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Epitope Mapping by HDX-MS Elucidates the Surface Coverage of Antigens Associated with High Blocking Efficiency of Antibodies to Birch Pollen Allergen

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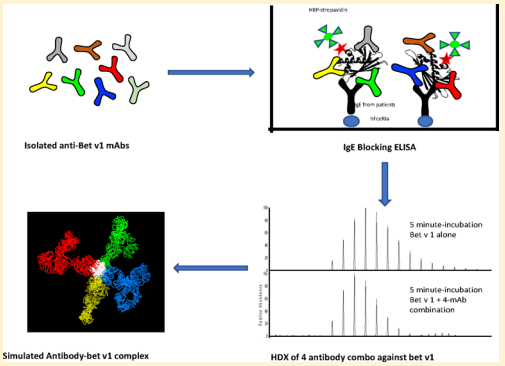
Supporting Information

ABSTRACT: Epitopes of a native pollen allergen protein, birch Bet v1, against four of the noncompeting anti-Bet v1 antibodies individually or in combination, were identified by solution-phase amide backbone H/D exchange (HDX) coupled with high-resolution Q-TOF or Orbitrap mass spectrometry. The HDX results indicates that the four anti-Bet v1 antibodies protected specific regions of Bet v1, explaining the difference in their blocking efficiency of each antibody against Bet v1 binding to polyclonal IgEs in Bet v1 allergic patients. An in-house HDX-MS system was further developed to explore the surface protection of Bet v1 in the presence of all four antibodies with 100% sequence coverage and high redundancy. The data demonstrated that four anti-Bet v1 antibodies were able to simultaneously bind to Bet v1 in solution to provide the most effective blocking for 9 of 10 tested IgE donors in an in vitro antibody-blocking assay. For the first time, we have applied HDX to elucidate the therapeutic advantage of combination antibodies compared with individual antibodies in treating Bet v1 induced allergy.



Birch pollen allergy affects an estimated 100 million individuals worldwide, with >95% of patients sensitized to the major birch allergen Bet v1.[1] Following exposure to allergen, type 1 or immediate hypersensitivity reactions are mediated by IgE antibodies that recognize distinct sites on the allergen, resulting in cross-linking and activation of Fc-epsilon receptors on immune cells and release of inflammatory mediators. Increased levels of IgG that compete with IgE for allergen binding[2] correlate with reduced allergic response following allergen-specific immunotherapy[3,4] and administration of allergen-blocking IgG monoclonal antibodies (mAbs), suggesting that Bet v1-specific IgG mAbs could be a possible treatment option.[5] However, individual IgG mAbs poorly block binding of patient-derived IgE to Bet v1, and combinations of mAbs targeting distinct epitopes are required to prevent IgE-mediated basophil degranulation.[6−8]

Site-directed mutagenesis has been commonly used for epitope mapping.[9] Alternative methods involve scanning libraries of overlapping peptides,[10,11] chimeric proteins,[12,13] and high-throughput mutagenesis.[14] High-resolution X-ray crystallography[15] can provide the most detailed representation of the epitopes, but it requires intensive effort and is not applicable to every antigen−antibody complex. For the case of Bet v1, Ferreira et al. used mutagenesis to implicate residues Thr10, Phe30, Ser57, Ser112, Ile113, and Asp125 of Bet v1 in binding to IgEs from 11 allergic subjects.[16] Similar epitopes including 11−30, 21−40, 51−70, and 111−130 of Bet v1 were reported by Katagiri et al. via peptide scanning methods. However, each of these methods has limitations in precisely defining the immunogenic epitopes and their spatial orientation against Bet v1. An alternative approach to epitope mapping uses amide hydrogen/deuterium exchange coupled with mass spectrometry (HDX-MS).[17−21] The technology has evolved from a MALDI-based approach[22] to LC/MS-based methods either with immobilized antibody[23] or direct analysis of antigen−antibody complex in solution.[21] Compared with other methods, HDX-MS is particularly useful for identifying discontinuous epitopes that are conformational in nature. Importantly, HDX examines the binding of antibody to native antigen in solution.

We previously demonstrated that a combination of two mAbs targeting the major cat allergen, Fel d 1, provided rapid, effective, and potentially long-lasting reduction in allergy symptoms in cat allergic patients.[24] Herein, a similar strategy was adopted to preclinically select potent, fully human IgG mAbs that block Bet v1 binding to IgE. Whereas individual mAbs blocked IgE binding for a minority of birch allergy donors, a combination of mAbs provided >80% blocking in 9 of 10 cases. We further delineated the epitopes of the individual mAbs and the Bet v1 surface covered by effective mAb combinations. The data support our approach of using a combination of mAbs to treat birch allergy.

EXPERIMENTAL PROCEDURE

Materials. Plasma from donors allergic to birch pollen was supplied by Plasmalab International (Everett, WA). Natural

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Bet v1 protein was supplied by Indoor Biotechnologies (Charlottesville, VA). Streptavidin/horseradish peroxidase conjugate was supplied by ThermoFisher Scientific (Waltham, MA). 3,3′,5,5′-tetramethylbenzidine (TMB)/H_2O_2 substrate was supplied by BD Pharmingen (San Diego, CA). Bovine serum albumin, phosphate-buffered saline with 0.05% Tween-20, sulfuric acid, phosphate-buffered saline tablets, deuterium oxide, guanidine hydrochloride, tris(2-carboxyethyl)phosphine, sodium hydroxide, and formic acid were supplied by Millipore-Sigma (St. Louis, MO). HPLC-grade water and acetonitrile were supplied by Burdick & Jackson (Mexico City, Mexico).

