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Supplementary Information for

Designer Covalent Heterobivalent Inhibitors Prevent IgE-Dependent Responses to Peanut Allergen

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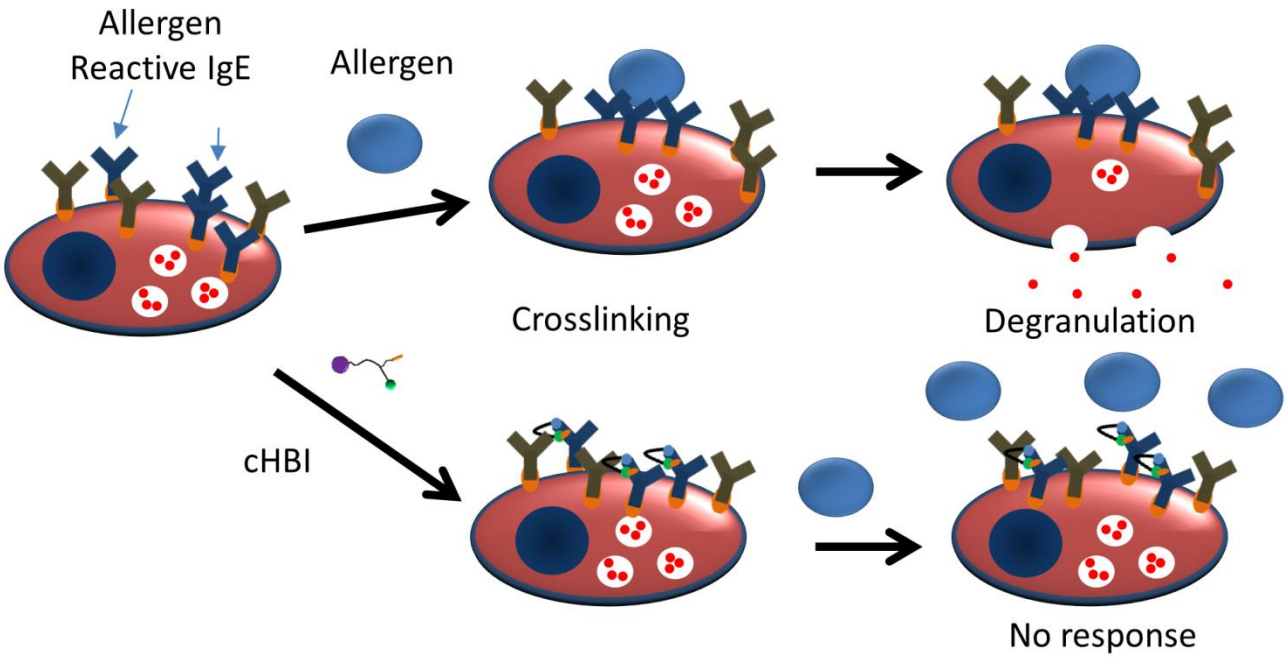
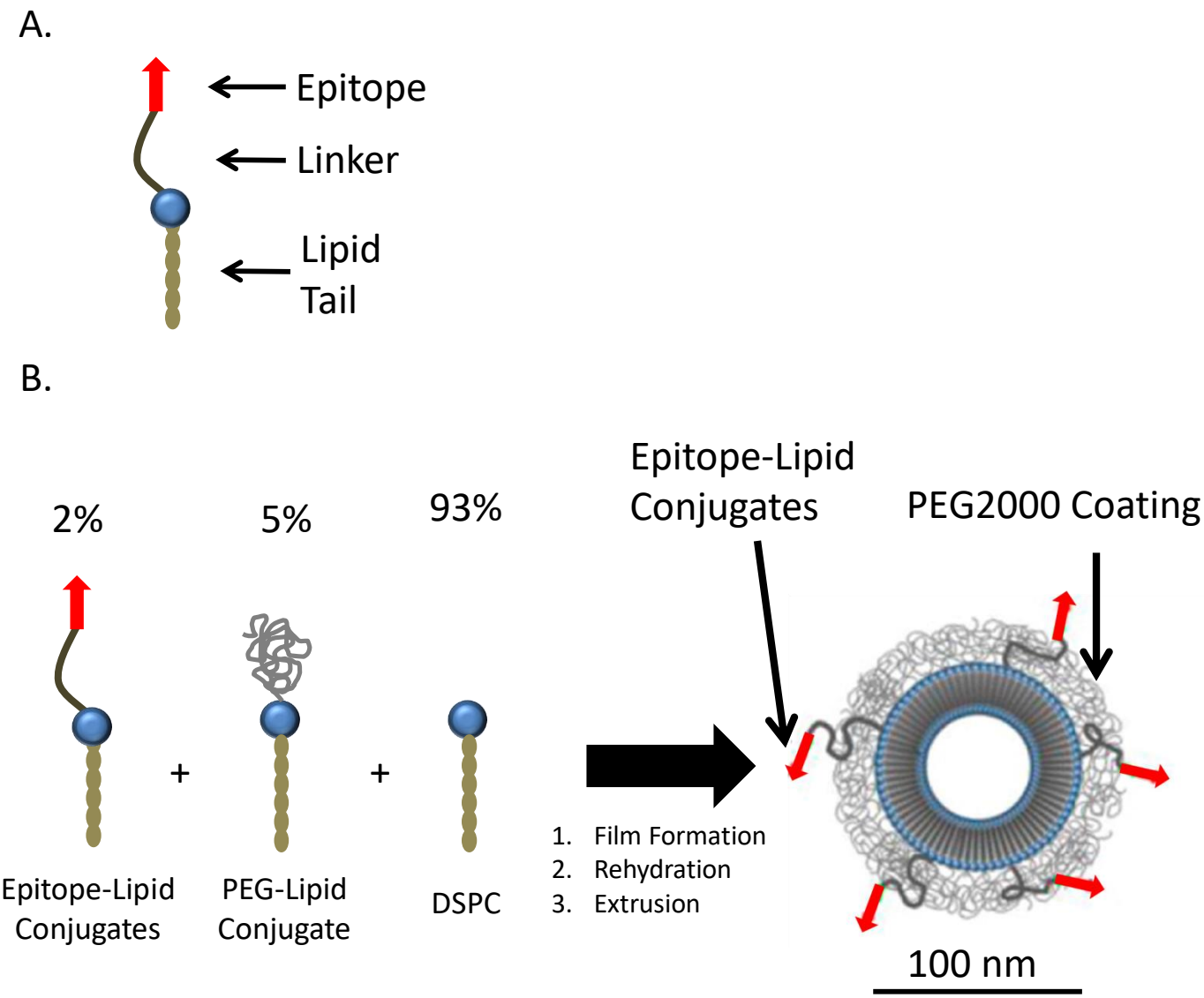


Figure S1. Schematic representation depicting IgE mediated degranulation (top) and how the developed cHBI irreversibly inhibit degranulation (bottom). By selectively and covalently binding to allergen sIgE, cHBIs irreversibly inhibit allergen-IgE interactions. Without the critical IgE-allergen binding, no degranulation occurs and therefore no allergic response.



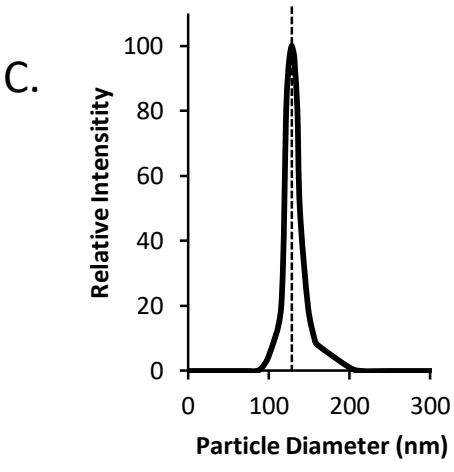


Figure S2. Nanoallergen Design and Characterization. (A) Cartoon of epitope-lipid structure, showing epitope attached to palmitic acid tail via an ethylene glycol (EG) tail. (B) Nanoallergens are assembled from three purified lipid components mixed at precise stoichiometries: epitope-lipid, PEG2000-lipid, and DSPC mixed at 2:5:93 % of total lipid respectively. Lipids were mixed, rehydrated and extruded through 100 nm pores to form 100 nm liposomes. (C) Dynamic light scattering (DLS) data show that nanoallergens were approximately 120 nm in diameter. The increased diameter (>100 nm) was a result of the PEG coating and epitope display. A representative DLS measurement is shown for a nanoallergen presenting Ara h 2 epitope 5 at 2% loading.

