodology described in this study can be directly applied to other allergen epitopes and more generally to other Ag-Ab interactions.

Human transgenic mouse model of anaphylaxis

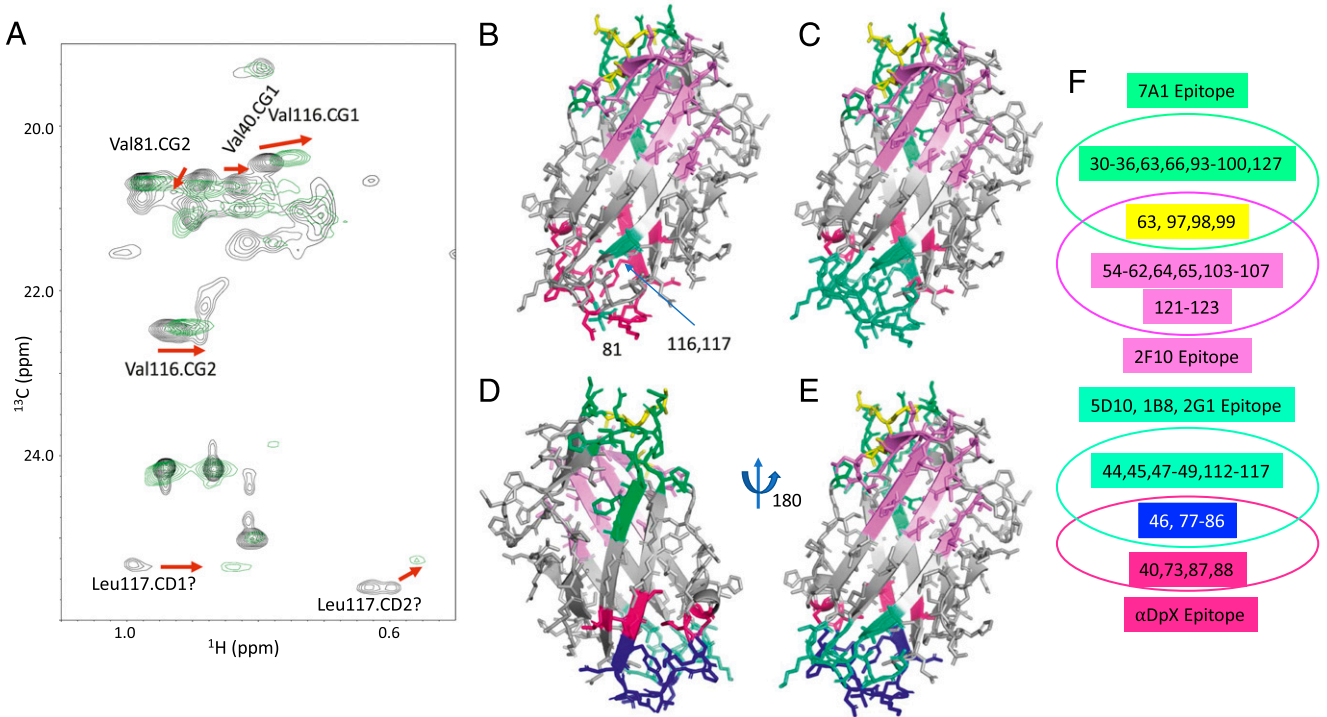
The *in vitro* immunoassays and NMR data support that the four hIgE mAbs recognize two separate epitopes defined by 5D10/1B8/2G1 and 2F10 mAbs. Theoretically, Abs recognizing nonoverlapping epitopes are sufficient to initiate allergic symptoms when they induce cross-linking of the high-affinity IgE receptor (Fc ϵ RI) by binding the allergen. To functionally test this hypothesis *in vivo*, a mouse model of passive systemic anaphylaxis was created to test Abs that bind these two epitopes. The model tested whether Fc ϵ RI was cross-linked by allergen and thus whether the purified hIgE passively transferred to the mice were capable of functioning alone and/or together. Mice were sensitized using the purified hIgE mAbs specific to Der p 2 3 d prior to challenge with 50 μg of purified nDer p 2 protein. Anaphylaxis was measured by the decrease in body temperature using implanted temperature probes. Fig. 7 shows that the hIgE mAbs that bind the predicted same antigenic site (1B8 and 2G1) do not induce a temperature decrease, whereas IgE mAbs that bind different antigenic sites (2F10 plus 2G1 or 2F10 plus 1B8) exhibited significant anaphylaxis. The temperature data from the different groups were compared by ANOVA, using a *p* value

of <0.05 to assess significance. From 15 to 35 min after challenge, both the 2F10 plus 1B8- and 2F10 plus 2G1-sensitized mice were significantly lower in temperature compared with the other three conditions, indicating anaphylaxis. After 35 min, the 2F10 plus 2G1 mice continued to be significantly lower for 85 min. The decrease in temperature displayed by the IgE groups 2F10 plus 2G1 and 2F10 plus 1B8 reflects systemic anaphylactic reactions that could have only occurred through their ability to simultaneously bind Der p 2 and cross-link Fc ϵ RI.

