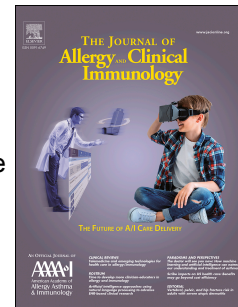


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Targeting immunodominant Bet v 1 epitopes with monoclonal antibodies prevents the birch allergic response

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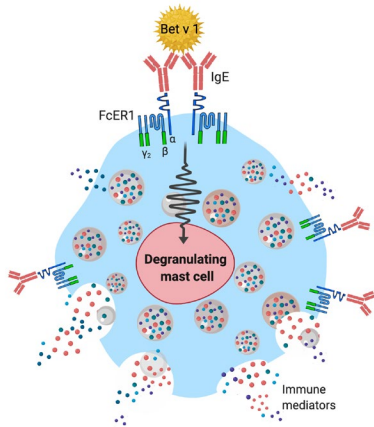
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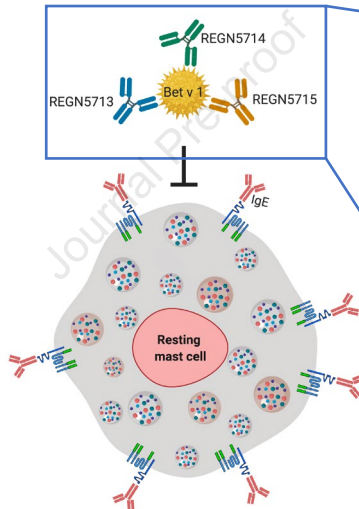


Targeting immunodominant Bet v 1 epitopes with monoclonal antibodies prevents the birch allergic response

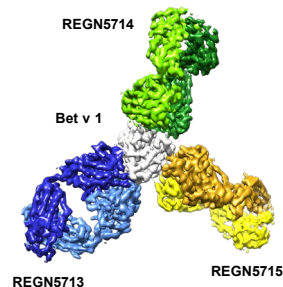
Activation of allergic signaling cascade by Bet v 1 in birch allergic subjects



REGN5713/14/15 binds to immunodominant epitopes on Bet v 1 preventing IgE engagement



Cryo-electron microscopy reveals a planar and roughly symmetrical complex formed by REGN5713/14/15 bound to Bet v 1



Substantial areas of Bet v 1 remain exposed in the complex suggesting that targeting specific epitopes of an allergen is sufficient to block the allergic response

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Abstract (246/250 words)

Background: Blocking the major cat allergen, Fel d 1, with monoclonal antibodies (mAbs) was effective in preventing an acute cat-allergic response.

Objective: We sought to extend the allergen-specific antibody approach and demonstrate that a combination of mAbs targeting Bet v 1, the immunodominant and most abundant allergenic protein in birch pollen, can prevent the birch-allergic response.

Methods: Bet v 1-specific mAbs, REGN5713, REGN5714 and REGN5715, were isolated using the VelocImmune platform. Surface plasmon resonance, X-ray crystallography and cryo-electron microscopy determined binding kinetics and structural data. Inhibition of IgE-binding, basophil activation and mast cell degranulation were assessed via blocking ELISA, flow cytometry and the passive cutaneous anaphylaxis mouse model.

Results: REGN5713, REGN5714 and REGN5715 bind with high affinity and non-competitively to Bet v 1. A cocktail of all three antibodies, REGN5713/14/15, blocks IgE binding to Bet v 1 and inhibits Bet v 1- and birch pollen extract-induced basophil activation ex-vivo and mast cell degranulation *in-vivo*. Crystal structures of the complex of Bet v 1 with Fabs of REGN5713 or REGN5715 show distinct interaction sites on Bet v 1. Cryo-electron microscopy reveals a planar and roughly symmetrical complex formed by REGN5713/14/15 bound to Bet v 1.

Conclusion: These data confirm the immunodominance of Bet v 1 in birch allergy and demonstrate blockade of the birch-allergic response with REGN5713/14/15. Structural analyses show simultaneous binding of REGN5713, REGN5714 and REGN5715 with substantial areas of Bet v 1 exposed, suggesting that targeting specific epitopes is sufficient to block the allergic response.

Capsule Summary (word limit 28/35)

A cocktail of monoclonal antibodies that bind simultaneously and non-competitively to Bet v 1, the major birch allergen, is sufficient to block initiation of the birch allergic response.

Clinical Implications (word limit: 30/30)

Bet v 1 is the immunodominant birch allergen and blockade with the Bet v 1-specific antibody cocktail, REGN5713/14/15, may be a novel and more convenient treatment approach for birch allergy.

Key words: Bet v 1; birch pollen allergy; monoclonal antibodies; IgE; X-ray crystallography; cryo-electron microscopy

Abbreviations used

AIT: Allergen immunotherapy

Å: Angstrom

Asn: Asparagine

AR: Allergic rhinitis

BPE: Birch pollen extract

C α _RMSD: Alpha carbon root mean square deviation

Cryo-EM: Cryogenic electron microscopy

ELISA: Enzyme-linked immunosorbent assay

Fab: Immunoglobulin fragment, antigen binding

Fc: Immunoglobulin fragment, crystallizable

Fc ϵ RI: human receptor for Fc-fragment of IgE

Fc ϵ R1a: mouse receptor 1a for Fc-fragment of IgE

Fcer1a^{hu/hu}: Notation for a humanized mouse in which the entire coding sequence for mouse *Fcer1a* gene has been replaced with the corresponding human sequence.

Fv: Immunoglobulin fragment, variable

HDX: Hydrogen deuterium exchange

ID: Intradermal

IgE: Immunoglobulin E

IgG: Immunoglobulin G

75	IV: Intravenous
76	KU _a /L: Kilounits of antibody per liter
77	mAb: Monoclonal antibody
78	MFI: Median fluorescence intensity
79	nBet v 1: Natural Bet v 1
80	rBet v 1: Recombinant Bet v 1
81	OAS: Oral allergy syndrome
82	PBMC: Peripheral blood mononuclear cells
83	PCA: Passive cutaneous anaphylaxis
84	rCar b 1: Recombinant Car b 1
85	SC: Subcutaneous
86	SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
87	SPR: surface plasmon resonance

INTRODUCTION

IgE-mediated allergy, or type 1 hypersensitivity, is an acute allergic reaction caused by allergen-induced crosslinking of IgE bound to Fc-epsilon receptors (FcεRs) on the surface of effector cells, which triggers the allergic cascade.¹ Pollen released from birch and related tree species (eg, alder, hazel, hornbeam) are a common cause of type 1 hypersensitivity effecting seasonal symptoms of AR and asthma-related emergency department visits.²⁻⁵ Birch allergy is estimated to affect over 100 million individuals worldwide⁶ with increasing incidence.⁷⁻¹⁰ Bet v 1, a 17 kDa protein, is the immunodominant and most abundant allergenic protein in birch pollen¹¹⁻¹⁴ with sensitization rates reaching 95% in some birch-allergic populations.¹⁵ IgE antibody levels to Bet v 1 have also been shown to be predictive of clinically relevant birch allergy.¹⁶

IgE antibodies against Bet v 1 can recognize major allergens from other tree species with structural similarity to Bet v 1. Notably, *in vitro* inhibition studies show pre-incubation of serum from individuals with pollen allergy with Bet v 1 results in a high degree of inhibition of IgE binding to alder, hazel, hornbeam and oak allergen extracts.¹⁷ Furthermore, it's estimated that 70% of individuals with birch allergy develop oral allergy syndrome (OAS), a secondary food allergy triggered by various raw fruits, vegetables, and nuts that contain allergenic proteins sharing ~50–80% homology with Bet v 1¹⁸⁻²⁰ highlighting the presence of similar IgE binding epitopes across structurally similar allergens.