Generation of Antibodies to Bet v1. VelocImmuneTM mice[25] were immunized with natural Bet v1 protein. Anti-Bet v1 antibody responses were monitored by enzyme-linked immunosorbent assay (ELISA). When the desired antibody response was reached, splenocytes were harvested, and Bet v1 reactive B cells were isolated by B cell sorting technology.[26] The light and heavy variable region genes from each antibody were isolated as described previously[27] and cloned into plasmid vectors containing a human kappa light chain constant region and human IgG4 heavy chain constant regions, respectively. The heavy chain contained a Ser228Pro substitution (EU numbering) in the hinge region, designated IgG4P, to promote stabilization of disulfide bonds between the two heavy chains. The mAbs were produced in stably transfected Chinese hamster ovary (CHO) cells. Lead mAbs were selected based on binding affinity, ability to bind noncompetitively to Bet v1, and potency in blocking IgE binding to Bet v1.

Antibody-Blocking Assay. The ability of each mAb or mAb combination to block Bet v1 binding to IgEs from birch allergic donors was determined using ELISA. Briefly, microtiter plates were coated overnight at 4 °C with 5 μg/mL human FcεR1a (the high-affinity receptor for IgE) extracellular domain protein that was produced with a C-terminal mouse Fc tag (hFcεR1a-mFc, Regeneron in-house protein). Plates were then blocked with 0.5% bovine serum albumin (BSA) for 1 h at room temperature (RT). Plasma from donors allergic to birch pollen was normalized for anti-Bet v1 IgE titers and then diluted 1:50 in phosphate-buffered saline with 0.05% Tween-20 (PBS-T), and IgEs were then captured on the receptor-coated plates. Biotin-labeled natural Bet v1 (0.1 nM) was premixed with anti-Bet v1 mAbs at a single concentration of 1 μg/mL or in 3-fold serial dilutions starting from 1 μg/mL of each mAb and incubated for 1 h at RT. The mAb/Bet v1 mixture was then added to the IgE-coated plate for 1 h. Plates were subsequently washed, and the amount of free natural Bet v1 bound to plate was detected using a streptavidin horseradish peroxidase conjugate added at a 1:10 000 dilution and incubated for 1 h at RT. Plates were washed with PBS-T in between each step of the ELISA protocol. To develop the colorimetric reaction, 3,3′,5,5′-tetramethylbenzidine (TMB)/H_2O_2 substrate was added to the plates and incubated for 20 min at RT. The reaction was stopped using 2 N sulfuric acid. The absorbance was subsequently measured on a spectrophotometer (Victor, PerkinElmer) at 450 nm. The maximum percent blocking was calculated using the highest mAb concentration used in each assay as described below

$$\text{percent blocking} = [(A_{450}\text{with no mAb}) - (A_{450}\text{with mAb})] / (A_{450}\text{with no mAb}) \times 100$$

Epitope Mapping of anti-Bet v1 mAbs Using Waters HDX-MS System. The epitope of each mAb or mAb combination was determined using a Waters HDX-MS platform, which consists of a Leaptec HDX PAL system for the deuterium labeling, a Waters Acquity M-Class (Auxiliary solvent manager) for the sample digestion and loading, a Waters Acquity M-Class (μBinary solvent manager) for the analytical column gradient, and a Synapt G2-Si mass spectrometer for peptide mass measurement. The D_2O labeling solution was prepared as PBS (tablet) in D_2O at pD 7.4 (10 mM phosphate buffer, 140 mM NaCl, and 3 mM KCl, equivalent to pH 7.0 at 25 °C). For deuterium labeling, 3.8 μL of natural Bet v1 alone (28 pmol/μL), Bet v1 premixed with each mAb, or a mixture of four mAbs with Bet v1 in equimolar concentrations was incubated with 56.2 μL of D_2O labeling solution for various time points (e.g., undeuterated control = 0 s, labeled for 1, 5, and 20 min) at room temperature. The deuteration was quenched by transferring 50 μL of the sample to 50 μL of prechilled 0.2 M tris(2-carboxyethyl)-phosphine (TCEP) and 6 M guanidine chloride, pH 2.5 (quench buffer), and the mixed sample was incubated at 1.0 °C for 2 min. The quenched sample was then injected into a Waters HDX Manager for online digestion using an immobilized pepsin/protease XIII column (NovaBioassays). The digested peptides were trapped onto a VanGuard precolumn (ACQUITY UPLC BEH C18 1.7 μm, 2.1 × 5 mm, Waters) at 0 °C and eluted to an analytical column (ACQUITY UPLC BEH C18 1.7 μm, 1.0 × 50 mm, Waters) for chromatographic separation using a 9 min gradient separation of 5−40% B (mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile). The main MS parameters were set as follows: analyzer mode, sensitivity mode; capillary voltage, 2.5 kV; source temperature, 80°; m/z range, 50−1700; MS1 and MSe scan time, 0.5 s; ramping MSe collision energy, 19 to 41 eV and lock mass scan rate, once per 30 s.

For identification of Bet v1 peptides, LC/MS^E data from an undeuterated sample were processed and searched against a database including Bet v1 and its randomized sequence via Waters ProteinLynx Global Server (PLGS) software. The identified peptides were imported to DynamX software and filtered by two criteria: (1) minimum products per amino acid: 0.3; and (2) replication file threshold: 3.[28,29] The deuterium uptake of each peptide in Bet v1 alone and Bet v1 with mAb was calculated based on centroid mass value at each time point with aligned retention time and high mass accuracy (<10 ppm) from triplicates. Glufib peptide (m/z 785.84) was used for lock mass spray at a scan frequency of once per 0.5 min. Any region of Bet v1 with significant protection upon mAb binding was defined as a component of the mAb's epitope.

Epitope Mapping of a Four-mAb Combination Using a Custom HDX-MS System. The epitope coverage of the four-mAb combination was further examined using a custom HDX-MS system (Figure S1), which consists of a liquid-cooling HDX autosampler (Novabioassays) for deuterium labeling, digestion and loading, a UHPLC system (Jasco) for peptide separation, and a Q Exactive Plus Hybrid Quadrupole−Orbitrap Mass Spectrometer (Thermo) for the peptide mass measurement.