Discussion

Defining allergenic epitopes recognized by hIgE Abs has long been a goal of allergen research that could ultimately lead to innovations in allergy therapeutics. To our knowledge, this study presents the first epitope mapping of four human monoclonal IgE Abs to Der p 2. This is one of the most important allergens associated with mite allergy and the development of diseases such as atopic dermatitis and asthma (45). The isolation of hIgE mAbs was possible thanks to the recent development of hIgE mAb technology (27). Sequencing of the DNA encoding IgE from individual human B cells has the advantage that the expressed Abs have a natural H and L chain pairing. In addition, the isolation of mAbs allows for studying clone-specific epitopes, which is not possible using polyclonal IgE from allergic subjects. This approach is a major advance in the Ab field, given the low frequency of IgE-producing B cells in blood. One of the limitations of the agnostic approach used in our hybridoma technology is that the specific B cell type encoding the IgE Ab cannot be defined. The Croote et al. (26) study that described IgE sequences against Ara h 2 found most of the B cells were plasmablasts. However, plasmablasts do not grow in the B cell culture system used in our hybridoma methodology.

The hIgE mAbs cloned from two individuals are inferred to be relevant to the IgE response in other patients from the following



information. The epitopes of three of the hIgE coming from two different patients (5D10, 1B8, and 2G1; Table I) overlapped with mIgG αDpX , which inhibited up to 37% of IgE Ab binding to Der p 2 in eight individually tested sera (33). Similarly, a previous study reported a maximum inhibition of IgE Ab binding of 10–50% for 1D8, 7A1, and αDpX for two individuals and

pooled sera from seven mite-allergic patients (31). This indicates the relevance of these regions to the patient IgE response in general. The overlap of the three hIgE mAb epitopes could indicate that the area recognized by these Abs is immunodominant for Der p 2. Two of the IgE mAbs (1B8 and 5D10) belonged to the same patient and turned out to have nearly identical H and L chain

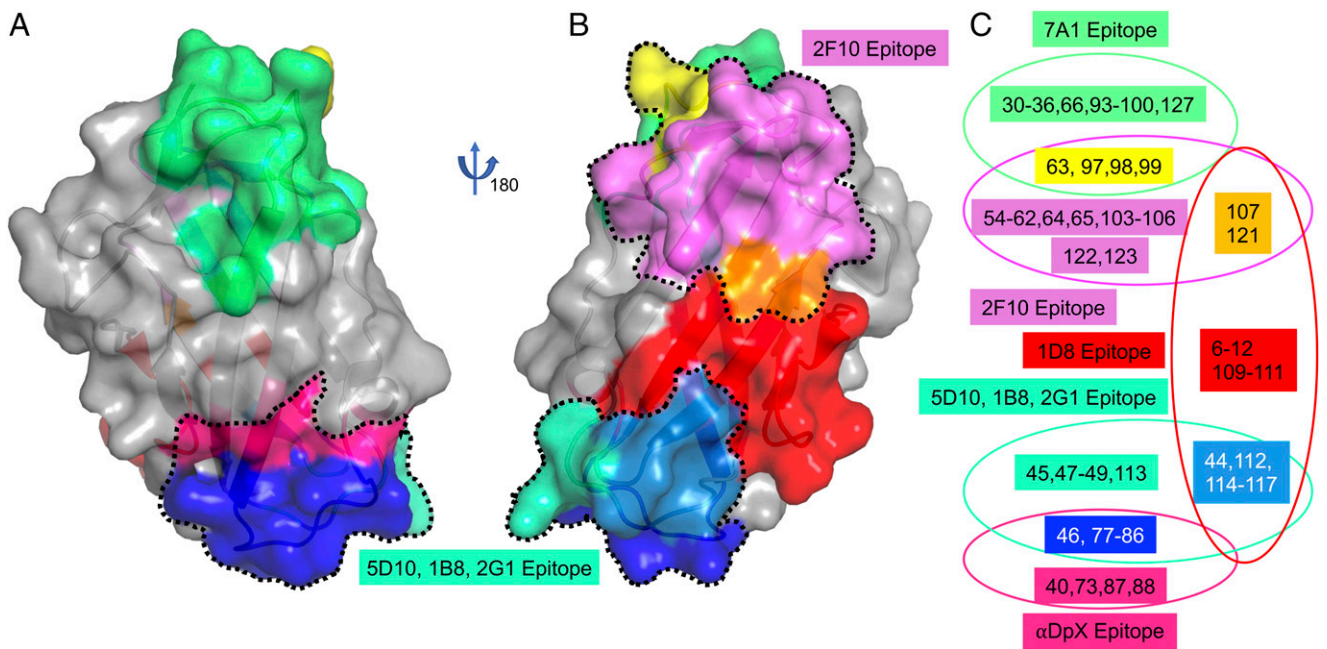
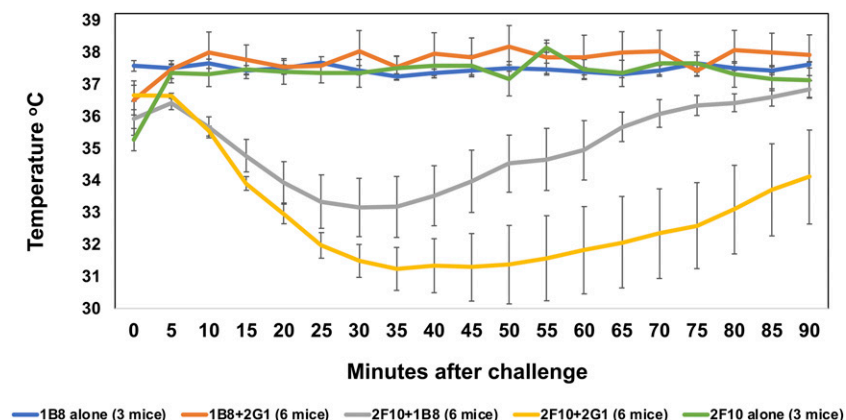