Allergen immunotherapy (AIT) is a treatment option for AR when first-line pharmacotherapy is insufficient or not well tolerated. The goal of AIT is to induce desensitization through administration of increasing doses of offending allergen over time, resulting in the generation of blocking IgG, which is thought to be one of the major protective mechanisms.²¹ The utility of AIT for birch allergy is well established.^{22,23} However, there are several limitations including adverse reactions, variable efficacy, and a lengthy treatment course.^{24,25} Passive administration of pre-selected allergen-specific blocking IgG mAbs may provide for a potentially faster and more convenient treatment approach as compared to AIT or when pharmacotherapies are insufficient, and has shown promise in early clinical studies, with a proof of mechanism study demonstrating clinical benefit in patients with cat allergy using a cocktail of 2 antibodies to Fel d 1, the primary cat allergen.²¹ In this study, X-ray crystal structure analysis and Hydrogen/deuterium exchange (HDX) epitope mapping revealed distinct binding epitopes for each antibody at opposing regions of Fel d 1 that when blocked, translated to clinical symptom relief. Such data supports a model in which coverage of potentially immunodominant IgE-binding epitopes may be sufficient to prevent initiation of the allergic response and is consistent with evidence that allergen-specific blocking IgG are an integral component of the protective mechanism of AIT.²⁶⁻²⁹

In the current study, we generated fully human, monoclonal IgG4 antibodies to natural Bet v 1 (nBet v 1), and employed *in-vitro* and *in-vivo* assays to preclinically identify an antibody cocktail that potently blocks Bet v 1 binding to IgE, thus preventing IgE-mediated effector cell activation. We further describe the structural characterization of the antigen-binding fragments (Fabs) from three antibodies bound to Bet v 1. This

132 information is critical to understand antibody-allergen interactions and helps identify
133 immunodominant epitopes, that when blocked, may lead to therapeutic symptom relief.

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METHODS

Generation of REGN5713, REGN5714, and REGN5715

REGN5713, REGN5714 and REGN5715 are fully human mAbs to nBet v 1. Briefly, VeloclImmune mice^{30,31} were immunized with nBet v 1 protein and antibody responses monitored by ELISA. Upon desired antibody response being reached, splenocytes were harvested, and Bet v 1-reactive B cells were isolated by B cell sorting technology.³² The light and heavy variable region genes from each antibody were isolated as described previously^{21,33}. Each mAb is a covalent heterotetramer consisting of 2 disulfide-linked human heavy chains, covalently linked through a disulfide bond to a human kappa light chain. The IgG4 constant domain of each antibody contains a serine to proline amino acid substitution in the hinge region that reconstructs the human IgG1 hinge sequence (CPPC) to promote stabilization of disulfide bonds between the 2 heavy chains³⁴ designated IgG4^P.

Surface plasmon resonance binding studies

The kinetics of Bet v 1-specific mAbs binding to allergen were determined on a Biacore 8K or Biacore 4000 biosensor (Cytiva, formerly GE Healthcare Life Sciences) at 25°C or 37°C. The CM5 or CM4 sensor chip was immobilized with mouse anti-human Fcγ mAb (anti-hFcγ) using standard amine-coupling chemistry.³⁵ Different Bet v 1-specific mAbs were captured on anti-hFcγ immobilized surfaces and varying concentrations of allergen were injected followed by a dissociation phase. At the end of each cycle, anti-hFcγ-captured Bet v 1-specific mAbs were removed using 12-second injection of 20 mM phosphoric acid. Surface plasmon resonance (SPR) binding

responses and calculation of binding kinetics is described in this article's Online Repository at www.jacionline.org.

The simultaneous binding of REGN5713, REGN5714, and REGN5715 to nBet v 1 was performed on a Biacore T200 biosensor (Cytiva, formerly GE Healthcare Life Sciences) as described in this article's Online Repository at www.jacionline.org.

Allergen-specific blocking ELISA

Plates were coated overnight at 4°C with 5 µg/mL human FcεR1a extracellular domain protein produced with a C-terminal mouse Fc tag in house, and then blocked with buffer (0.5% BSA in PBS) for 1 hour. Plasma from individuals with birch allergy (PlasmaLab International, Everett, WA) were normalized as indicated in Table E1, further diluted 1:50, and IgE captured on the receptor-coated plates. Biotinylated nBet v 1 (0.1 nM or 1 nM) was pre-mixed with Bet v 1-specific mAbs in 3-fold serial dilutions starting at 37.7 pM for 1 hour and then added to the IgE-coated plate for 1 hour. Plates were subsequently washed and nBet v 1 detected using a streptavidin horseradish peroxidase conjugate (1:10,000 dilution, Thermo Scientific). TMB substrate (BD Biosciences, San Jose, CA) was added for 20 minutes and the reaction stopped using 2N sulfuric acid. Absorbance was measured on a spectrophotometer at 450 nM. Calculation of % blocking can be found at this article's online Repository at www.jacionline.org.

Flow cytometry-based basophil activation assessed by phosphorylated Erk

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood drawn from individuals with self-reported birch allergy (BioIVT, Hicksville, NY and HemaCare,

Northridge, CA), and stimulated according to this article's Online Repository at www.jacionline.org.

Flow cytometry data were analyzed using FlowJo (BD Biosciences, San Jose, CA) and presented as the median fluorescence intensity (MFI) of phosphorylated Erk staining within the basophil gate. Percent Max Inhibition was calculated as previously described.²¹

Animals

All procedures were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee of Regeneron Pharmaceuticals. WT Balb/c mice were purchased from Jackson Labs (Bar Harbor, ME) and *Fcer1a*^{hu/hu} mice were generated in a hybrid 129S6/C57BL/6 background.³⁶

Passive cutaneous anaphylaxis (PCA) mouse model

Antisera used for passive intradermal (ID) sensitization in wild type (WT) Balb/c mice were generated via an immunization regimen described in this article's Online Repository at www.jacionline.org.

Plasma samples from a subset of 5 individuals with birch-allergy used in the blocking ELISA were utilized for passive ID sensitization in *Fcer1a*^{hu/hu} mice (Table E2). Samples were sent to Phadia Immunology Reference Laboratory (Portage, MI) for total IgE, common silver birch- and Bet v 1-specific IgE ImmunoCAP testing. Antiserum injection was normalized to a Bet v 1-specific IgE ImmunoCAPTM of 10 KU_a/L.

PCA methodology in WT Balb/c or *Fcer1a*^{hu/hu} mice is described in this article's Online Repository at www.jacionline.org.

Crystallographic sample preparation and crystallization

The methodology for crystallographic sample preparation and crystallization, data collection and structure determination of recombinant Bet v 1 (rBet v 1) + REGN5713 Fab and rBet v 1 + REGN5715 Fab is described in this article's Online Repository at www.jacionline.org.

Cryo-EM sample preparation and data collection

The preparation of complexes of nBet v 1 and the three Fab fragments from REGN5713, REGN5714, and REGN5715 for cryogenic electron microscopy (cryo-EM) and cryo-EM data processing is described in this article's Online Repository at www.jacionline.org.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 6.0 and 7.0. For experiments in which the endpoint (ng Evans Blue Dye/mg ear tissue) was measured in 2 independent experiments, a 2-way ANOVA was used to evaluate the interaction between experimental groups (treatment) and experiment on endpoint values. If the interaction between the 2 independent variables (experimental group and experiment) was not statistically significant, data were combined and statistical significance determined by 2-way ANOVA followed by Bonferroni's multiple comparison post hoc test. A threshold of $P < .05$ was considered statistically significant.