The concentrations of the samples and the deuterium labeling buffer were as described above. HDX reactions for Bet v1 itself or Bet v1 mixed with the combination of all four anti-Bet v1 mAbs were performed in a 96 well PCR plate by adding a 10 μL aliquot of each sample to 90 μL of the labeling solution (1× PBS buffer in D_2O at pD 7.4). The samples were prepared in duplicates and incubated at 0 °C for 0 (undeuterated control), 1, 5, and 20 min. After incubation, the HDX reaction was quenched by adding 100 μL of prechilled buffer

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Table 1. Single Antibodies and Antibody Combinations Blocking Bet v1 Binding to Allergen-Specific IgEs from 10 Bet v1 Allergy Donors[a]

antibodies	percent blocking (at 1 μg/mL each antibody)									
donor ID:	1	2	3	4	5	6	7	8	9	10
MAB1	44	53	43	53	67	70	**89**	**80**	**82**	62
MAB2	62	79	52	38	**81**	66	51	74	49	61
MAB3	24	17	36	23	28	24	17	23	14	23
MAB4	27	16	−46	−4	15	9	16	4	13	12
MAB1 + MAB2	79	**93**	72	77	**91**	**91**	**98**	**94**	**94**	**96**
MAB1 + MAB3	58	62	51	77	**83**	75	**93**	**87**	**87**	77
MAB1 + MAB4	61	61	−6	63	75	69	**94**	**81**	**89**	64
MAB2 + MAB3	70	**85**	73	57	**90**	78	65	**83**	60	79
MAB2 + MAB4	74	**88**	21	45	**84**	69	65	75	61	76
MAB3 + MAB4	47	31	16	28	41	29	33	29	28	47
MAB1 + MAB2 + MAB3	**83**	**92**	65	**91**	**96**	**89**	**98**	**94**	**94**	**90**
MAB1 + MAB2 + MAB4	83	**91**	16	**85**	**91**	79	**96**	**90**	**95**	78
MAB1 + MAB3 + MAB4	71	69	3	**82**	**88**	71	**95**	**86**	**93**	76
MAB2 + MAB3 + MAB4	78	**88**	39	63	**91**	79	72	**83**	68	**85**
MAB1 + MAB2 + MAB3 + MAB4	**86**	**93**	44	**89**	**95**	**84**	**98**	**94**	**97**	**87**
biotinylated Bet v1 (no antibody) at 0.1 nM	0	0	0	0	0	0	0	0	0	0
isotype control	−3	−2	−5	−4	−1	−4	−2	−4	−2	−3

[a]Allergy donor plasma was normalized to either to 1:20 (six donors), 1:10 (three donors), or 1:9 (one donor) anti-Bet v1-specific IgE titer and then diluted 1:50 for this assay. A maximum blocking over 80% (labeled in bold) was considered to be significant.

(4 M guanidine chloride, 0.6 M TCEP, adjusted to pH 2.3 with 1 M NaOH). A 100 μL aliquot of each quenched sample was injected onto an immobilized pepsin/protease XIII column (NovaBioassays) for online digestion and HPLC separation.

The digested peptides were trapped onto a 1.0 × 50 mm C8 column (NovaBioassays) at −9 °C. After the column was desalted for 3 min, the trapped peptides were eluted by a 25 min gradient with a UHPLC system (Jasco) at −9 °C. Mobile phase A was 0.5% formic acid/95% water/4.5% acetonitrile and had a melting point below −9 °C. Mobile phase B was 0.1% formic acid in acetonitrile. The column was initially equilibrated with 100% mobile phase A. Post sample injection and trapping, the gradient began with a 0.5 min hold at 0% mobile phase B followed by an increase to 8% mobile phase B over 2.5 min and an increase to 28% mobile phase B over the next 14 min for peptide separation. The column was then washed by an increase to 95% mobile phase B over 3 min following by a decrease to 2% mobile phase B in 0.5 min. The gradient ended with a 4.5 min hold at 2% mobile phase B. The separated peptides were analyzed by mass spectrometry in LC/MS/MS or LC/MS modes. The main MS parameters were set as follows: resolving power, 70 000 (m/z 200) in MS scan and 35 000 in MS/MS scan; spray voltage, 3.8 kV; capillary temperature, 325°; AGC target, 3e6 in MS scan and 1e5 in MS/MS scan; maximum injection time, 100 ms for MS scan and 50 ms for MS/MS scan; MS/MS loop count, 6; m/z range, 300−1500; and stepped NCE; 15−26−36.The LC/MS/MS data of undeuterated Bet v1 sample were searched against a database including Bet v1 and its randomized sequence using a Byonic search engine (Protein Metrics). The identified peptide list was then imported together with the LC/MS data from all deuterated samples into the HDX workbench software (Scripps Florida, version 3.3) to calculate the deuterium uptake level of individual peptides in each sample.

RESULTS AND DISCUSSION

mAb Selection, IgE-Blocking Activity, and Combination Treatment. One of the challenges in developing IgG-based therapy targeting Bet v1 is to block binding of the polyclonal IgE response in patients. Since the IgE repertoire could be highly variable across patients, it is critical to start with a diversified panel of anti-Bet v1 mAbs to select the best combination for therapeutic purpose. In our study, we generated a panel of fully human anti-Bet v1 mAbs in VelocImmune mice.[25,30] Twenty mAbs were selected for detailed evaluation based on their epitope diversity by cross-competition for binding to Bet v1, affinities, and blocking properties. Their potency in blocking the binding of Bet v1 to IgE from two donors with birch allergy was evaluated in an IgE-blocking assay. Consistent with previous reports, individual mAbs were able to partially block IgE binding to Bet v1 by 8.5−64% (Table S1), which underlines the polyclonality of the IgEs. Subsequently, seven mAbs that exhibited >40% inhibition were tested in all possible combinations for blocking activity against IgEs from five allergic donors. Through a series of such combinatorial screens, a subset of four noncompeting anti-Bet v1 antibodies (MAB1, MAB2, MAB3, and MAB4) were selected for detailed characterization. Interestingly, while individually MAB1 and MAB2 could block >80% Bet v1 binding to IgE in three and one patients, respectively, and no significant inhibition was observed for either MAB3 or MAB4 individually, the combination of these four mAbs exhibited over 80% blocking for 9 of 10 donors, suggesting the feasibility of the mAb cocktail approach as a therapeutic modality for birch allergy (Table 1). In addition, two- (MAB1 + MAB2) and three- (MAB1 + MAB2 + MAB3) mAb combinations afforded >75% blocking for 9 of 10 donors.