FIGURE 6. Epitope mapping summary. **(A)** and **(B)** show Der p 2 rendered as a semitransparent surface and ribbon diagram. **(C)** is a Venn diagram of the residues in the epitopes examined in this study. The coloring of the diagram is consistent with the structure rendering. Dashed lines highlight the proposed hIgE epitopes of 5D10/1B8/2G1 and 2F10.

FIGURE 7. Human FcεRI transgenic mouse anaphylaxis. Mice were sensitized using purified hIgE mAbs (see legend) specific to Der p 2 3 d prior to challenge with 50 μg of purified nDer p 2 protein. Anaphylaxis is measured as drop in body temperature using implanted temperature probes. Error bars represent the SE in the mean.



variable gene segment rearrangements. A recent study of IgG Abs generated during peanut oral immunotherapy demonstrated that three different patients generated Abs against Ara h 2 with very similar sequences (46). This suggested convergent selection by B cells and likely indicated that a specific epitope of Ara h 2 was being targeted, as seems to be the case for this Der p 2 area.

The fourth hIgE mAb bound to a site different from epitopes for the other three IgE and two IgG mAbs. The identification of the separate epitope was confirmed with a mouse model of passive systemic anaphylaxis. The decrease in temperature displayed by the mice given IgE groups 2F10 plus 2G1 and 2F10 plus 1B8 reflects significant systemic anaphylactic reactions that could have only occurred through their ability to cross-link FcεRI. This shows that the structural and immunoassay data are able to predict which Abs are capable of functioning together to activate mediator release and which are not. Because 1B8 and 2G1 bind the same site, they would be predicted to not have the ability to cross-link FcεRI in the presence of allergen.

The addition of the NMR strategy used in this study allowed the direct observation of conformational changes in specific methyl groups in the allergen when bound to the Abs. A similar strategy has been used previously in smaller Fab–Ag complexes (23, 47). Previously, detection of ^{15}N -labeled allergens (Art v 1 and Bet v 1) in complex with polyclonal IgE was attempted, but the relaxation of ^{15}N is much faster, leading to poorer signal-to-noise, hence, the changes were difficult to discern (48, 49). Additionally, the ^{15}N data in those studies was acquired with an excess of free allergen, which likely dominated the spectra (50). Methyl labeling and perdeuteration offers superior sensitivity for NMR detection of the allergen–Ab complex, which is in a m.w. range that is typically difficult to observe by NMR. This is a tradeoff between fewer observables (only Ile, Val, and Leu methyl resonances) with easier detection versus more probes (typically ^{15}N backbone atoms) at significantly lower sensitivity. With fewer observables, it is worth commenting that the proposed epitopes in this study likely include more residues than observed in crystal structure epitopes. Therefore, these regions should be considered as the framework for further experiments.