Table S1. Ara h 2 and Ara h 6 Epitopes used in Nanoallergen Study

Protein	Epitope#	Sequence	Number	References
Ara h 2	1	NLRPCEQHLMQKIQRD	41-56	Mueller et al., 2011. M
Ara h 2	2	SDRLQGRQQ	117-126	Mueller et al., 2011,
Ara h 2	3	HASARQQWEL	18-27	Albrecht, M, 2009.
Ara h 2	4	RQQEQQFKRELRNLPQQ	123-139	Mueller et al., 2011,
Ara h 2	5	DPYSP^{OH}SDRRGAGSS	70-83	Bublin et al., 2015
Ara h 6	1	MRRERGRGQDSSSS	24-37	K Otsu, Dreskin, 2014.
Ara h 6	2	KPCEQHIMQRI	45-55	K Otsu, Dreskin, 2014.
Ara h 6	3	GEQEQYDSYNFGSTRSSDQ	38-56	Mishra et al. 2014
Ara h 6	4	QDRQ	90-93	Bublin et al., 2015
Ara h 6	5	SCERQVD	34-40	Bublin et al., 2015

*Note -that all cysteines were replaced with serines and that P^{OH} denotes a hydroxylated proline

Table S2. List of patient sera, their sIgE levels for peanuts as determined by ImmunoCAP and food challenge data.

Serum Number	Peanut sIgE (kU/L)	Cumulative reactive dose (mg protein)	Symptoms
1	84.4	No food challenge data. Serum obtained from commercial source (PlasmaLabs)	
2	99.3		
3	50		
4	124.5		
5	451	143	GI pain, urticaria, flushing, pruritus, coughing, congestion, wheezing
6	432	143	Throat itching, urticaria, erythema, nausea, emesis, dizziness
7	396	443	Throat itching, nausea, GI pain, flushing, vomiting, sneezing, elevated heart rate
8	320	443	Urticaria, GI pain
9	215	443	Throat itching, urticaria, GI pain
10	265	443	Nausea, nasal congestion, flushing, conjunctival injection, wheezing, elevated heart rate and systolic bp, pruritus, urticaria
11	100	No food challenge data, subject withdrew from study prior to challenge	
12	148	443	Flushing, abdominal pain, diarrhea, nausea
13	122	443	Oral pruritus, urticaria, abdominal pain, emesis, nasal congestion, sneezing
14	145	143	Oral pruritus, nasal congestion, abdominal pain, nausea, decrease in heart rate
15	151	473	Urticaria, erythema, pruritus, abdominal pain, flushing, sneezing, coughing
16	650	443	Abdominal pain, emesis

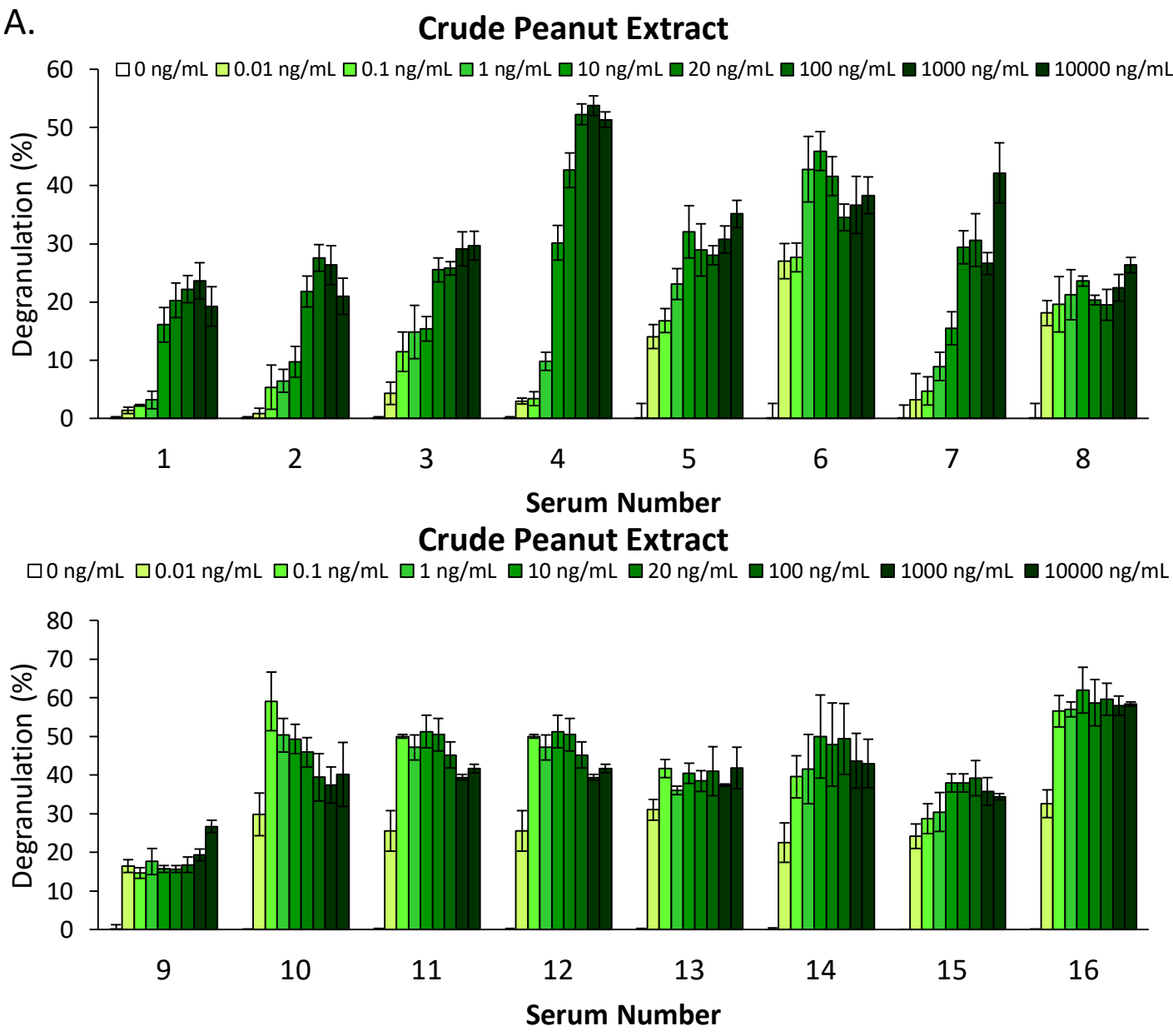
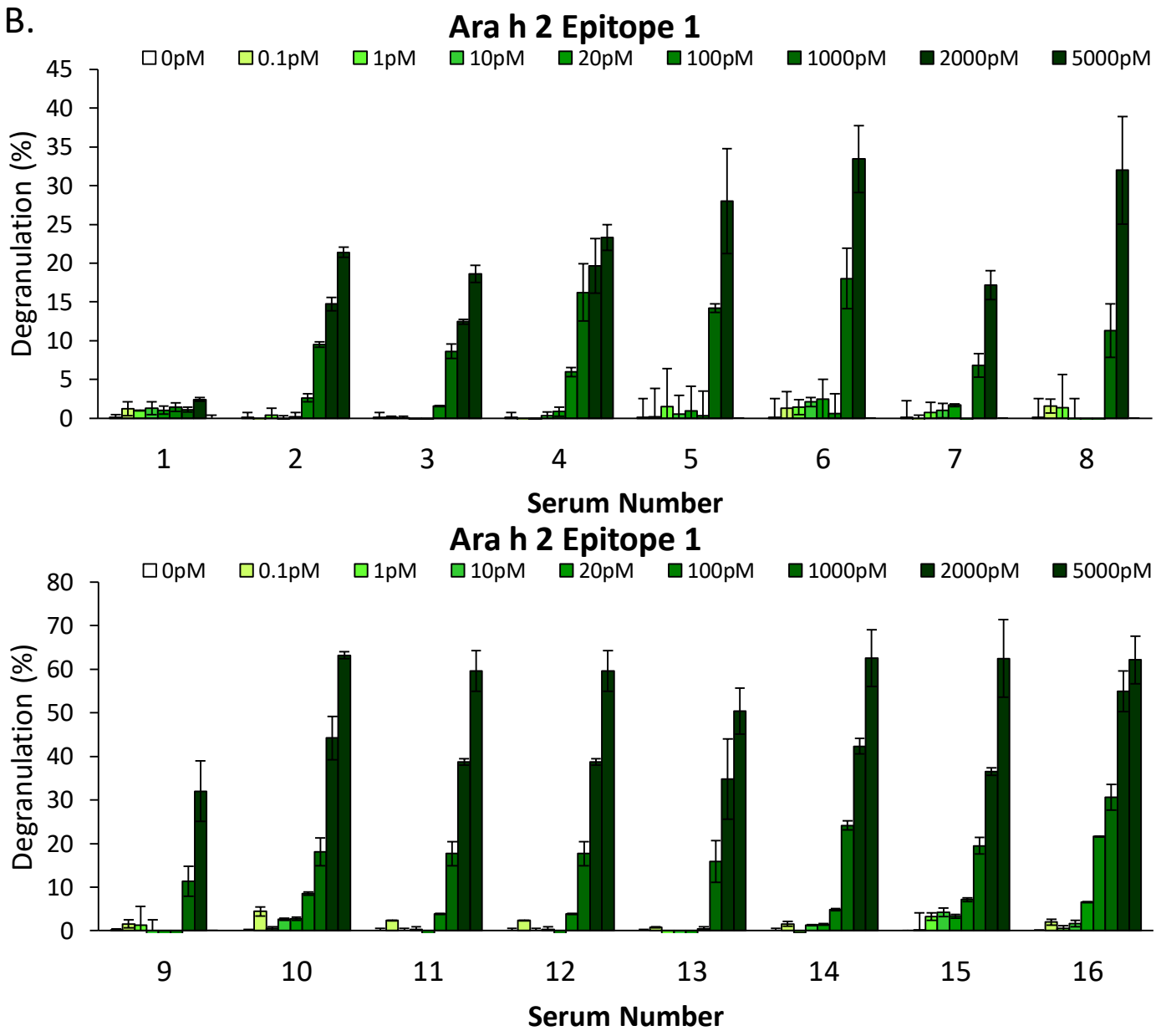


Figure S3. RBL cell degranulation assays using patient sera/nanoallergen combinations for Ara h 2. Nanoallergens were loaded with each of the ten epitopes (2% loading) at a time. RBL-SX38 cells were primed with patient sera 1-16 overnight. Then primed RBL-SX38 cells were incubated with varying concentrations of the (A) crude-peanut-extract or (B-F) nanoallergens displaying Ara h 2 epitopes or (G-K) nanoallergens displaying Ara h 6 epitopes for 1.5 hours. Degranulation was then determined with a beta-hexosaminidase assay. Error bars indicate ±SD of triplicate experiments.