Dose-dependent activity of each mAb and their combinations were further assessed for Donor 5 (Figure 1). A significant improvement in maximum blocking activity was observed upon combining MAB1 and MAB2. While MAB3 and MAB4 did not show any blocking activity when used individually, the addition of either or both to MAB1 and MAB2 did not provide any additional benefits of IgE blocking, suggesting a limited IgE repertoire in this donor. Since the four antibodies do not cross-compete for Bet v1 binding, the differences in their blocking potency are likely attributed to their different epitopes against Bet v1. To address these questions, HDX-MS was

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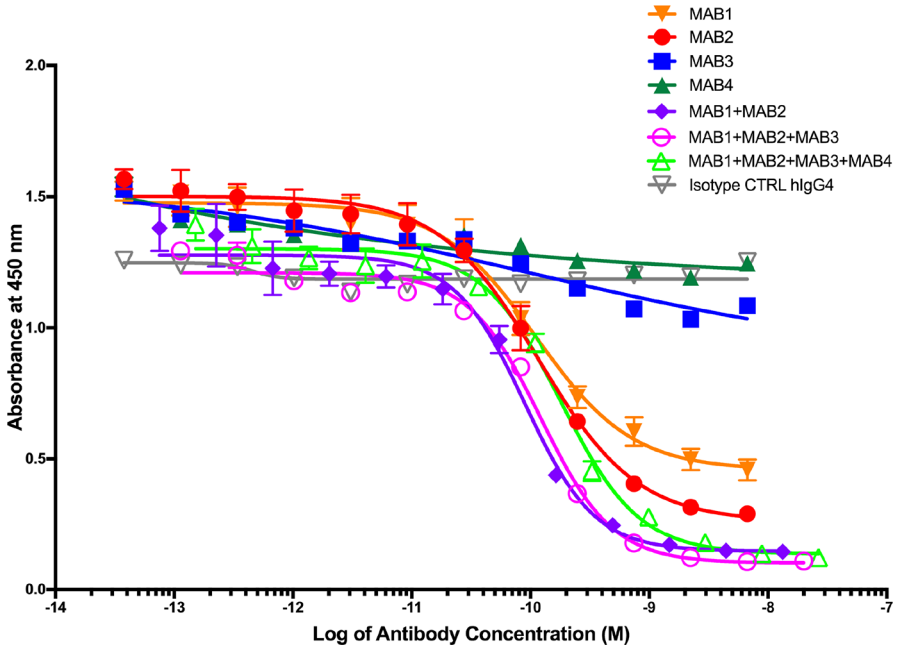

Figure 1. Dose-dependent response of individual mAbs and mAb combinations in blocking Bet v1 binding to IgE from Donor 5.

applied to investigate the epitopes of the four anti-Bet v1 mAbs both individually and in combination.

HDX-MS Epitope Mapping of Individual anti-Bet v1 mAbs. Bet v1 comprises a seven stranded antiparallel β-sheet and a long C-terminal α-helix. The secondary structure motifs adapted from the published structure[31] are labeled on the model shown in Figure 2a. The epitope of each mAb was identified by HDX-MS using the Waters HDX system with a standard procedure of an 8 min LC/MS run. The protected regions of Bet v1 by each anti-Bet v1 antibody were summarized in Figure 2 with detailed uptake profiles of representative peptides shown in Figure 3. For each of the antibodies, three peptides were selected to represent the epitope regions. For all of the identified epitopes, a significant reduction of D-uptake level was observed for the corresponding peptides upon antibody binding. An average of 30 peptides from Bet v1 with ~90% sequence coverage was achieved in each experiment. The differential heat map was generated by Dynamx 3.0, and the peptides showing significant protection upon mAb binding are shown in Figure S2. The D-uptake vs time plots for all peptides for MAB1, MAB2, MAB3, and MAB4 experiments are summarized in Figure S3a–d. The regions of Bet v1 with the strongest protection/binding against each antibody were defined as the primary epitope.

MAB1 has a specific epitope against region 23–43, as supported by multiple overlapping peptides (Figures 2b and 3a). The primary epitope of MAB2 is against 44–56 of Bet v1, which covers β2 and β3, while a modest protection was also observed from region 23–43 covering α2 and β2 (Figures 2c and 3b). As shown in Figures 2d and 3c, the epitope for MAB3 includes the N-terminus of the protein from 2 to 19 and 23 to 43 represented by multiple overlapping peptides, covering β1, α1, α2, and β2. As shown in Figure 2e, MAB4 induced protection was detected from three discontinuous regions, represented by three peptides from Figure 3d. Region 57–70, which covers the loop connecting β3 and β4, was detected as the primary epitope, while region 81–96 covering the β5 sheet (orange) and region 23–43 (yellow) were also mildly protected.