The data are likely to be useful in the design of improved therapeutics, including hypoallergens that specifically target these human epitopes. The recent study of an x-ray crystal structure of Der p 2 in complex with IgG mAb 7A1 has provided a model for how knowledge of residues involved in IgE Ab binding led to the expression of modified allergens with reduced IgE Ab binding capacity (23). The modification of only one IgE epitope is not expected to be sufficient to significantly reduce mediator release activity when other epitopes are involved in the full IgE response (51). The current study extends epitope mapping to the identification of two additional hIgE binding sites that will be further explored to create

hypoallergens. Other studies have already proposed mutations of Der p 2 that might be useful for immunotherapy (20, 42, 43, 52, 53). Reginald and Chew (54) studied 21 single alanine mutations of Der p 2 and concluded that five mutations substantially affected serum IgE binding: N10, E25, K77, K96, and E102. Of these, N10 is in the 1D8-predicted epitope, K77 is in the 5D10/1B8/2G1-predicted epitope, K96 is in the 7A1 epitope, and E102 is adjacent to the 2F10 epitope. This suggests again that the mAbs examined in this study are relevant to the patient IgE repertoire of allergic subjects. Thus, these IgE mAbs are representative of the human polyclonal IgE Ab response. The information gained from the NMR epitope mapping analysis should be useful for the design of hypoallergens for immunotherapy.

Disclosures

A.P. is an employee and M.D.C. is the president of Indoor Biotechnologies. A.P. (contact principal investigator) and M.D.C. are principal investigators of the study. The other authors have no financial conflicts of interest.