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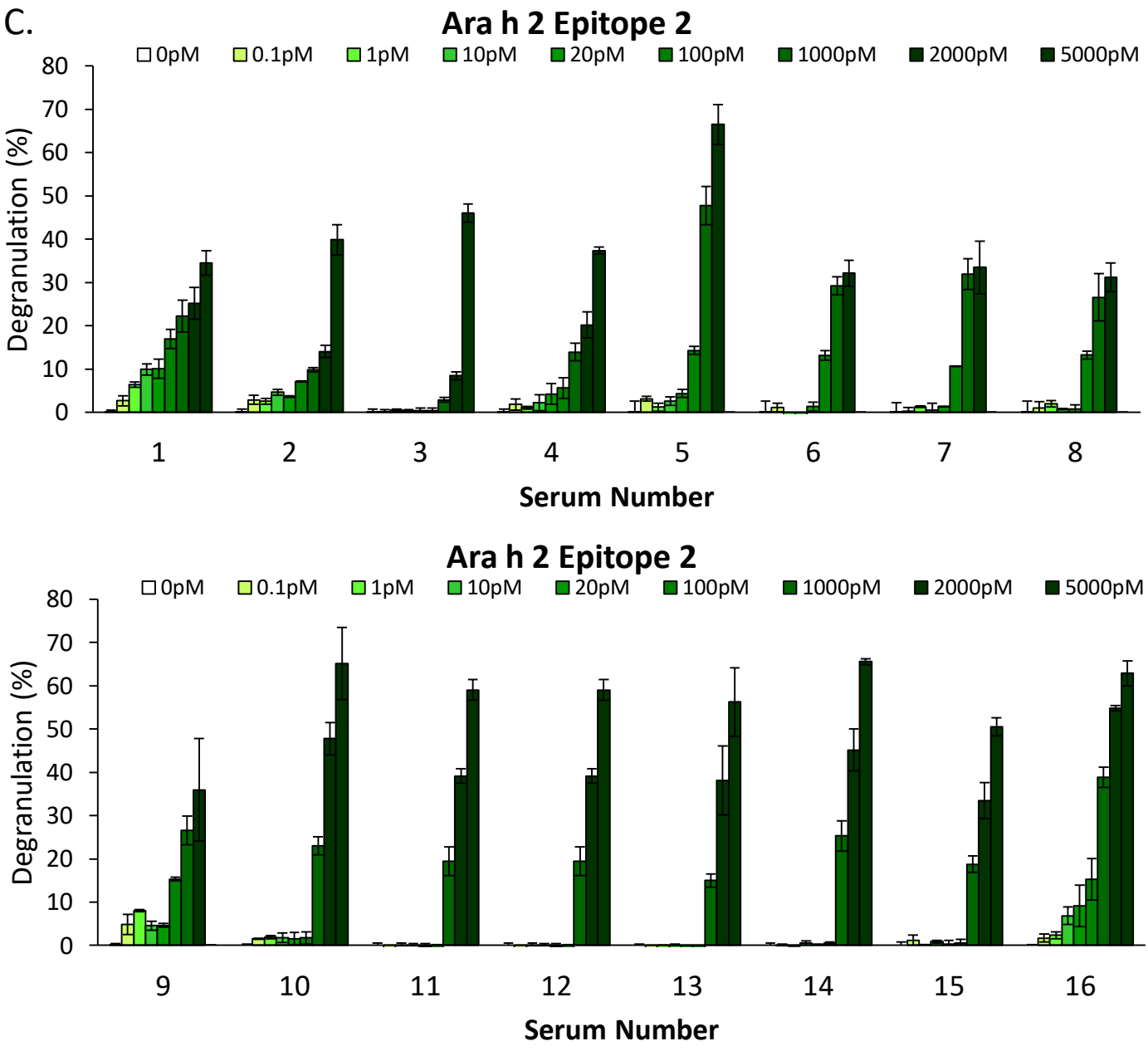

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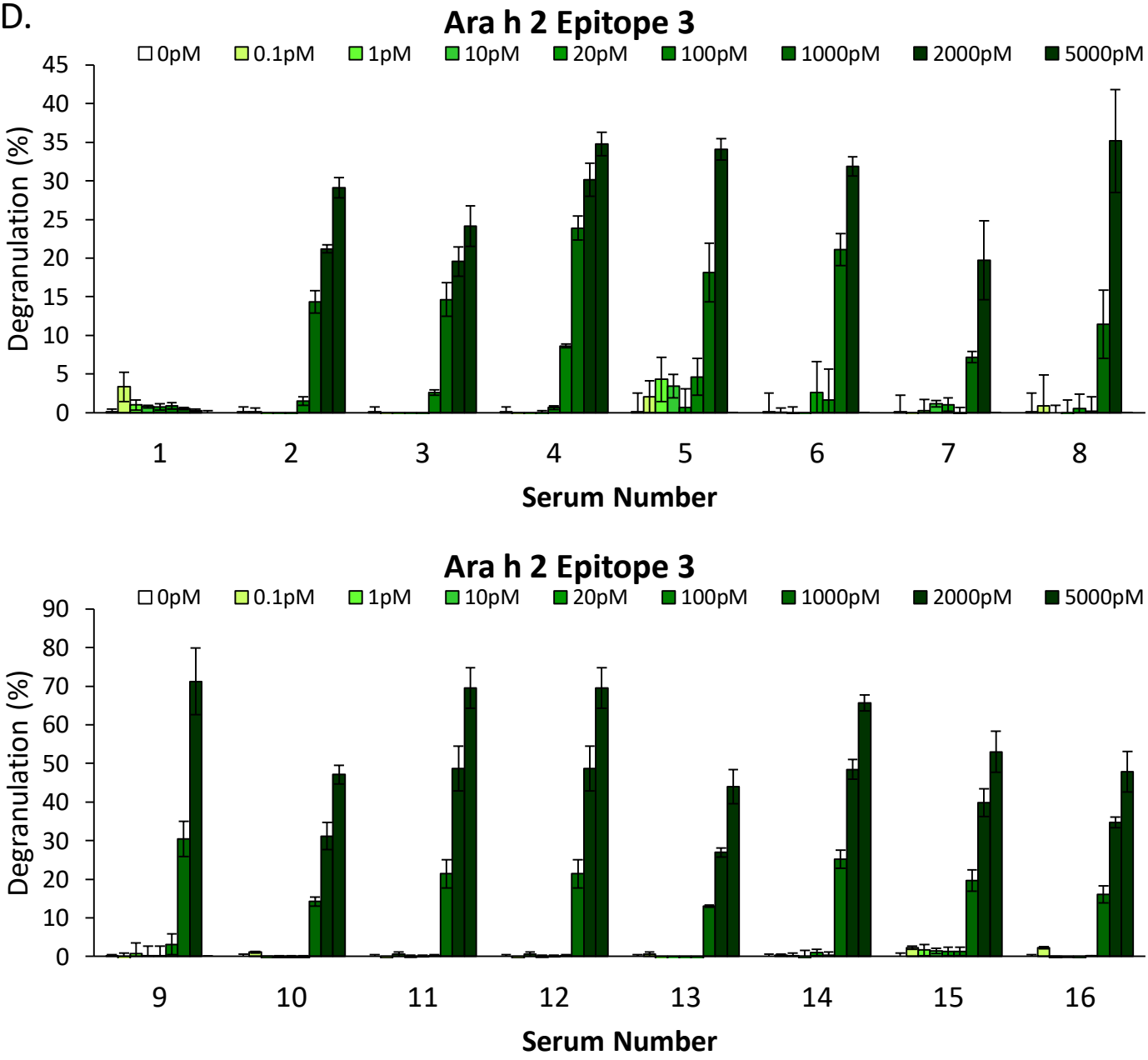