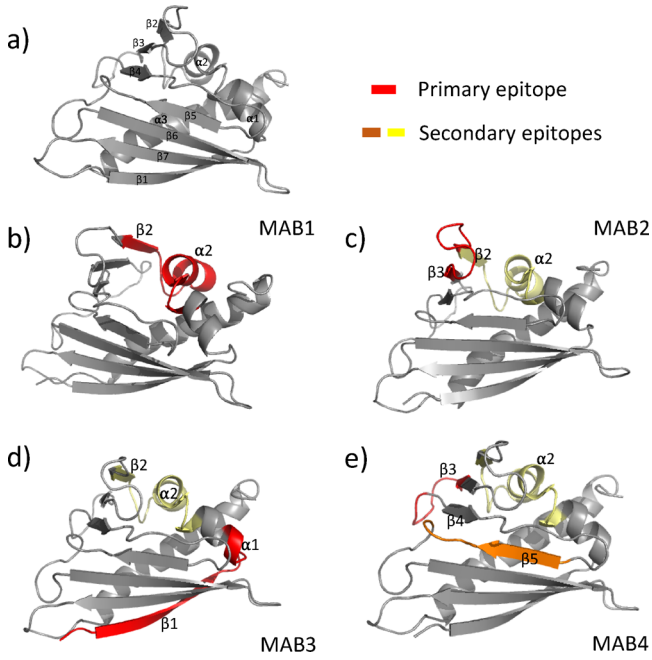

Figure 2. Bet v1 epitopes recognized by each individual mAb. The primary protected regions are labeled in red, and the secondary regions are labeled in yellow or orange. (a) the Bet v1 model is adapted from pdb 1BV1,[31] the helixes are labeled as α1 to α3, and the β sheets are labeled as β1 to β7. (b) The epitope of MAB1 is labeled in red, mainly covering α2 and β2. (c) The primary and secondary epitopes of MAB2 are labeled in red and yellow. The MAB2 primary epitope is mainly covering β3. (d) The epitopes of MAB3 are labeled in red and yellow. The primary epitope is mainly covering β1 and part of α1. (e) The epitopes of MAB4 are colored in red, orange, and yellow. Red is the primary epitope covering β4 and the loop connecting β3 and β4.

HDX-MS Epitope Mapping of the Bet v1 Surface Covered by the Four-mAb Combination. HDX-MS of Bet v1 in the presence of the combination of four mAbs was first

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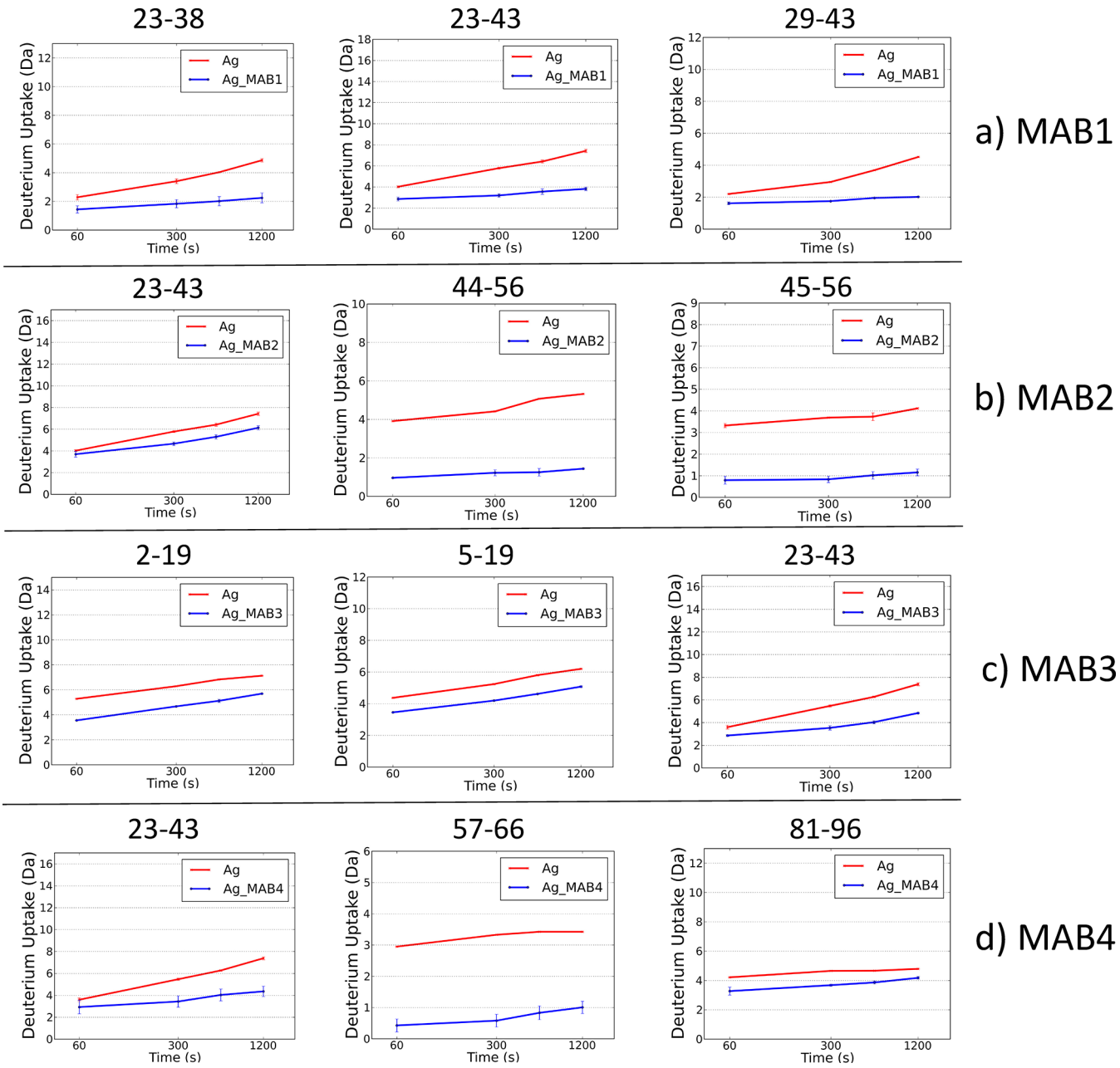