RESULTS

Three Bet v 1-specific mAbs, REGN5713, REGN5714 and REGN5715 simultaneously bind to nBet v 1 with high affinity and block nBet v 1 binding to polyclonal human IgE

Fully human Bet v 1-specific IgG4^P mAbs were generated using the VelocImmune human antibody mouse platform.^{30,31} Since the IgE response to allergens is highly polyclonal and crosslinking of FcεRI requires recognition of more than one epitope, multiple IgE binding sites on the allergen would likely need to be blocked to prevent FcεRI crosslinking. Therefore, from the nearly 1,000 mAbs generated, 20 were characterized in various combinations based on their binding affinity, epitope diversity and ability to block Bet v 1 binding to IgE from birch allergic human plasma and 3 lead Bet v 1 mAb candidates, REGN5713, REGN5714 and REGN5715, were selected.

All three mAbs bound to nBet v 1 with high affinity (Fig 1, A) and sequentially, irrespective of order of addition (Fig 1, B, Fig E2). Binding to structurally similar allergens from related trees and foods implicated in OAS (Fig E3) revealed that all three mAbs bound to the major hornbeam allergen, Car b 1 and REGN5715 bound to major allergens from alder (Aln g 1), apple (Mal d 1), and hazel (Cor a 1) as summarized in Table E3. No binding was observed for major allergens from oak (Que a 1), carrot (Dau c 1.2), or celery (Api g 1 or Api g 2) (data not shown). Finally, we evaluated dual and triple mAb cocktails alongside each single mAb in a blocking ELISA to identify optimal mAb combinations for *in-vivo* studies. REGN5713/15 and REGN5713/14/15 were most effective in blocking nBet v 1 binding to polyclonal IgE from 16 individuals with birch

allergy achieving 91 and 94 median percent blocking across all donors (Fig 2, A–B, Table E1). Therefore, REGN5713/15 and REGN5713/14/15 were further evaluated for their capacity to block allergic effector cell activation.

Bet v 1-specific mAbs inhibit basophil activation

Using a flow cytometry-based, functional phosphoflow assay^{21,37}, basophils from 10 individuals with birch allergy showed concentration-dependent activation by both nBet v 1 and BPE. Bet v 1-specific mAbs tested individually did not sufficiently block basophil activation across multiple donors, which was consistent with the inferior capacity to block IgE binding (Fig 2). REGN5713/15 and REGN5713/14/15 demonstrated dose-dependent inhibition of basophil activation across all donors and blocked >90% of nBet v 1-induced basophil activation in 4/10 and 9/10 donors, respectively (Fig 2, C). Notably, while REGN5713/15 blocked basophil activation, there was a subset of donor samples that required addition of REGN5714 to achieve maximal blockade, suggesting that blocking an additional IgE binding epitope may more potently inhibit basophil activation across multiple donors (Fig 2, D, Fig E4). Nevertheless, it cannot be ruled out that some individuals may harbor IgE antibodies specific to areas of Bet v 1 not covered by REGN5713/14/15.

Although the relative composition of allergenic and non-allergenic proteins in birch pollen may vary across species, Bet v 1 is present in all birch species.^{11,13} Thus, we assessed the ability of Bet v 1-specific mAbs to block basophil activation induced by BPE using blood from 4 individuals with birch allergy. REGN5713/15 and REGN5713/14/15 achieved >90% blockade of BPE-induced basophil activation in 2 and 3 donors, respectively (Fig 2, C and Fig E5). Collectively, these data demonstrate that

Bet v 1-specific mAbs block basophil activation induced by nBet v 1 as well as a heterologous mix of proteins present in BPE suggesting the immunodominance of Bet v 1 in the birch allergic response.

Bet v 1-specific mAbs inhibit nBet v 1-, rCar b 1- and BPE- induced mast cell degranulation in vivo in the PCA mouse model

The PCA model measures local mast cell activation-induced vascular permeability and has been used to study hypersensitivity.^{21,38,39} To characterize the capacity of Bet v 1-specific mAbs to block nBet v 1- and BPE-induced type 1 hypersensitivity *in-vivo*, mice were passively sensitized with IgE-containing antisera generated in mice against nBet v 1 or BPE from three different birch species: *B. pendula*, *B. nigra*, and *B. populifolia*. BPE were analyzed by SDS-PAGE and ELISA and showed comparable concentrations of Bet v 1 across extracts (Fig E6). REGN5713/14/15 completely blocked nBet v 1-induced mast cell degranulation and significantly reduced BPE-induced degranulation relative to isotype control for the 3 tested birch species (Fig 3, A). Together, these data demonstrate that a combination of Bet v 1-specific mAbs inhibits both nBet v 1- and BPE-induced mast cell activation *in vivo*.

Car b 1, the major allergenic protein in hornbeam pollen, shares ~75% amino acid identity with Bet v 1 and SPR studies showed that REGN5713, REGN5714, and REGN5715 bound to rCar b 1 with nanomolar affinity (Table E3, Fig E3). Mice were sensitized using antisera generated in mice against either nBet v 1 or rCar b 1 and upon systemic allergen challenge, both REGN5713/15 and REGN5713/14/15 significantly blocked degranulation induced by either nBet v 1 or rCar b 1 relative to isotype control with more potent blocking achieved using REGN5713/14/15 as compared to

REGN5713/15 (Fig 3, *B*). These results highlight that Bet v 1 mAb cocktails block effector cell activation to a Bet v 1-related allergen suggesting cross-protection against allergens from birch related tree species.

Bet v 1-specific mAbs block mast cell degranulation induced by birch allergic human polyclonal IgE in the PCA mouse model

Allergen-specific antibody efficacy in the PCA model using mouse antisera has been shown to translate to efficacy in individuals with allergy.²¹ Utilizing *Fcer1a*^{hu/hu} mice³⁶ we optimized the model for use with human allergic donor plasma containing polyclonal allergen-specific IgE. Briefly, *Fcer1a*^{hu/hu} mice were sensitized with IgE containing plasma from 5 individuals with birch allergy (Table E2) and challenged systemically with nBet v 1. REGN5713/14/15 inhibited >90% of degranulation across 4/5 allergic donor plasma tested, while REGN5713/15 achieved the same magnitude of inhibition in 1/5 allergic donor plasma tested as summarized in Fig 4, emphasizing enhanced efficacy of the triple mAb combo. Collectively, the three-antibody cocktail, REGN5713/14/15, achieved maximal blocking efficacy across all assay formats evaluated, indicating that REGN5713/14/15 may inhibit the birch allergic response more potently than REGN5713/15.