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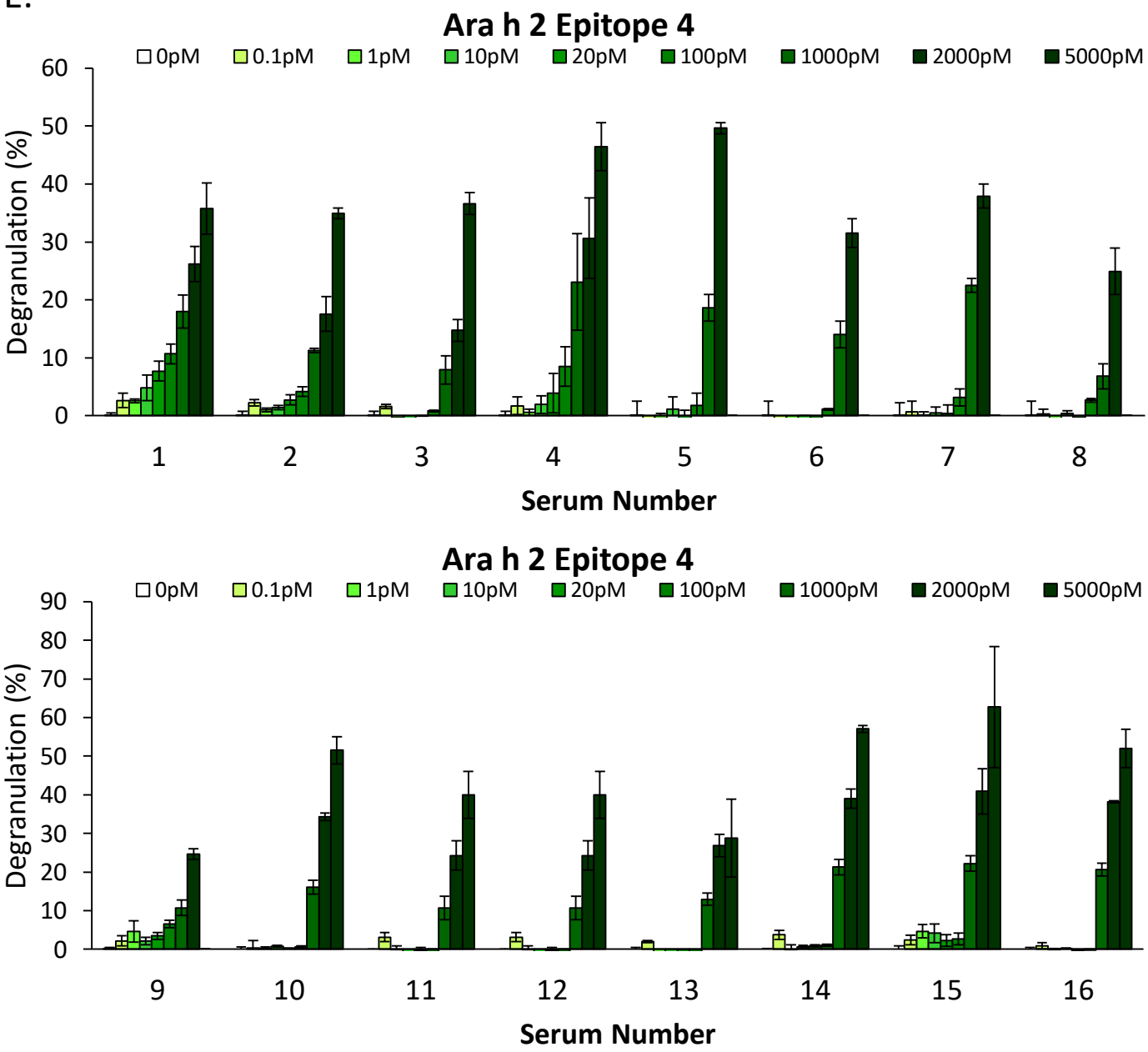

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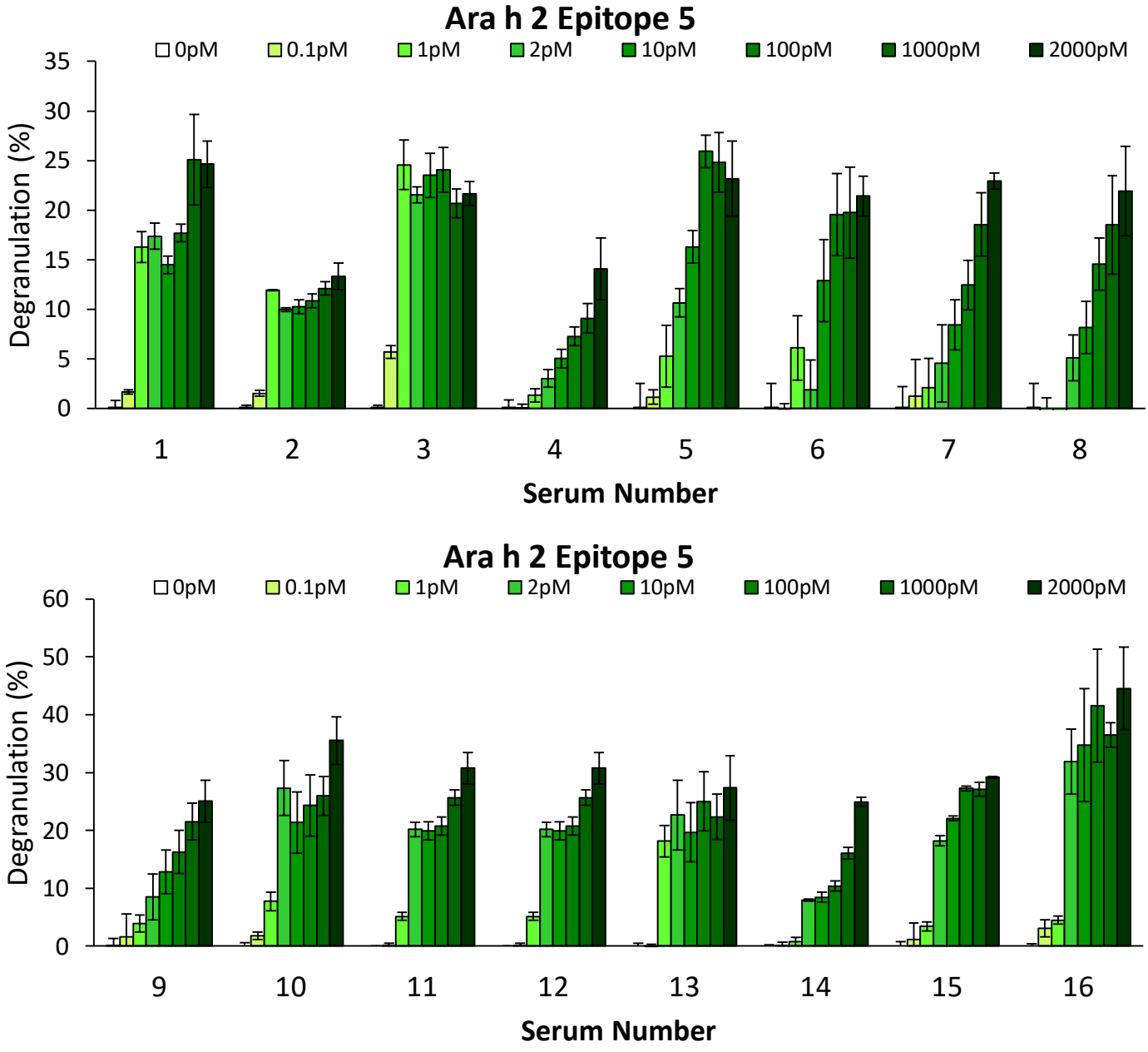