Figure 3. Peptides with different deuterium uptake profiles between Bet v1 alone (red) and Bet v1 in complex with each anti-Bet v1 mAb (blue). (a) For MAB1, the primary epitope is defined as 23 to 43, represented by peptides 23−38, 23−43, and 29−43. All three peptides show similar D-uptake protection upon MAB1 binding. (b) The primary epitope for MAB2, 44 to 56 is represented by peptides 44−56 and 45−56. Both of the peptides show greater than 2 Da D-uptake differences when binding to MAB2. Peptide 23−43 is also showing modest protection, considered as the secondary epitope of MAB2. (c) The N-terminus region 2 to 19 is protected upon MAB3 binding, with similar D-uptake profiles in peptides 2−19 and 5−19. Region 23−43 is also protected and considered as a secondary epitope for MAB3. (d) Three discontinued regions were protected by MAB4. Region 57 to 70 (peptide 57−66) is showing the largest protection and considered the primary epitope. Region 81 to 96 (peptide 81−96) and region 23−43 (peptide 23−43) are also with modest protection.

performed using a Waters HDX system coupled with Q-TOF. The regions of Bet v1 protected by combination include peptides 2−19, 23−43, 44−70, and 81−96, which cover the epitopes of each individual mAb (Figures S2e and S3e). The data suggest that all four mAbs were able to simultaneously bind to Bet v1 in solution without competing with each other. For example, two peptides from region 23−43 are showing highest protection when four mAbs are present, compared with individual antibodies (Figure S3f). However, only 19 Bet v1 peptides were identified, resulting in a 76% sequence coverage with regions 97−105 and 127−153 undetected (Figure S2e). The reason is that with the digested peptides from the mixture of four mAbs, there were significantly stronger interference peaks with reduced sequence coverage of Bet v1, less confidence in peptide identification, and a decreased accuracy in determining deuterium uptake.

To overcome these limitations, we developed an in-house HDX system (Figure S1) with a −9 °C chromatography chamber. A subzero chromatography has been reported by other studies to provide significant reduction in the systematic

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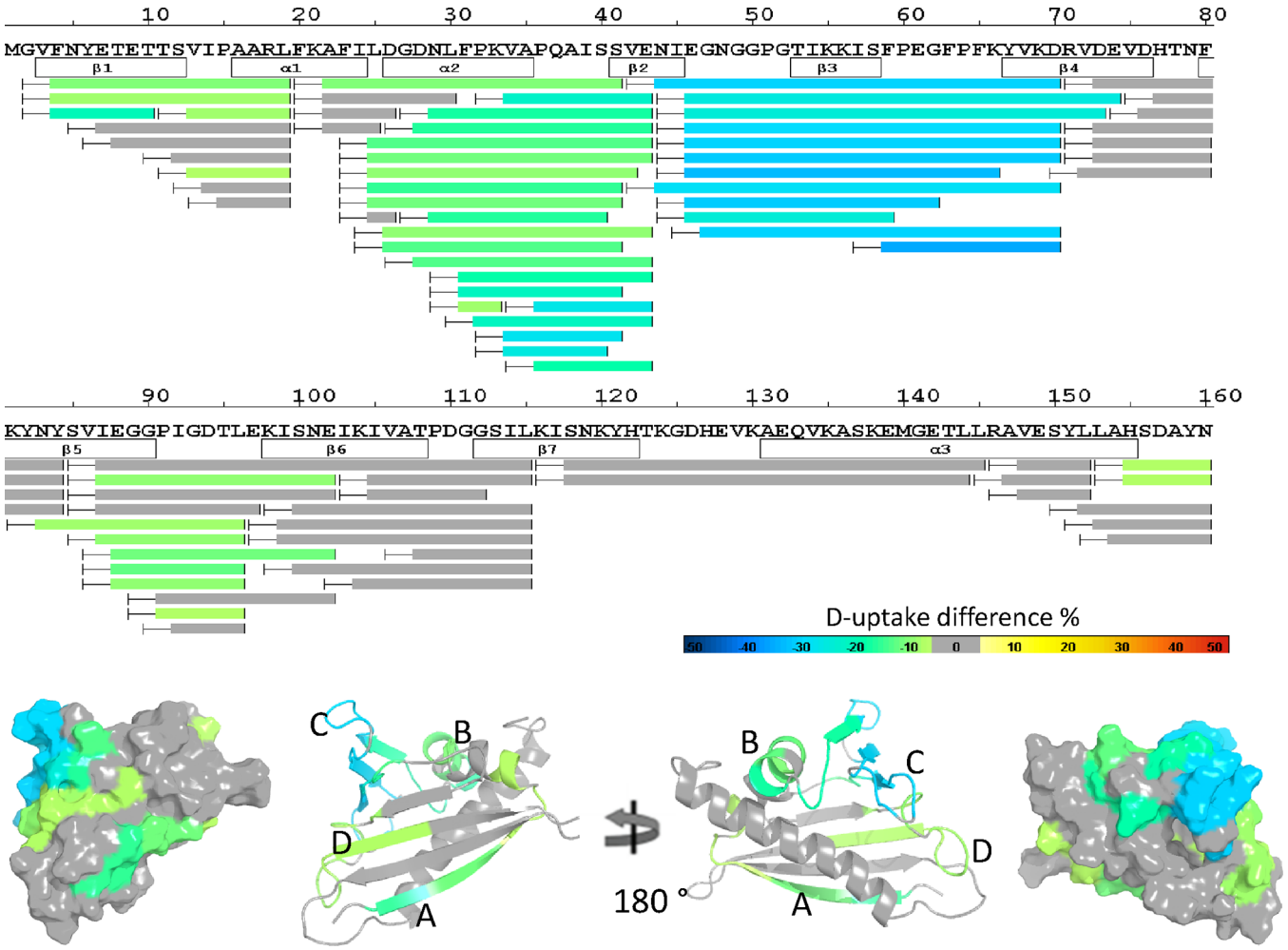

Figure 4. Heat map of HDX-MS epitope mapping results of Bet v1 in the presence of the four-mAb combination. Each line represents an identified peptide, and the darker color is corresponding to greater D-uptake protection upon antibody binding. Four major regions of the molecule, including 2−19 (A), 23−43 (B), 44−70 (C), and 81−96 (D) are showing significant D-uptake protection when binding to the four mAbs. The protected regions are also labeled on the 3D model.

back-exchange.[32,33] Therefore, a longer LC gradient (25 min) could be applied to improve the peptide separation. A Q Exactive plus mass spectrometer was also used to acquire the LC/MS/MS spectra under the data-dependent mode with increased resolution to distinguish the actual peptides from the interference peaks. Using the in-house HDX-MS system, we achieved a sequence coverage of 100% with a total number of 67 peptides identified. All D-uptake vs time plots for the combination experiment performed with the in-house system are summarized in Figure S5. The improved sequence coverage helps to better define the Bet v1 surface that is occluded by the four mAbs.