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Figure S1

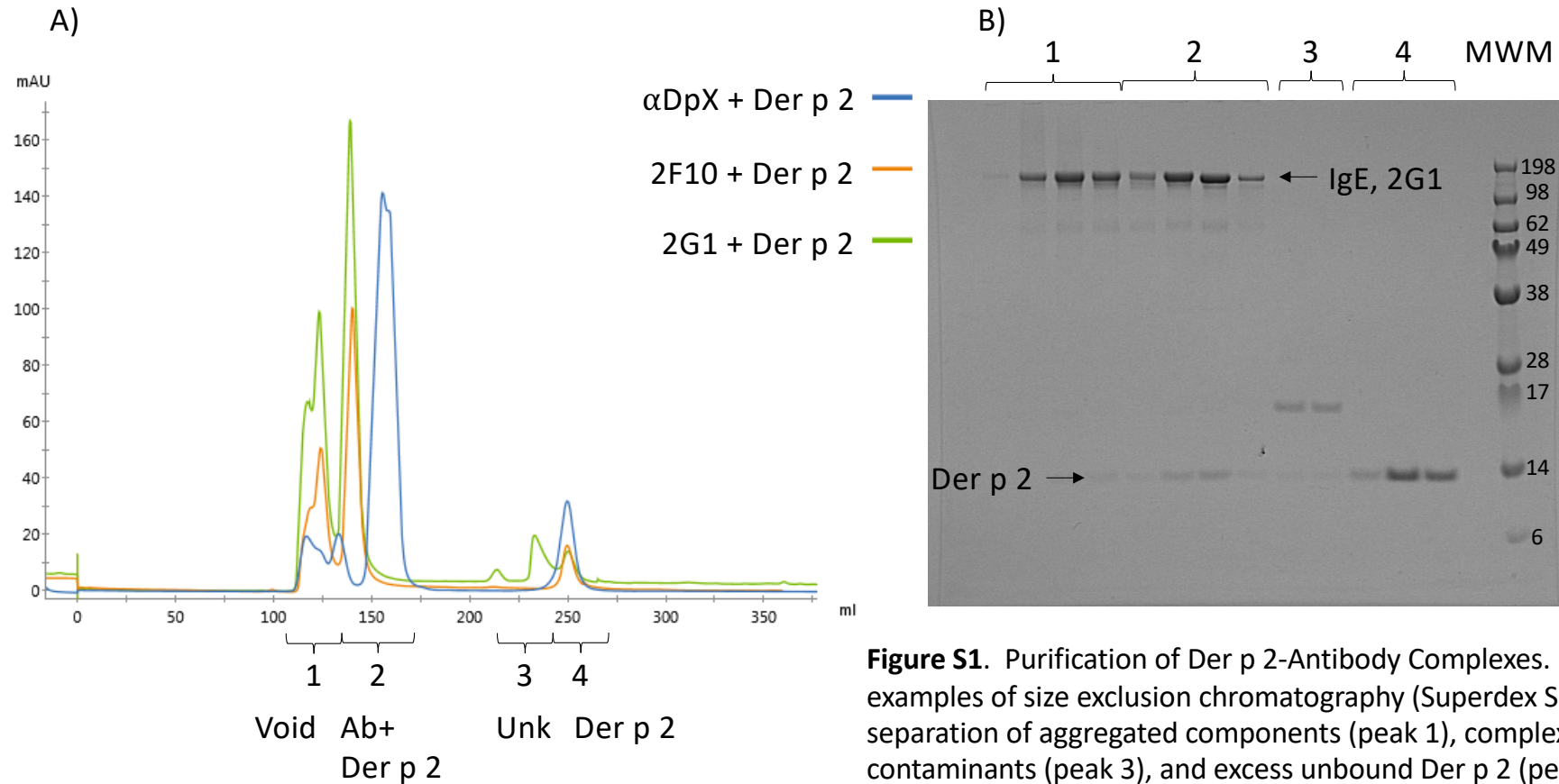


Figure S1. Purification of Der p 2-Antibody Complexes. **A)** Panel A shows examples of size exclusion chromatography (Superdex S200-26/600) separation of aggregated components (peak 1), complexes (peak 2), contaminants (peak 3), and excess unbound Der p 2 (peak 4). Note that the IgG (e.g. αDpX, 150 kDa) complexes would elute later than the IgE (e.g. 2F10, 2G1, 190 kDa) complexes. **B)** Representative SDS-PAGE of peaks from Superdex column using the 2G1 complex purification as an example. Peak 2 was concentrated for NMR samples.

Figure S2

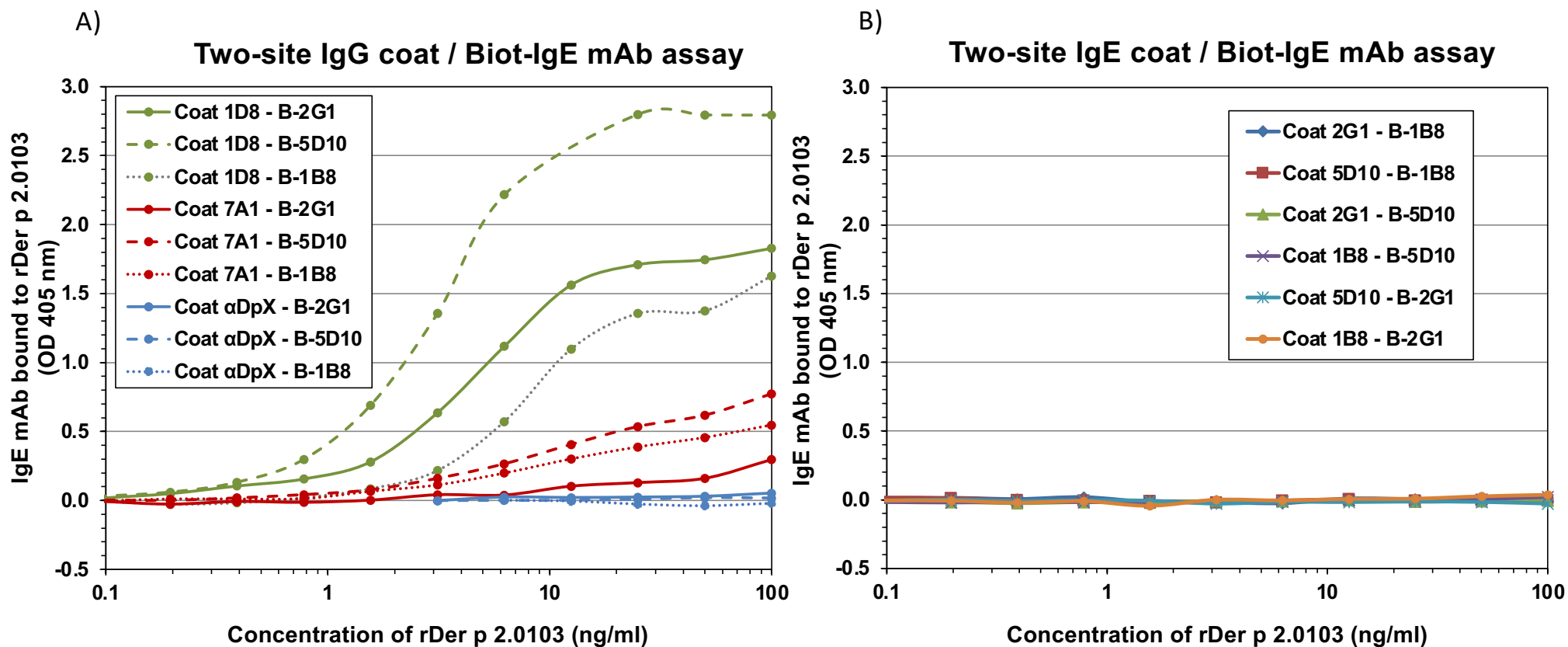


Figure S2. Two-site ELISA indicating **A)** the overlap of the three human IgE mAb 1B8, 2G1 and 5D10 with the murine mAb α-DpX, and **B)** the overlap of the three IgE mAbs 2G1, 1B8 and 5D10.