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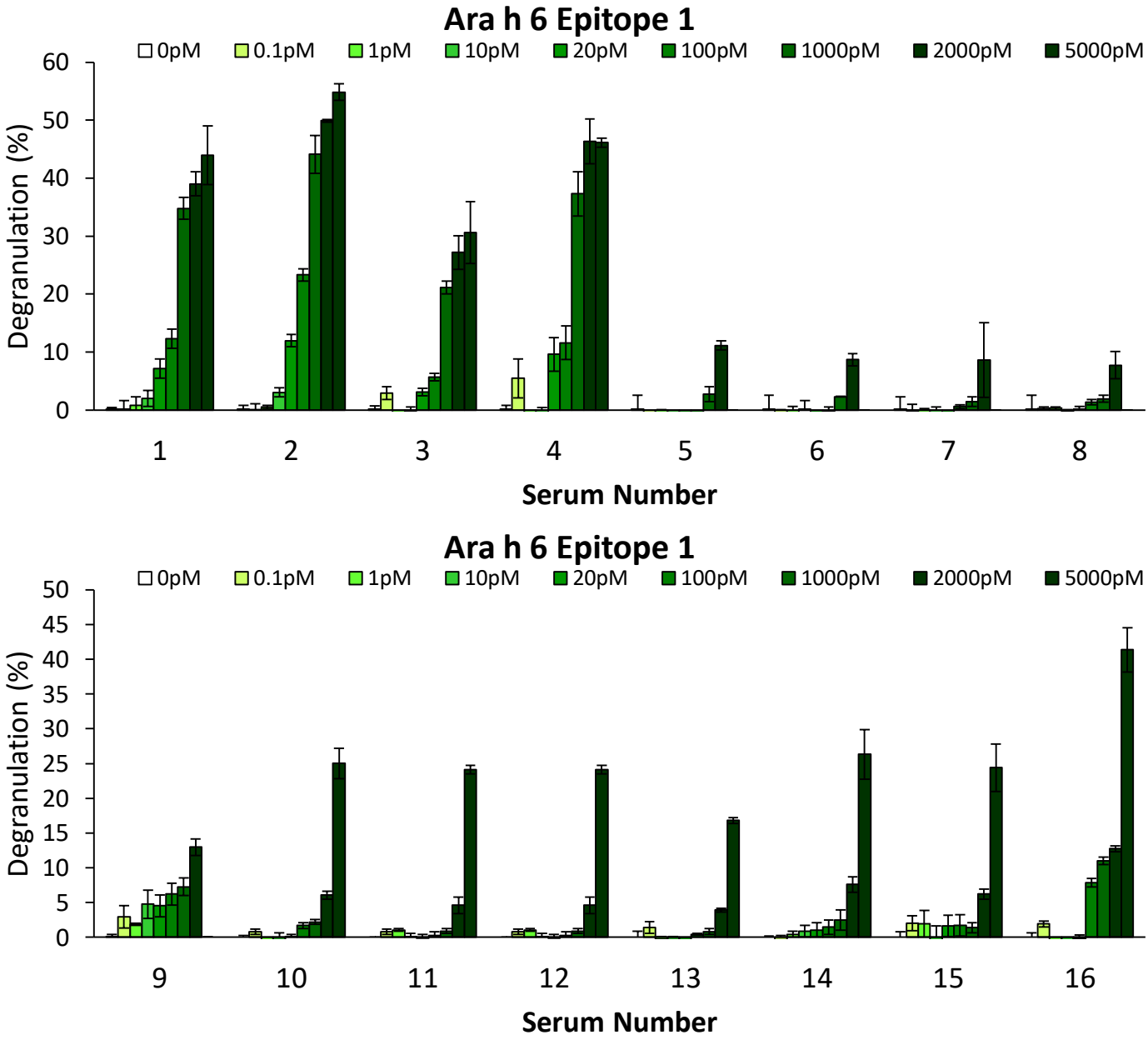

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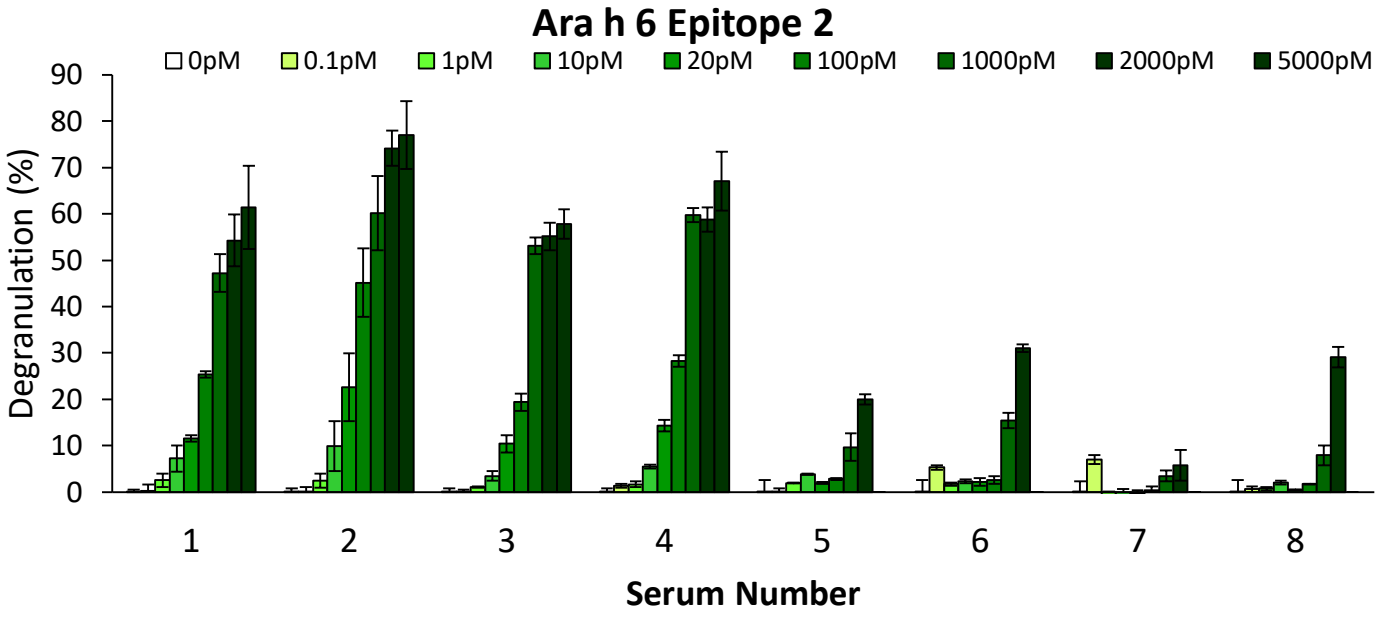