The data were consolidated, and the differential %D values for each residue were calculated using HDX workbench software (Figure 4). Consistent with the data acquired from the Waters HDX system, four major regions including 2−19 (A), 23−43 (B), 44−70 (C), and 81−96 (D) of Bet v1 were defined as binding sites for the four-mAb combination (Figure 5). Mass spectra for selected peptides representing the four major regions are shown in Figure S4. No bimodal distribution has been observed for any peptide from Bet v1, suggesting that all four antibodies were able to bind one Bet v1 molecule simultaneously. Each of the four regions was also identified as the primary epitope of each antibody. MAB1, which showed the best blocking activity as a single mAb, protects region 23−43 of Bet v1. In combination with MAB2, which primarily protects region 44−56 with a minor protection observed in region 23−43 of Bet v1, the combination of both antibodies improved the overall blocking efficiency to 7 out of 10 subjects. This observation suggests that region 23−56 including the α2, β2, and β3 domains of Bet v1 may contain immunodominant IgE epitopes. Adding MAB3 to further protect region 2−19 of Bet v1 moderately increased blocking activity for some donors relative to the MAB1 + MAB2 combination and showed moderate decreases for other donors. MAB4, which had distinct epitopes (regions 57−70 and 81−96), did not block IgE binding when used alone and modestly increased blocking when added to the three-antibody combination. Notably, the high-level blocking was observed even though the C-terminus of Bet v1 (99−161) including α3, β6, and β7 remained unprotected in the presence of all four mAbs.

Our data are consistent with the X-ray crystal structure of Bet v1 in complex with a murine anti-Bet v1 mAb, BV16, that partially inhibits binding of IgE binding to Bet v1. The structure identifies residues 42−52 of Bet v1 as the primary contact site, with additional residues in 70−97 providing secondary sites of contact.[6] Similarly, based on

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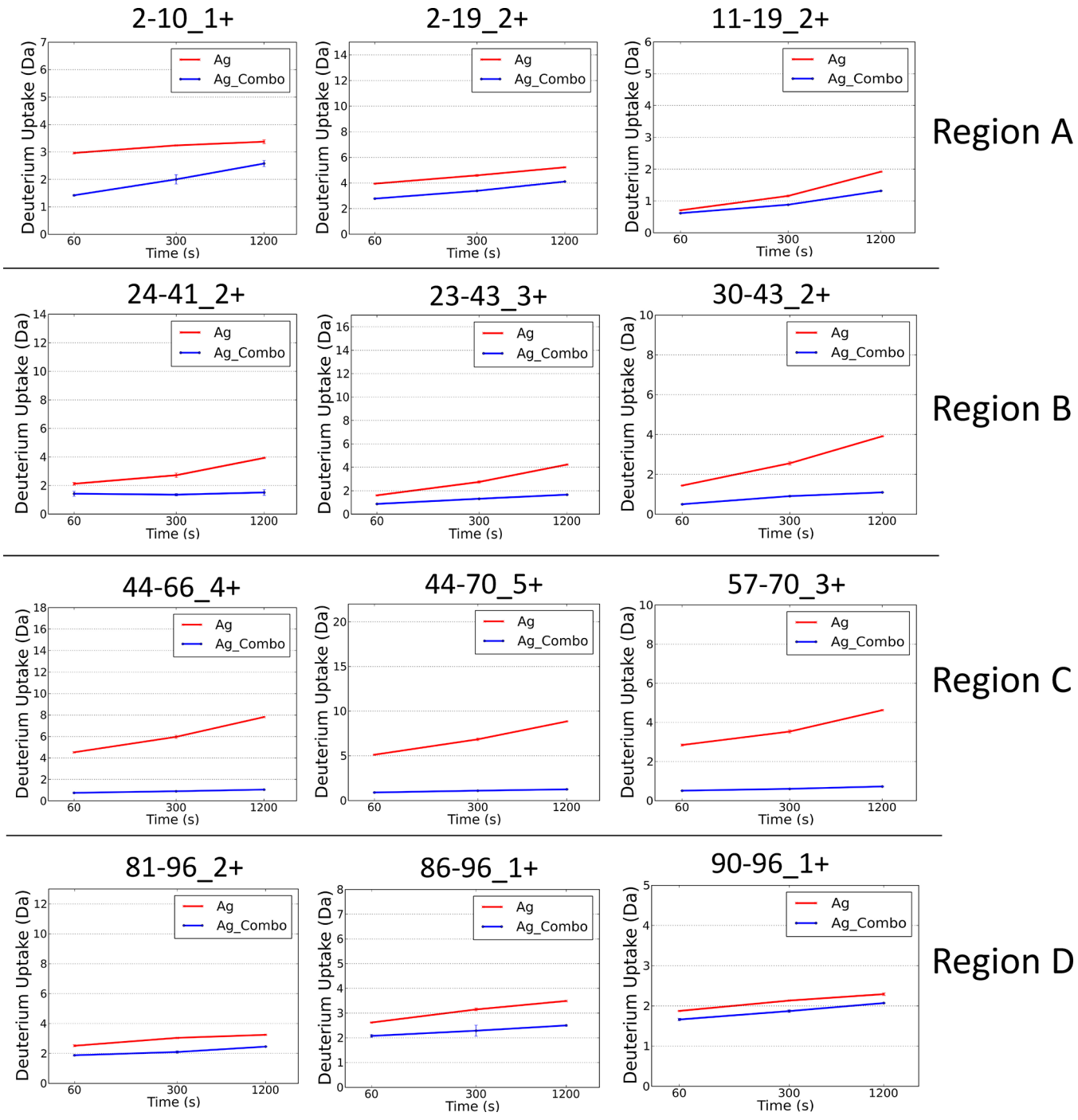