Structural analysis of Bet v 1-antibody complexes

To understand the structure of the antibody-allergen interactions, we examined the complexes formed by Bet v 1 bound to REGN5713, REGN5714, and REGN5715 Fab fragments, singly and in combination, using X-ray crystallography and cryo-EM. X-ray crystal structures of rBet v 1 + REGN5713 Fab and rBet v 1 + REGN5715 Fab, as well

as a high-resolution cryo-EM structure of nBet v 1 bound by all 3 Fabs were obtained (Table 1 and Fig 5). Importantly, the X-ray and cryo-EM structures agreed closely with each other, with $C\alpha_RMSD < 1 \text{ \AA}$ for Bet v 1 and the Fv portion of the relevant Fab for each method. The Bet v 1 molecule in our structures shows no major difference compared to previously reported unbound Bet v 1,⁴⁰ with $C\alpha_RMSD < 1 \text{ \AA}$. REGN5713 contacts a broad flat surface of Bet v 1, using both the Fab heavy and light chains. The light chain primarily binds to a loop formed by residues 32–37 in Bet v 1, while the heavy chain binds Helix a3⁴⁰ covering residues 138–153. REGN5714 uses both heavy and light chains to bind a broad, flat epitope covering one face of the main beta sheet in Bet v 1, primarily strands b1, b5, b6, and b7. Asn 82, at the center of this epitope, is part of an N-glycosylation motif (Asn-X-Ser) which would disrupt antibody binding; however, nBet v 1 is an intracellular protein and not glycosylated.^{41,42} REGN5715 binds a tightly focused epitope: a loop formed by residues 42–50. This binding is almost exclusively through the Fab heavy chain, with only one peripheral contact made by a light chain residue. Detailed Bet v 1 interactions with each Fab are shown in Fig 6.

The cryo-EM structure of nBet v 1 bound to REGN5713/14/15 Fabs reveals that the Fabs are arranged rather symmetrically around the perimeter of Bet v 1, forming a 3-pointed star with the allergen in the center (Fig 5). The contact patches of the 3 antibodies do not overlap, consistent with our SPR data showing that all 3 antibodies simultaneously bind to Bet v 1 with no interference. The hydrophobic putative ligand-binding pocket in Bet v 1⁴³ is intact in our structure, and not perturbed by antibody binding. We observe some EM density in the pocket but cannot interpret it as any particular ligand. The Fab-nBet v 1 cryo-EM structure informed a simple model (Fig E7)

332 portraying the interaction of intact IgGs with the allergen. The Bet v 1 surface area
333 buried by the 3 Fabs is approximately 30%. Of note, space is available on nBet v 1 to
334 place the Fc and second Fab arm of each antibody. The 3 antibody Fabs mostly
335 occlude the surface of Bet v 1 around the allergen's "equator" but leave large regions of
336 exposed surface at the 2 "poles".

DISCUSSION

The treatment of allergic symptoms with potent, pre-selected, allergen-specific IgG mAbs is a potential therapeutic paradigm first evaluated with the treatment of cat hair-induced AR using mAbs specific to the major cat allergen, Fel d 1.²¹ Using a similar approach, we demonstrate that multiple IgG mAbs targeting non-overlapping epitopes of Bet v 1, the major birch allergen, effectively block Bet v 1 binding to birch allergic polyclonal IgE and prevent the Bet v 1- and BPE-induced allergic response in a series of preclinical model systems. Across all assays, REGN5713/14/15 was most effective indicating it may inhibit the birch allergic response more potently than REGN5713/15. Furthermore, X-ray crystal structures of the REGN5713 and REGN5715 Fab fragments bound to rBet v 1 presented along with cryo-EM of nBet v 1 bound to REGN5713/14/15 Fabs reveal the 3-dimensional architecture of the complete three antibody complex, providing the first published report of an allergen:antibody complex using cryo-EM.

Together with the results from the proof-of-mechanism study using a cocktail of cat allergen-specific IgG antibodies²¹, our results are consistent with studies demonstrating that allergen-specific IgG is a critical component of AIT desensitization^{27-29,44} and a protective feature of natural allergen tolerance.⁴⁵ Through direct competition, allergen-specific IgG antibodies prevent IgE engagement and initiation of the allergic cascade. Blocking IgG antibodies may also decrease T-cell activation by preventing facilitated antigen presentation.⁴⁶ Although not evaluated in the current study, it's possible that these Bet v 1-specific mAbs will also inhibit allergen-IgE complex binding to low affinity receptors (FcεRII/CD23) present on B cells as demonstrated previously using Fel d 1 antibodies.⁴⁷

In addition to influencing cellular and humoral processes, treatment of a specific allergy may lower the overall “allergic threshold”. In the polyallergic patient, exposure to one offending allergen may not be enough to elicit symptoms, however additive exposure to multiple allergens may exceed the patient’s allergy threshold resulting in effector cell activation. In this context, it’s possible that blocking one major driver of allergy, thereby reducing allergen load, may result in symptomatic relief.⁴⁸⁻⁵⁰ Furthermore, limiting antigen presentation and allergic cell activation over time may work in concert to reduce downstream effects of the early-phase allergic response leading to an overall reduction of type 2 inflammation. We report binding of REGN5713, REGN5714 and REGN5715 to Bet v 1 homologous allergens in alder, hazel and hornbeam pollen. We further show blockade of rCar b 1-induced mast cell degranulation via administration of Bet v 1 mAb cocktails suggesting the potential to provide symptomatic relief to patients sensitized to related tree species extending beyond the birch pollen season. Furthermore, REGN5715 binds to the major apple allergen, Mal d 1 presenting potential benefit of Bet v 1-specific mAbs for the subset of birch allergic patients with OAS. It will be interesting to test the clinical implications of Bet v 1 antibodies at preventing symptoms to related allergens and foods that cause OAS.

Passive administration of neutralizing allergen-specific antibodies also avoids the need for repeated administration of allergen/extract/peptides + adjuvant required with immunotherapy regimens and broadens the potential patient population to include asthmatics that are contraindicated to undergo AIT⁵¹. Pollen types in the US and Europe, including birch, have been associated with an increase in over-the-counter medicines for AR and peaks in asthma-related emergency department visits^{4,52-55}

Treatment with anti-IgE, omalizumab, has also been shown to prevent seasonal peaks in asthma exacerbations suggesting benefit to asthmatics via blockade of the IgE-mediated response.³ Therefore, a Bet v 1 therapeutic could reach beyond AR to offer symptomatic relief for asthma globally and should be further investigated.

Epitope specificities and recognition profiles of allergen-specific IgG and IgE are a current area of intense research and there is mounting evidence that most IgE antibodies recognize specific epitopes, suggesting certain areas of an allergen may be immunodominant.⁵⁶⁻⁵⁸ Previously we reported Bet v 1 surface coverage of four individual Bet v 1-specific mAbs using HDX-MS that revealed distinct, non-overlapping epitopes for each antibody.⁵⁹ Interestingly, although four mAbs are capable of binding simultaneously to Bet v 1, data presented here suggest three antibodies are sufficient to achieve near maximal blockade of allergic effector cell activation strengthening the idea that specific epitope coverage may be sufficient to prevent initiation of the allergic response. More specifically, our structural data show that simultaneous binding of REGN5713 and REGN5715 antibody fragments leaves more than half of the surface area of Bet v 1 exposed. REGN5714 binds in this region, but does not completely cover it, leaving two substantial patches of allergen uncovered. Since REGN5713/15 dual mAb cocktail reduces effector cell activation to levels near those of the triple cocktail, the structural data suggest that REGN5713/15 blocks epitope 'hot spots' on Bet v 1 that drive the majority of IgE specificity. However, the addition of REGN5714 had substantial impact in a subset of donors evaluated in the basophil assay and boosted blocking percentages for all five human plasma samples tested in the PCA model suggesting the triple mAb cocktail may provide benefit to a broader population with birch allergies.