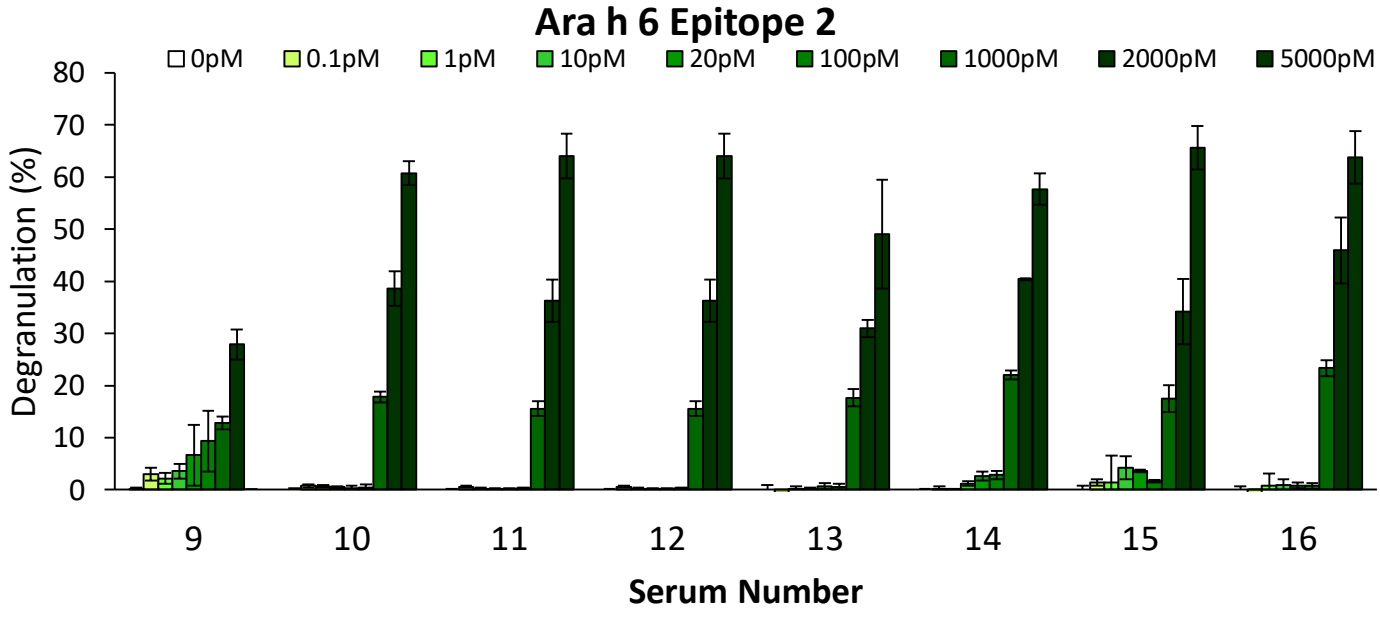

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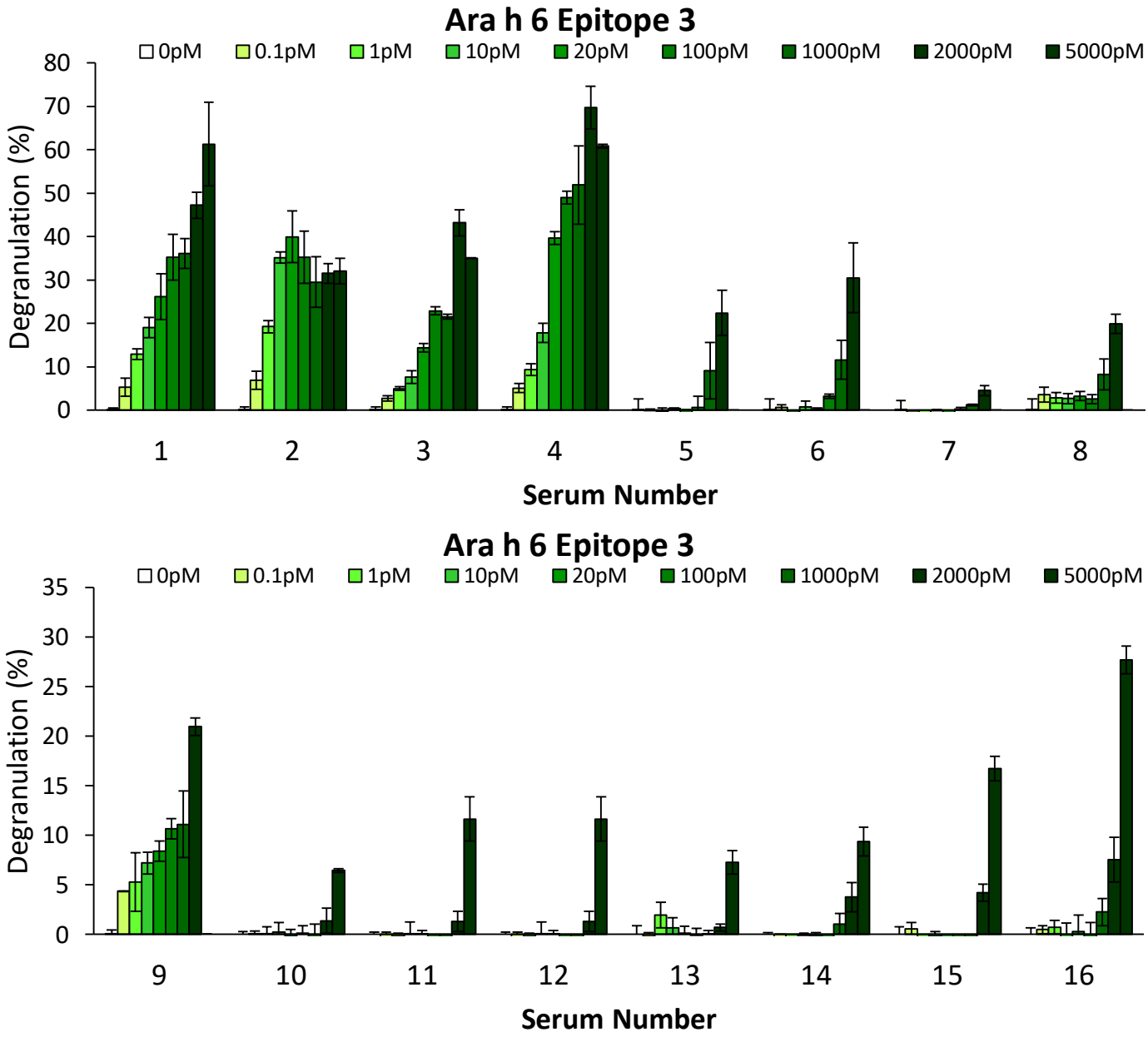

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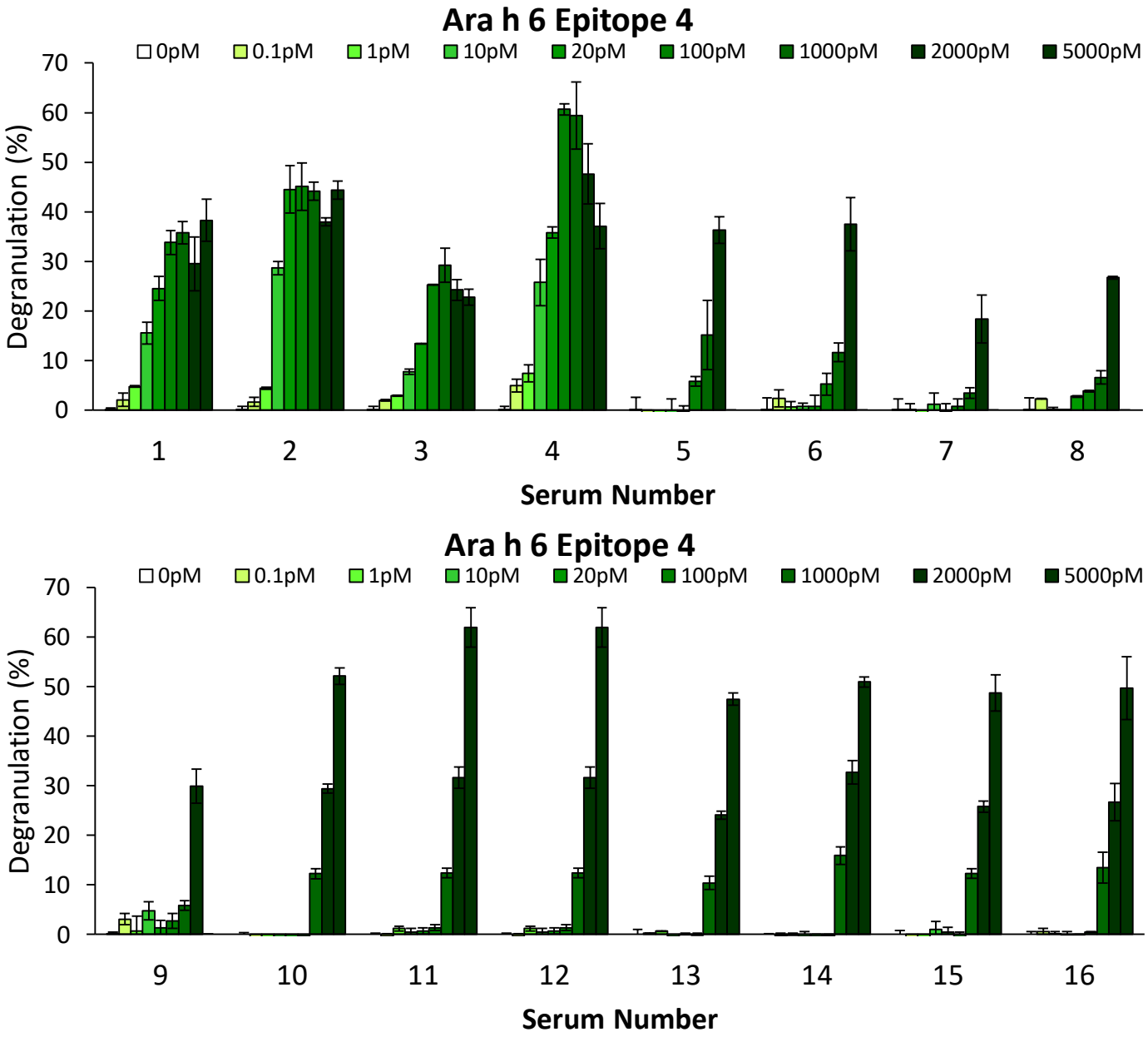

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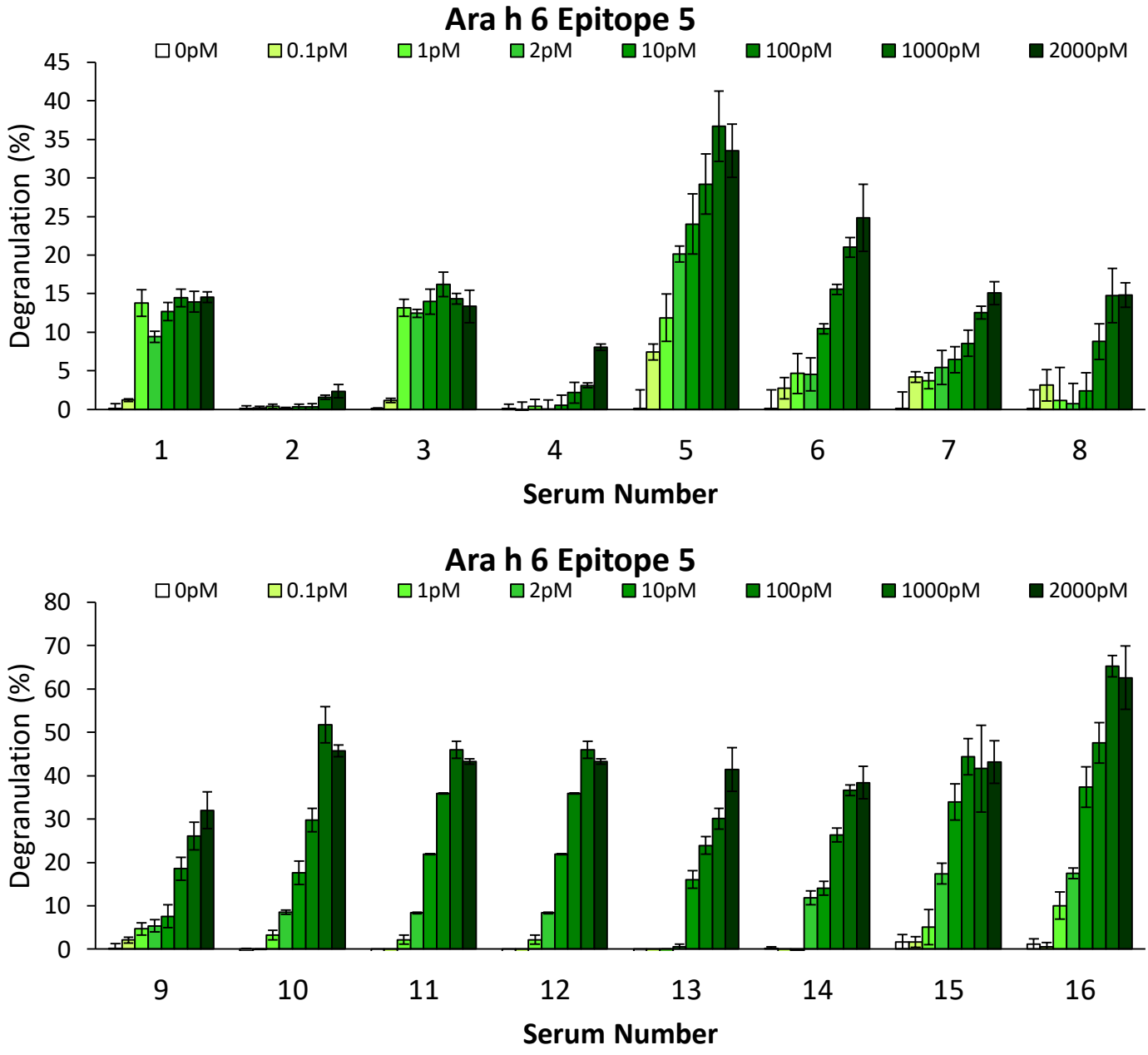