Figure S3

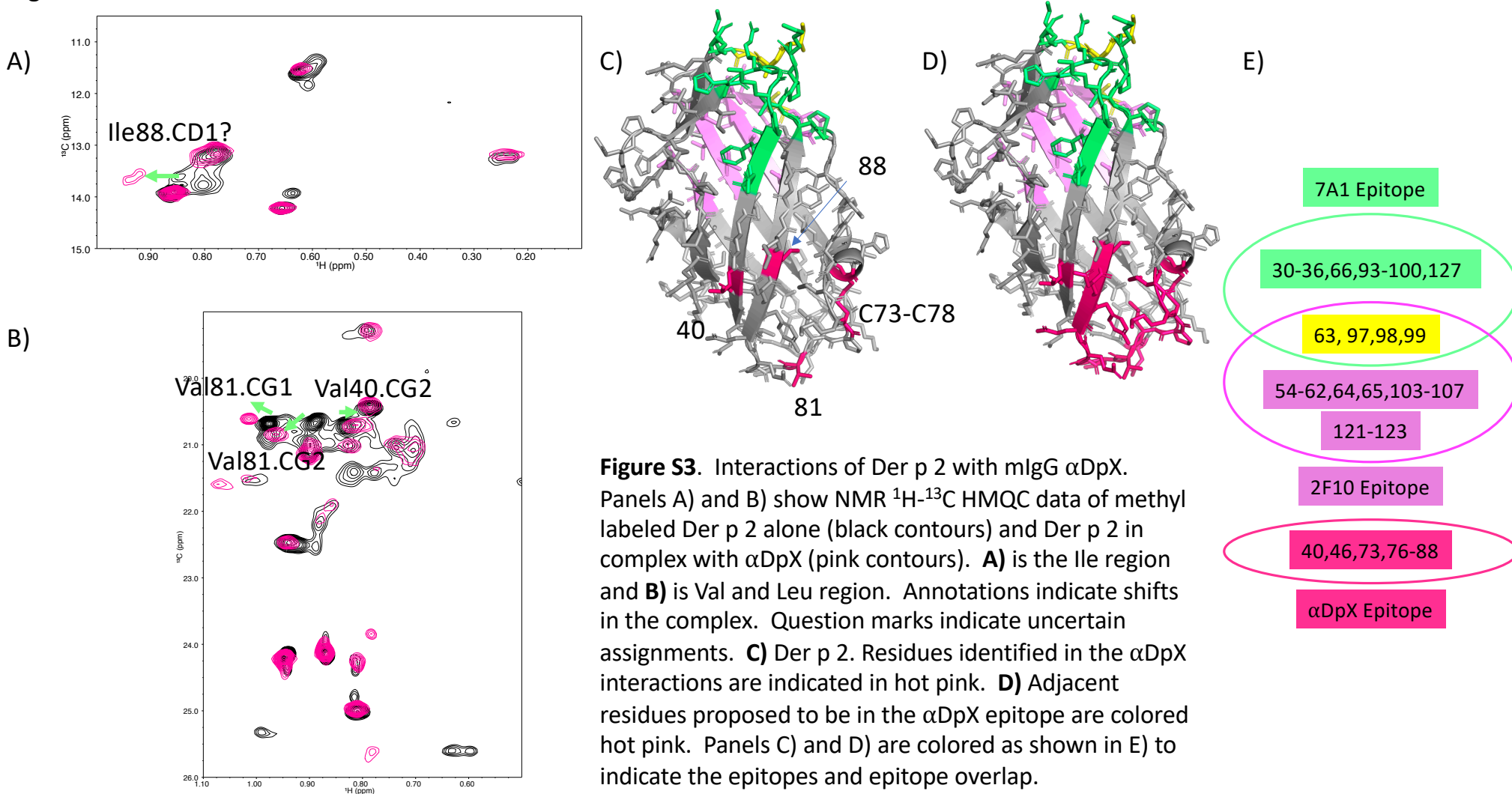


Figure S3. Interactions of Der p 2 with mIgG α DpX. Panels A) and B) show NMR ^1H - ^{13}C HMQC data of methyl labeled Der p 2 alone (black contours) and Der p 2 in complex with α DpX (pink contours). **A)** is the Ile region and **B)** is Val and Leu region. Annotations indicate shifts in the complex. Question marks indicate uncertain assignments. **C)** Der p 2. Residues identified in the α DpX interactions are indicated in hot pink. **D)** Adjacent residues proposed to be in the α DpX epitope are colored hot pink. Panels C) and D) are colored as shown in E) to indicate the epitopes and epitope overlap.