As we begin to understand more about immunological memory and the source of pathogenic IgE,³⁶ the difficulty in isolating IgE producing cells has been realized.^{60,61} To aid in selection and development of blocking therapeutics, mapping the binding sites of blocking IgG antibodies presents an alternative approach to provide insight into “important” IgE binding sites. In this study, the X-ray crystallography and cryo-EM were used to reveal distinct amino acid contacts and enable an understanding of the conformational nature of the antibody:allergen interaction and thus the major IgE binding sites. Our structures reveal that REGN5715 binds the phosphate-binding loop (P-loop) of Bet v 1, an area also recognized by the BV16 antibody, demonstrated to be a dominant IgE-binding epitope.⁴⁰ Other important IgE binding sites have been reported including regions 29-58 and 73-103 of Bet v1.⁶² REGN5713 covers part of the 29-58 region with K33, P36 and Q37 as direct contact residues with Bet v 1 (Fig 6, C), and REGN5714 covers part of the 73–103 region making contacts with D73, E74, Y84, S100, and E102 (Fig 6, C). Bet v 1 residues P60, F63, P64 and K66 are important for clone M0418 IgE scFv binding,⁶³ but do not make direct contact with any of our antibodies. However, REGN5713 may prevent IgE from binding to this epitope by steric interference at a site away from the allergen surface.

Taken together, the Velocimmune human antibody mouse platform presents an unbiased and reproducible approach for the generation of allergen-specific blocking antibodies. Coupled with downstream binding kinetics, functional assays, and structural interrogation, we have developed a streamlined process that allows for the selection for high-affinity, non-competing, allergen-specific blocking antibodies. A combination of REGN5713, REGN5714 and REGN5715 is demonstrated to block Bet v 1 binding to the

majority of birch-specific IgE resulting in blockade of allergic effector cell activation. The utility of this approach to achieve symptomatic relief in individuals with birch allergy has been realized in a proof-of-principle clinical study evaluating the ability of REGN5713/14/15 to block acute allergic symptoms triggered by nasal provocation with birch pollen extract (Gevaert et al., co-submission). Together with the positive results achieved in a similar clinical study using 2 distinct monoclonal IgG antibodies targeting the major cat allergen, Fel d 1 (REGN1908/09) in patients with cat allergy²¹ our findings support the notion that passive administration of allergen-specific mAbs may possibly offer a new and more convenient treatment approach for allergy and presents the potential of establishing a repertoire of anti-allergen therapeutics for personalized allergy management.

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Data and materials availability: The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files or are available upon reasonable requests to the authors. The crystal and cryo-EM structures presented in this study have been deposited in the Protein Data Bank and the

461 Electron Microscopy Data Bank with PDB IDs 7MXL, 7N0U, 7N0V, and EMDB ID EMD-
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660 **TABLE 1. X-ray (A) and cryo-EM (B) data collection and refinement statistics**

A.	Bet v 1 + REGN5713 Fab	Bet v 1 + REGN5715 Fab
Data Collection Statistics		
Space group	P3 ₁ 21	P6 ₁ 22
Unit cell (Å / °)	140.766 140.766 94.294 / 90.0 90.0 120.0	133.105 133.105 145.420 / 90.0 90.0 120.0
Data collection location	Beamline 5.0.2, Advanced Light Source, LBNL	Beamline 5.0.2, Advanced Light Source, LBNL
Resolution (Å)	100 - 3.0 3.05 - 3.00	100 - 3.7 3.76 - 3.70
Unique reflections	22121 1100	8574 404
R _{merge}	0.112 1.066	0.3 2.68
R _{pim}	0.039 0.373	0.037 0.384
CC _{1/2}	--- 0.73	--- 0.9
Completeness (%)	100 100	99.9 100
Average redundancy	9.3 9	68.1 46.6
I/σ(I)	17.7 1.4	23 2
Refinement Statistics		
Resolution (Å)	100 - 3.0	100 - 3.7
Reflections used (free)	20835 (1039)	7733 (380)
R factor	0.240	0.327
R _{free}	0.280	0.378
<u>RMSD</u>		
bonds (Å)	0.003	0.002
angles (°)	0.65	0.62
<u>Ramachandran statistics</u>		
Most favored (%)	98.1	97.9
Additional allowed (%)	1.9	2.1
Outliers (%)	0	0
Rotameric outliers (%)	2.4	0
Asymmetric unit contents	1 REGN5713 Fab + 1 Bet v 1	1 REGN5715 Fab + 1 Bet v 1
PDB code	7N0U	7N0V

B.	Bet v 1 – REGN5713,14,15 Fabs PDB 7MXL, EMD-24073
Data collection and processing	
Magnification	130,000
Voltage (kV)	300
Electron exposure (e ⁻ /Å ²)	44.63
Defocus range (μm)	-1.2 to -2.2
Pixel size (Å)	1.04
Symmetry imposed	C1
Map resolution (masked) (Å)	3.2
FSC threshold	0.143
Refinement	
Map sharpening <i>B</i> factor (Å)	-108.6
Model composition	
Non-hydrogen atoms	10867
Protein residues	1422
Ligands	0
CC map vs model (%) (mask)	0.85
R.m.s. deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.624
Validation	
MolProbity score	1.95
Clashscore	11.24
Poor rotamers (%)	0.08
Ramachandran plot	
Favored (%)	94.29
Allowed (%)	5.71
Disallowed (%)	0.00

Figure Legends

FIG 1 SPR binding studies showing REGN5713, REGN5714 and REGN5715 has high affinity to nBet v 1 and can simultaneously bind to nBet v 1 in a non-competitive manner. (A) SPR binding sensorgrams and individual binding kinetic parameters for nBet v 1 binding to anti-hFcγ-captured REGN5713, REGN5714, or REGN5715 at 37°C on a Biacore 8K biosensor. Multiple concentrations of nBet v 1 ranging from 0.78 nM to 25.0 nM (2-fold serial dilution) were injected in duplicate over REGN5713, REGN5714, or REGN5715 surfaces for 3 minutes followed by a 20-minute dissociation phase. The real time binding sensorgrams are shown in black and the fits generated by globally fitting binding sensorgrams to a 1:1 binding model with mass transport limitation are shown in red. (B) Representative binding sensorgrams generated using the REGN5713 immobilized surface, showing a stepwise increase in the binding response upon sequential injection of REGN5714 and REGN5715 over the surface-immobilized REGN5713:nBet v 1 complex. REGN5713 was first coupled to the CM5 sensor surface using standard amine coupling chemistry. nBet v 1 was later injected over the sensor surface, followed by the sequential injections of the other 2 Bet v 1-specific mAbs in different orders.

FIG 2. Bet v 1-specific antibodies block Bet v 1 binding to polyclonal human IgE and inhibit Bet v 1-induced basophil activation in whole blood from birch-allergic donors. (A) Summarized median percent blocking of nBet v 1 binding to polyclonal human IgE and fraction of donors blocked at >90% compared to isotype control in the blocking ELISA. (B) Representative ELISA data from 1 of 16 donors testing antibody blocking 0.1 or 1 nM Bet v 1 binding to Bet v 1-specific polyclonal IgE. Individual

blocking percentages for all antibody combinations in all 16 donors evaluated are shown in Table E1. (C) Summarized median percent blocking of 100 pM nBet v 1- or BPE- (normalized to Bet v 1 concentration) induced basophil activation and fraction of donors blocked at >90% compared to isotype control in the phospho-Erk phosphoflow assay. (D) Representative graph of nBet v 1 and BPE dose response and anti-Bet v 1 antibody blocking of 100 pM Bet v 1-induced basophil activation from 1 of 10 birch-allergic donors in the phospho-Erk Phosphoflow assay. Individual graphs for the 9 additional donors tested and flow cytometry gating strategy are shown in Fig E4.