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Table S3- EC_{50} and D_{max} data from Nanoallergen Analysis.

Allergen		Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6	Serum 7	Serum 8
CPE(ng/mL)	Dmax	21.9±1.1	25.5±3.4	32.6±4.7	53.8±1.9	34.62±3.3	39.9±3.0	39±7.5	26.4±1.3
	EC50	4.1±1.3	5.9±4.5	1.9±2.4	5.9±1.2	0.061±0.05	>0.01	9.9±10.2	>0.01
Ara h 2 Epitope 1 (pM)	D_{max}	N/A	21.4±0.7	18.6±1.1	27.1±2.0	25.6±2.3	33.4±2.3	17.6±1.9	31.9±6.9
	EC_{50}	N/A	>1000	>1000	590±150	985±190	995±202	>1000	>1000
Ara h 2 Epitope 2 (pM)	D_{max}	34.5±2.8	39.9±3.5	46.0±2.1	37.4±0.8	73.9±7.7	32.2±1.1	33.6±0.6	30.9±1.5
	EC_{50}	134±118	>1000	>1000	>1000	430±160	133±20	160±14	138±31
Ara h 2 Epitope 3 (pM)	D_{max}	N/A	34.7±3.6	27.3±3.0	39.8±3.5	42±10.7	35.5±2	19.7±5.1	35.1±6.7
	EC_{50}	N/A	1350±270	880±220	570±170	940±550	840±80	>2000	>1000
Ara h 2 Epitope 4 (pM)	D_{max}	35.7±4.4	35±0.92	36.6±1.9	46.4±4.2	48±1.4	31.5±2.5	38±1.4	24.9±4
	EC_{50}	370±150	>1000	>2000	790±190	>1000	>1000	950±140	>1000
Ara h 2 Epitope 5 (pM)	D_{max}	21.5±2.1	11.4±0.6	22.7±0.7	14.1±3.1	24.9±1.0	20.9±1.4	22.9±0.8	20.9±3.2
	EC_{50}	0.52±0.4	0.2±0.2	0.12±2.1	98.2±10	0.88±0.17	6.9±2.5	19.1±10.3	6.9±5.9
Ara h 6 Epitope 1 (pM)	D_{max}	50.5±3.6	59.6±4.2	35.7±4.8	52.1±8.8	11.1±0.8	8.7±1.1	8.6±6.4	7.7±2.4
	EC_{50}	381.6±99	19±66	610±207	320±220	>1000	>1000	>1000	>1000
Ara h 6 Epitope 2 (pM)	D_{max}	73.25±2.8	78.5±5.7	61.9±3.6	70.3±4.2	20.0±1.1	31.0±2.8	N/A	29.1±2.2
	EC_{50}	330±61	82.8±31	193±50	150±44	>1000	>1000	N/A	>1000
Ara h 6 Epitope 3 (pM)	D_{max}	61.3±9.6	34.1±2.0	35.0±2.9	64.1±6.6	22.4±5.2	30.5±8.1	N/A	19.9±2.2
	EC_{50}	63.3±27	0.6±0.3	45.3±21	18.5±11	>1000	>1000	N/A	>1000
Ara h 6 Epitope 4 (pM)	D_{max}	35.2±1.8	43.2±2.7	25.7±1.5	51.1±5.2	37.1±2.6	37.3±2.4	18.4±4.8	26.7±0.26
	EC_{50}	10.2±2.8	4.2±1.7	17.7±4.2	9.8±4.6	1070±100	>1000	>1000	>1000
Ara h 6 Epitope 5 (pM)	D_{max}	13.3±0.9	N/A	13.4±2.6	14.3±0.5	27.3±4.8	22.4±4.3	15.1±1.5	16.5±2.8
	EC_{50}	0.28±0.16	N/A	0.29±0.47	0.34±0.1	0.88±0.98	25±6.52	9.2±4.33	84±60

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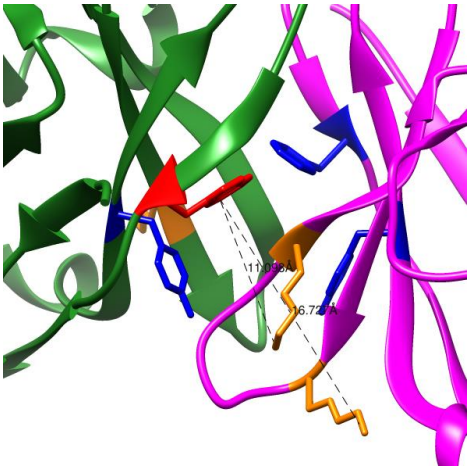
Allergen		Serum 9	Serum 10	Serum 11	Serum 12	Serum 13	Serum 14	Serum 15	Serum 16
CPE(ng/mL)	Dmax	26.7±1.6	59.1±7.6	51.3±3.3	57.3±3.3	51.7±2.3	49.9±9.1	39.2±4.6	66.5±4.1
	EC50	>0.01	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
Ara h 2 Epitope 1 (pM)	D_{max}	36.4±1.6	65.8±4.2	68.6±6.5	86.4±19.4	54.6±3.8	65.4±3.1	64.5±6.7	64.8±5.7
	EC_{50}	>1000	1500±118	1800±250	2000±800	1500±150	1300±90	1500±220	350±140
Ara h 2 Epitope 2 (pM)	D_{max}	35.9±11.8	69.4±3.2	66.8±2.1	72.9±1.1	59.7±2.6	68±1.7	61.5±3.1	65.6±3.7
	EC_{50}	146.7±140	1400±90.3	1650±76	1300±28	1600±100	1300±50	1800±150	320±80
Ara h 2 Epitope 3 (pM)	D_{max}	72±8.6	51.4±2.6	74.3±0.7	74.7±2.0	50.5±8.9	68.8±0.7	56.1±3	50.7±1.8
	EC_{50}	>1000	1650±119	1500±20	1250±50	1900±500	1300±20	1300±100	1400±70
Ara h 2 Epitope 4 (pM)	D_{max}	24.6±1.4	56.8±1.2	46.3±5.3	90.1±14.5	53.2±2.9	66.8±7.0	65.0±7.6	56.3±21.8
	EC_{50}	>1000	1650±50	1900±320	1700±500	2000±150	1600±270	1400±220	1300±750
Ara h 2 Epitope 5 (pM)	D_{max}	23.3±4.4	28.3±3.4	24.3±2.2	21.4±1.6	22.7±1.5	20.6±2.1	26.4±1.3	37.6±1.7
	EC_{50}	5.9±7.4	2.6±2.0	1.4±0.3	1.4±0.2	0.7±0.5	28±19	1.6±0.2	1.5±0.1
Ara h 6 Epitope 1 (pM)	D_{max}	12.9±1.2	25±2.2	24.0±0.6	0	16.5±0.4	26.3±3.6	24.4±3.4	48.7±26.8
	EC_{50}	86±55	>2000	>2000	N/A	>2000	>2000	>2000	2800±1700
Ara h 6 Epitope 2 (pM)	D_{max}	27.9±2.8	69±5.3	78±1.9	69.1±1.9	50.5±6	70.6±7.6	68.8±3.9	68.8±1.9
	EC_{50}	>1000	1800±76	2200±76	1400±60	1900±322	1700±320	1720.6±150	1400±60
Ara h 6 Epitope 3 (pM)	D_{max}	26.1±3.9	0	11.7±2.2	11.3±1.1	0	9.4±1.4	16.7±1.3	27.7±1.4
	EC_{50}	146±102	N/A	>2000	N/A	N/A	N/A	>2000	>2000
Ara h 6 Epitope 4 (pM)	D_{max}	29.9±3.5	6.25±3.2	80.1±6.0	61.4±4.6	51.5±1.4	58.7±1.5	69.4±12.6	52.5±2.0
	EC_{50}	>1000	2100±160	2500±300	1850±70	2000±80	1750±70	2800±800	1700±100
Ara h 6 Epitope 5 (pM)	D_{max}	23.4±4.9	47.5±2.2	44.4±2.2	47.2±3.4	27.0±2.1	39.2±3.3	43.6±1.2	65.9±7.5
	EC_{50}	6.3±7.7	54±16	76±22	82±30	85±21	120±60	19±3.5	90±50