Figure 5. Peptides with different deuterium uptake profiles between Bet v1 alone (red) and Bet v1 in the presence of the four-mAb combination (blue).

the IgE-blocking activities of mAbs raised to defined Bet v1 peptides, Geiras et al. proposed that important IgE binding sites cluster within a small patch defined by residues 49−58 and 73−103 located on one face of Bet v1.[34] Since our HDX data clearly demonstrated that the four anti-Bet v1 IgGs can bind to Bet v1 simultaneously in solution, we conclude that the IgE response in the tested panel of donors was primarily directed at the Bet v1 N-terminal region, with minimal response to the C-terminus. In our recent Phase 1b study of mAbs to the cat allergen Fel d 1 (18KDa), we observed that a single dose of two anti-Fel d 1 mAbs significantly reduced clinical symptoms in response to nasal challenge, supporting that blocking only certain immunogenic regions of allergens are required to prevent IgE cross-linking. The findings help define the feasibility of allergen-specific mAb therapy of allergic diseases.

In summary, we have successfully applied HDX to characterize the interactions between Bet v1, and all four antibodies in either single or the combination format. (Figure 6). To our knowledge, this study is the first to show that IgE binding to Bet v1 could be blocked with high breadth and potency using human IgG mAbs in combination. Furthermore, for the first time, we have reported an optimized HDX system to obtain the epitopes of an antigen together with four mAbs.

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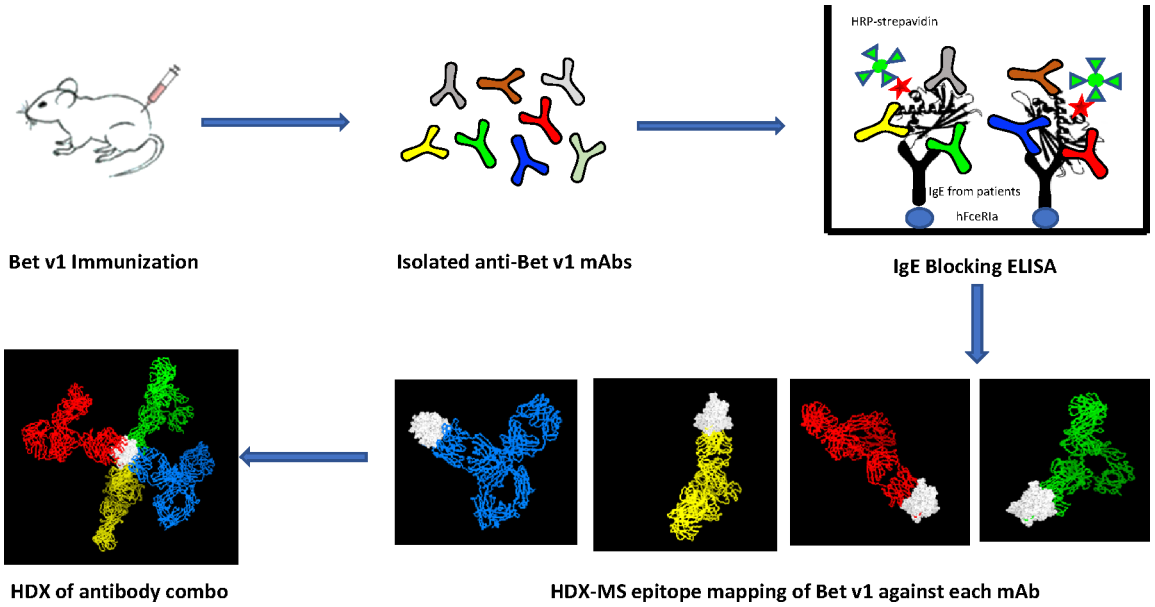

Figure 6. Overall workflow of selecting an optimized mAb combination for birch allergy. VelocImmuneTM mice were immunized with natural Bet v1 protein to produce specific antibodies against Bet v1. Monoclonal antibodies were isolated, and their blocking capabilities against serum IgEs were tested using IgE-blocking ELISA. Different formats of antibody combination were also evaluated, and a four antibody combination was identified to have the best blocking efficiency. HDX was then used to map the epitope of each antibody against Bet v1. To further understand the behavior of four antibodies and Bet v1 complex in solution, an in-house HDX system was built to investigate the Bet v1 surface protection. The data suggested that the four antibodies can bind simultaneously on Bet v1 (17 KDa) without competing with each other. The epitope information was used to simulate one of the possible antibody–antigen interactions using a docking algorithm.[35]

Determining the mAbs' epitopes not only provides molecular insight into their synergistic IgE-blocking activity but also provides a valuable approach to designing combinations of mAbs to other potent allergens.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b01864.

Additional experimental results for individual mAbs' partial blocking on IgE binding to Bet v1 (Table S1); The in-house HDX system coupled with QE plus mass spectrometer (Figure S1); the differential heat map for individual and combination mAb's HDX-MS experiments with the Waters HDX-MS system (Figure S2); D-uptake vs time plots for all peptides comparing the antigen and the antigen with MAB1, MAB2, MAB3, MAB4, and four combination antibodies based on the data set collected with the Waters system (Figure S3); Example mass spectra for the protected regions in the four combination antibody experiment performed on the in-house system (Figure S4); and D-uptake vs time plots for all peptides comparing the antigen and the antigen with four combination antibodies for the data collected with the in-house system (Figure S5) (PDF)

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Notes

The authors declare no competing financial interest.

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