FIG 3 Bet v 1-specific mAbs inhibit nBet v 1-, rCar b 1- and BPE- induced mast cell degranulation *in vivo* in the PCA mouse model. (A) In the PCA model, using antisera generated in mice against three different commercially available BPE, REGN5713/14/15 significantly reduced BPE-induced mast cell degranulation by 75-98% relative to isotype control mAb (n=10 mice per group). B) Similarly, using antisera generated in mice against nBet v 1 or rCar b 1 protein, REGN5713/15 and REGN5713/14/15 significantly reduced nBet v 1- or rCar b 1-induced mast cell degranulation as compared to isotype control antibody (n=5 mice per group in two independent experiments). Statistical analysis was performed using 2-way ANOVA followed by Bonferroni's multiple comparisons post hoc test. **** signifies $P < .0001$; statistical significance relative to isotype control group is shown.

FIG 4. Bet v 1-specific mAbs block mast cell degranulation induced by birch allergic human polyclonal IgE in the PCA mouse model. *Fcer1a*^{hu/hu} mice (n = 4-5 mice per group in 2 independent experiments) were utilized to evaluate the ability of REGN5713/15 or REGN5713/14/15 to block mast cell degranulation induced by

polyclonal human IgE from 5 different birch allergic plasma donor samples. The fraction of donors blocked at >90% as compared to isotype control are summarized in the table. Statistical analysis was performed using 2-way ANOVA followed by Bonferroni's multiple comparisons post hoc test. **** signifies $P < .0001$; statistical significance relative to isotype control group is shown. An interaction between the duplicate experiments from donor 5 was found to be statistically significant, and thus statistical analysis could not be performed on the combined data.

FIG 5. Crystal and cryo-EM structures for Bet v 1 in complex with Fabs. (A) Cryo-EM structure of Bet v 1 + REGN5714 at 3.2 Å. The Fab-Bet v 1 structure is shown in cartoon representation on the left, with a closeup of the interacting region shown in the box on the right. Residues involved in the Bet v 1 + antibody interaction are shown in stick form and labeled. (B) X-ray crystal structure of Bet v 1 in complex with REGN5713 Fab at 3.0 Å. Depiction is the same as in panel A. The complete structure view on the left is aligned so that the Bet v 1 molecule has the same orientation as in panel A. (C) Crystal structure of Bet v 1 in complex with REGN5715 Fab. Depiction is same as panel A and B. D) Cryo-EM map of the Bet v 1 + 3 Fab complex and E) structural model of the same complex in cartoon representation.

FIG 6. Interactions between Bet v 1 and REGN5713, REGN5714 and REGN5715.

(A) Bet v 1 from the cryo-EM complex structure is shown in molecular surface representation, with colored patches on the white surface corresponding to contacts with an antibody (contacts defined as interatomic distances <4.5 Å for non-hydrogen atoms). Contacts are colored according to the color of the contacting Fab chain, as in Fig 5 (B) Contacts are mapped onto the amino acid sequence of Bet v 1; a dot above a

731 residue indicates a contact with an antibody chain. The colors of the dots match the
732 colors in panel A and Fig 5, C) Table showing the residues in Bet v 1 interacting with
733 each antibody, colors of the table match previous panels.

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Figure 1:

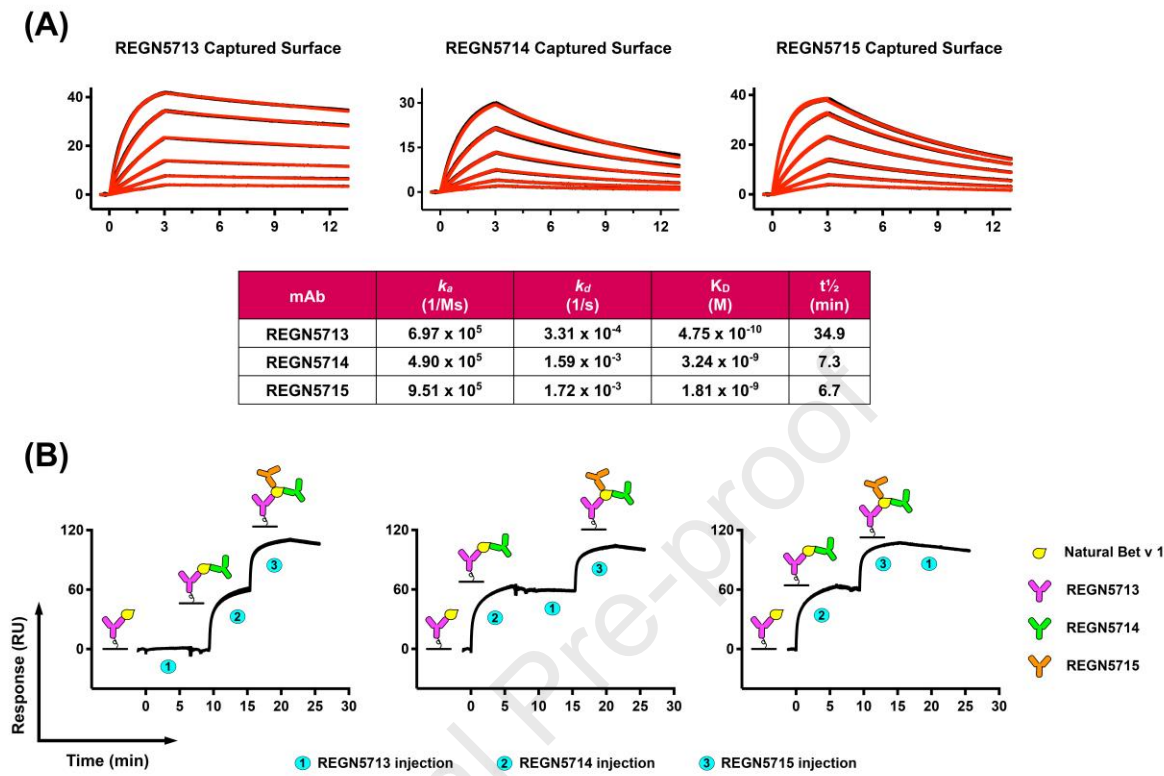
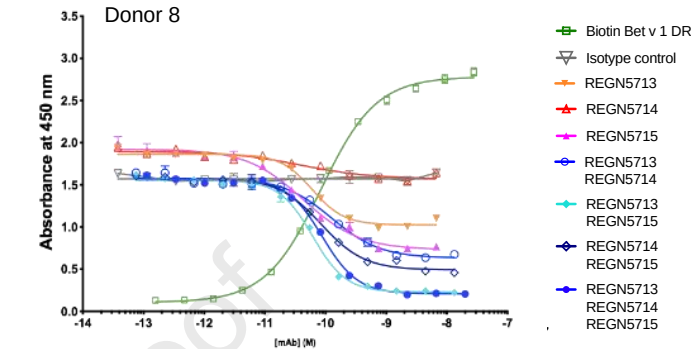


Figure 2:

A.

Blocking ELISA Assay		
Bet v 1 mAb or mAb combination	Donors Blocked at >90% vs Isotype control	Median % Blocking
REGN5713	0/16	57
REGN5714	0/16	19
REGN5715	0/16	65
REGN5713/14	1/16	73
REGN5714/15	0/16	75
REGN5713/15	10/16	91
REGN5713/14/15	12/16	94

B.



C.