Figure S4

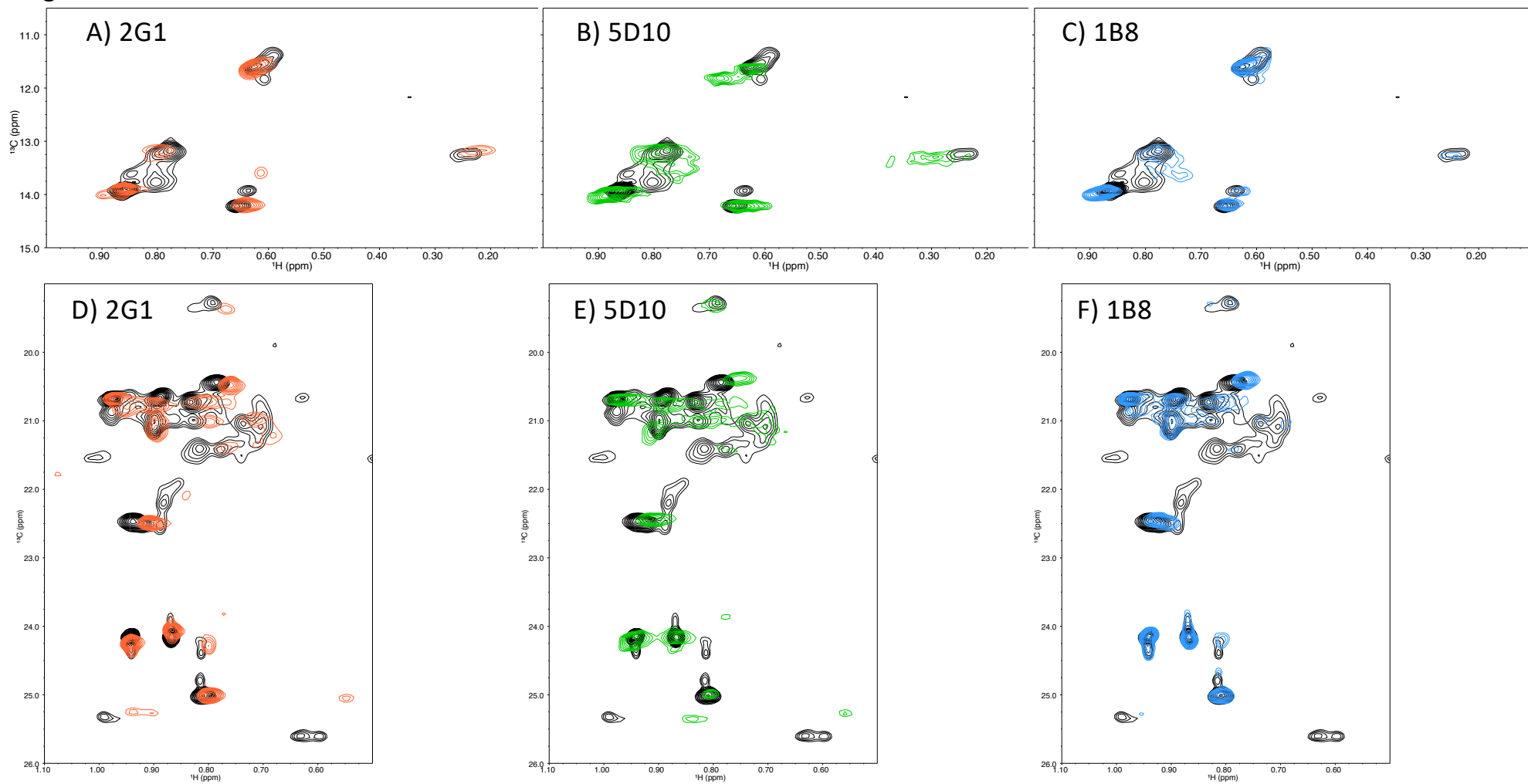


Figure S4. Interactions of Der p 2 with mIgE 2G1, 5D10, and 1B8. NMR ^1H - ^{13}C HMQC data of methyl labeled Der p 2 alone (black contours) and Der p 2 in complex with 2G1 (orange contours, A) and D)), 5D10 (green contours, B) and E)), and 1B8 (blue contours, C) and F)) are shown. A), B), and C) are the Ile region and D), E), and F) are the Val/Leu region.