Phospho-flow Basophil Activation Assay				
Bet v 1 mAb or mAb combination	Birch Pollen Extract		Bet v 1	
	Donors Blocked at >90% vs Isotype control	Median % Blocking	Donors Blocked at >90% vs Isotype control	Median % Blocking
REGN5713	0/4	73	2/10	50
REGN5714	0/4	63	1/10	55
REGN5715	1/4	83	1/10	67
REGN5713/15	2/4	91	4/10	86
REGN5713/14/15	3/4	92	9/10	97

D.

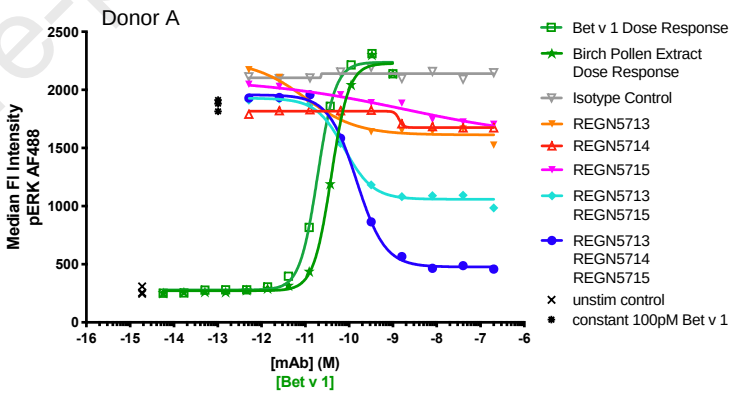


Figure 3:

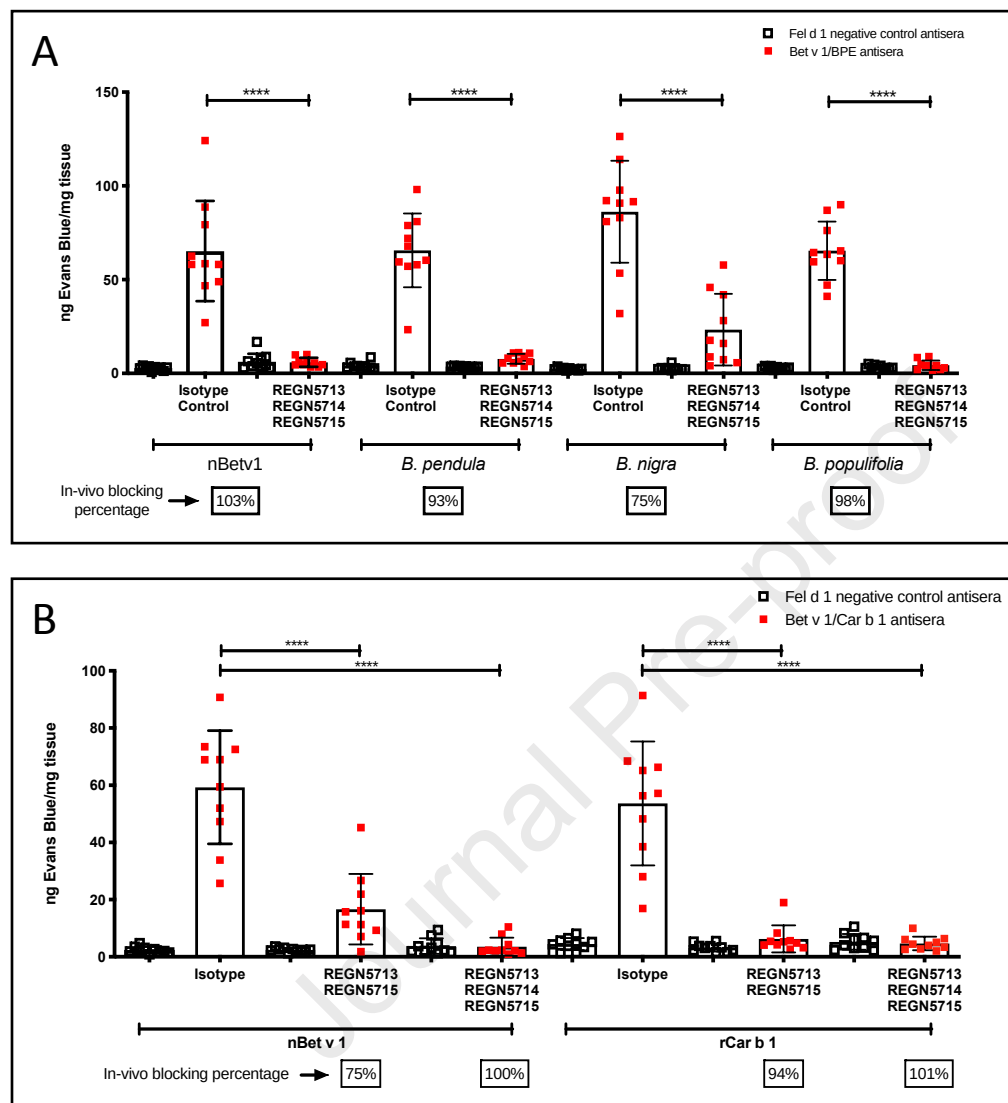


Figure 4:

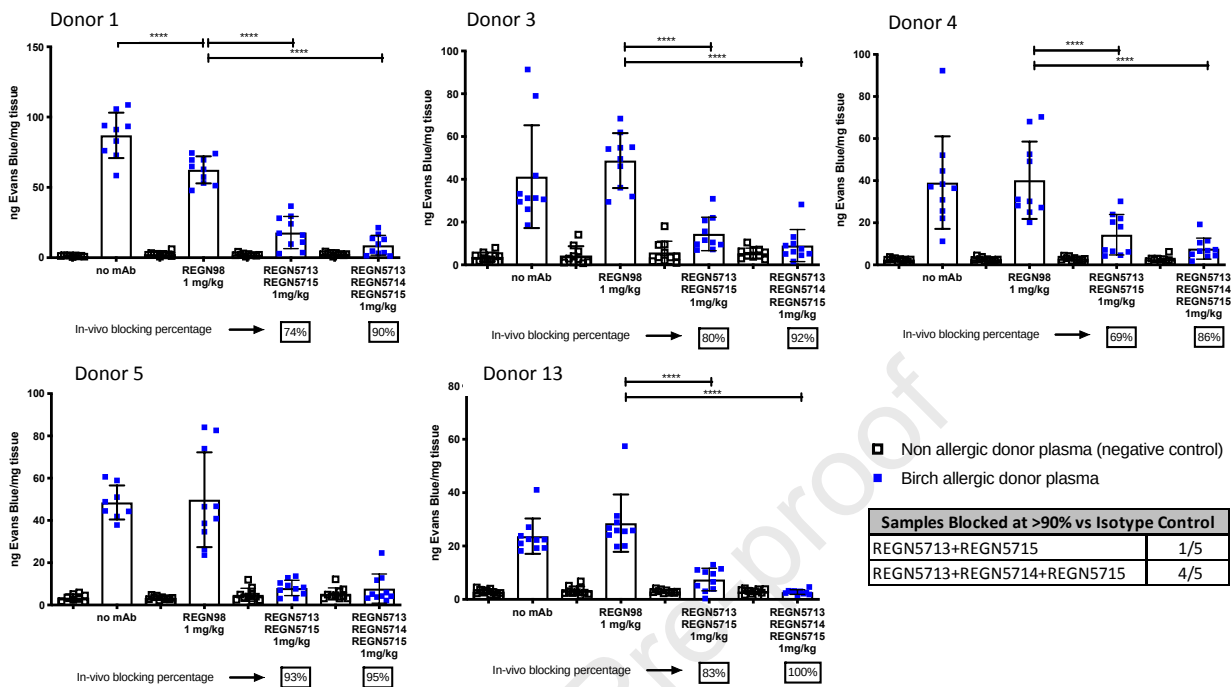


Figure 5:

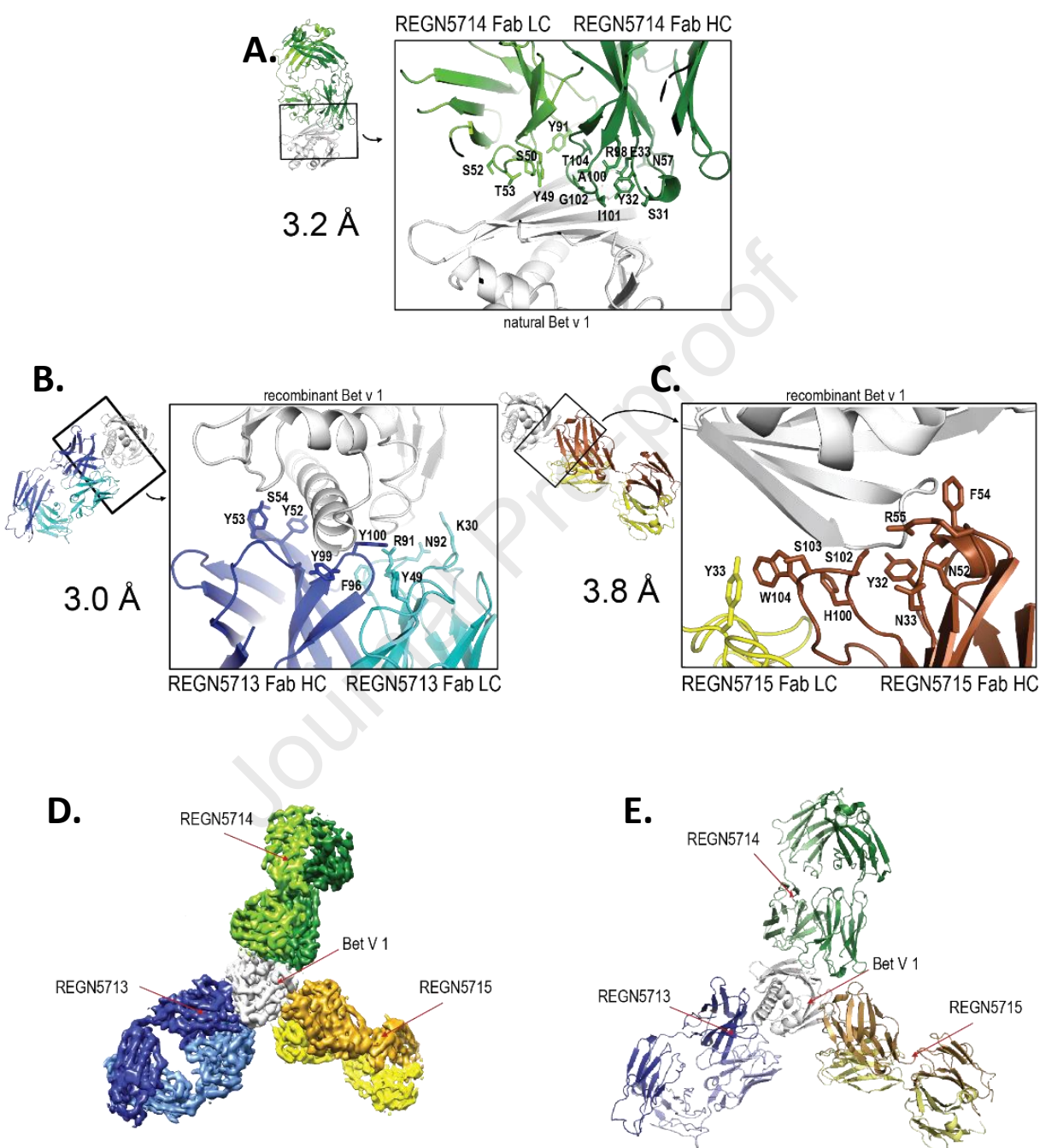
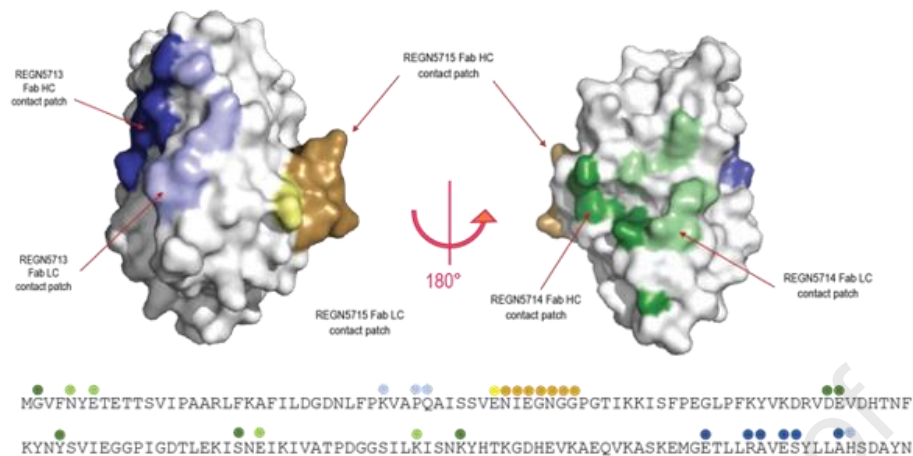


Figure 6:



C.

Bet v 1 Residue	Antibody residues interacting with this Bet v 1 residue				Bet v 1 mAb
Gly 2	Asn57 - H				REGN5714
Asn 5	Tyr91 - L	Thr104 - H			REGN5714
Glu 7	Tyr91 - L	Ser50 - L			REGN5714
Lys 33	Arg91 - L	Tyr100 - H			REGN5713
Pro 36	Phe32 - L				REGN5713
Gln 37	Arg91 - L	Asn92 - L	Tyr50 - H		REGN5713
Glu 43	Tyr33 - L	Trp104 - H			REGN5715
Asn 44	Ser102 - H	Ser103 - H			REGN5715
Ile 45	Trp104 - H				REGN5715
Asp 46	Tyr32 - H	His100 - H			REGN5715
Gly 47	His100 - H				REGN5715
Asn 48	Tyr32 - H	Asn33 - H	Asn52 - H	Arg55 - H	REGN5715
Gly 49	Phe54 - H				REGN5715
Gly 50	Phe54 - H				REGN5715
Asp 73	Ser31 - H				REGN5714
Glu 74	Tyr32 - H	Arg98 - H			REGN5714
Tyr 84	Ile101 - H				REGN5714
Ser 100	Ile101 - H				REGN5714
Glu 102	Tyr49 - L	Gly102 - H			REGN5714
Lys 116	Ser52 - L	Thr53 - L			REGN5714
Lys 120	Glu33 - H	Ala100 - H			REGN5714
Glu 142	Ser54 -H				REGN5713
Arg 146	Ser54 -H	Tyr53 - H			REGN5713
Ala 147	Ph3 - H				REGN5713
Glu 149	Tyr53 - H				REGN5713
Ser 150	Try100 - H				REGN5713
Ala 154	Tyr99 - H				REGN5713
His 155	Try100 - H	Tyr49 - L			REGN5713

Journal Pre-proof