Figure S5

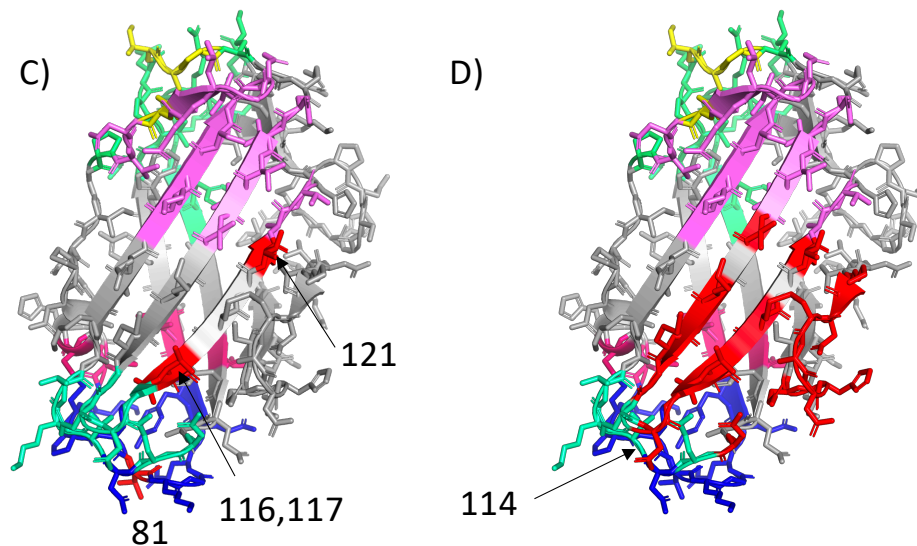
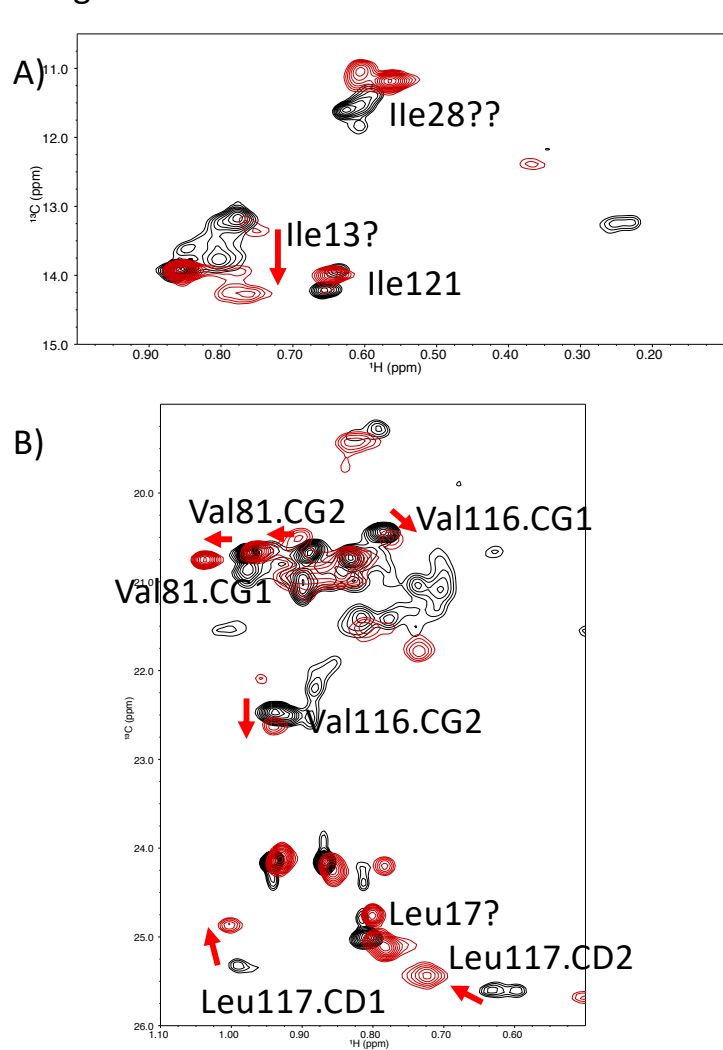


Figure S5. Interactions of Der p 2 with mIgG 1D8. Panels A) and B) show NMR ^1H - ^{13}C HMQC data of methyl labeled Der p 2 alone (black contours) and Der p 2 in complex with 1D8 (red contours). **A)** is the Ile region and **B)** is Val and Leu region. Annotations indicate shifts in the complex. Question marks indicate uncertain assignments. **C)** Der p 2. Residues identified in the 1D8 interactions are indicated in red. **D)** Adjacent residues proposed to be in the αDpX epitope are colored hot pink. Panels C) and D) are colored as shown in E) to indicate the epitopes and epitope overlap.