Table S4. Cross reactivity of Ara h 2 Epitope 5 and Ara h 6 Epitope 5 with epitopes from other Ara h proteins. Ara h 2 Epitope 5 (left column) or Ara h 6 Epitope 5 (right column) were compared to the primary sequences of Ara h 1, Ara h 3 and Ara h 2 or Ara h 6 using an online sequence homology calculator (https://web.expasy.org/)and homologous sequences were listed. Red signifies an exact sequence match, yellow indicates a conservative mutation in same amino acid group.

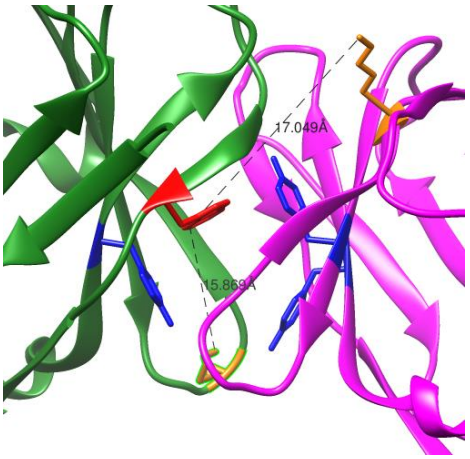
Protein	Sequence	Ara h 2 Epitope 5
Ara h 2	86-99	DPYSPSDRRGAGSS
Ara h 1	68-81	DPRCVYDPRGHTGT
Ara h 1	173-186	NPFYFPSRRFSTRY
Ara h 3	198-211	LPYSPYSPQSQPRQ
Ara h 3	305-318	EYDEEDRRRGRGSR
Ara h 6	60-73	EQYDSYDIRSTRSS

Protein	Sequence	Ara h 6 Epitope 5
Ara h 6	34-40	SCERQVD
Ara h 1	45-51	SCQQEPD
Ara h 3	249-255	EQAFQVD
Ara h 3	254-260	VDDRQIV
Ara h 2	32-38	RCQSQLE
Ara h 2	159-165	RCDLEVE

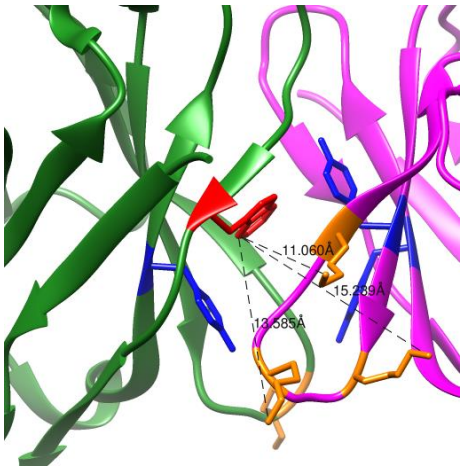
A.



B.



C.



D.

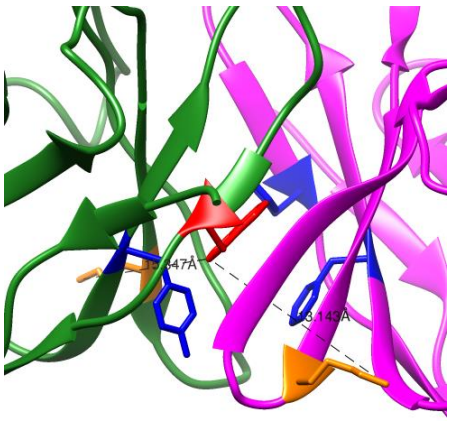


Figure S4. Crystal structures of NBS sites of various antibodies with lysines highlighted in orange. (A)Rituximab, (B) Cetuximab , (C) Trastuzumab, (D) Anti-DNP IgE SPE-7. Note that the light chain is shown in purple and the heavy chain in green, while the tryptophan of the NBS pocket is red and the tyrosine resides are shown in blue.

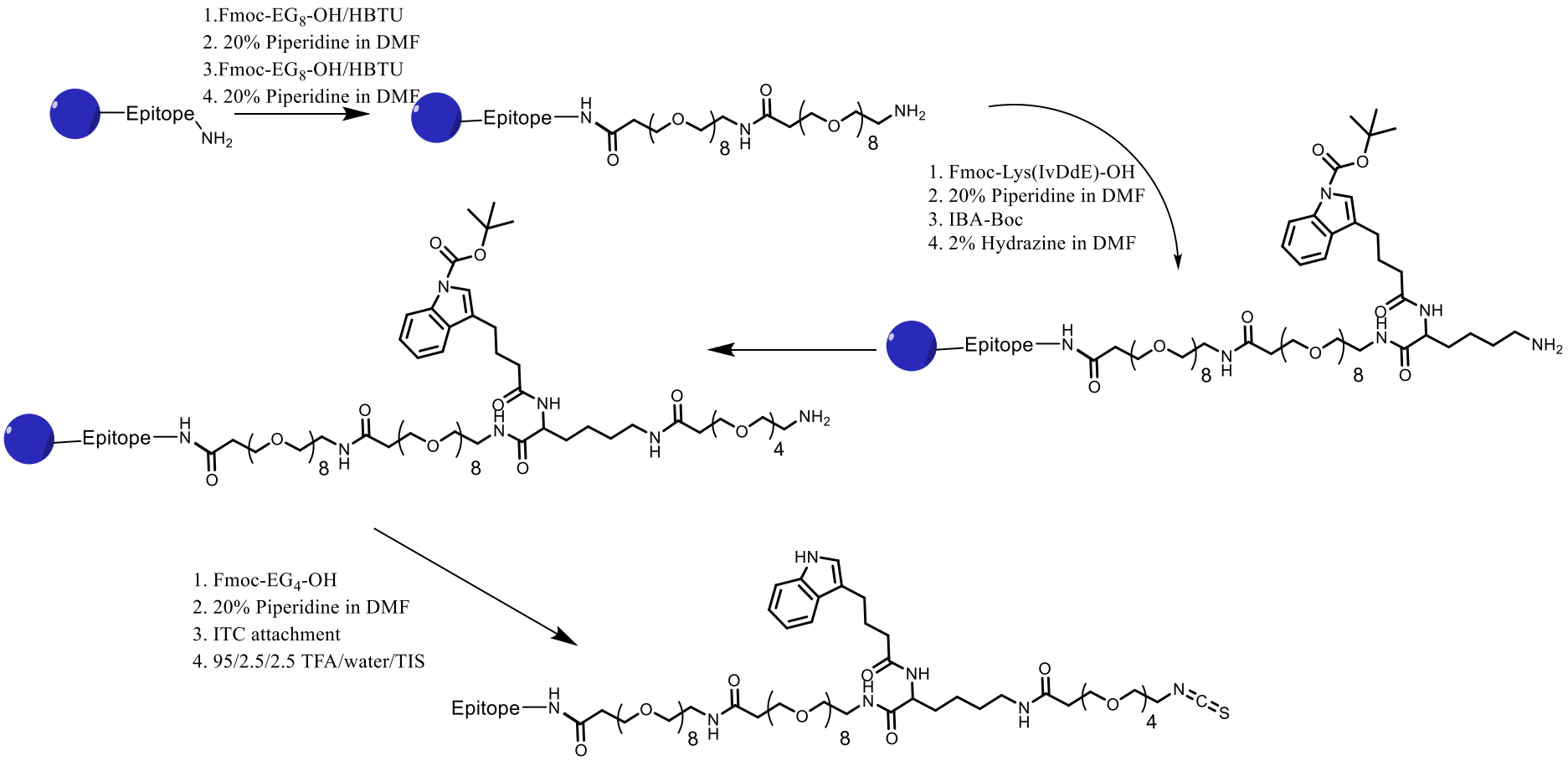


Figure S5. Synthetic scheme of cHBI molecules. Epitopes were synthesized as linear peptides on a rink amide resin using standard Fmoc chemistry. To the amine terminus, an ethylene glycol spacer (Fmoc-EG_8-OH) was added followed by addition of Fmoc-lys(IvDde)-OH. Fmoc was removed with piperidine and the NBS ligand (IBA) was attached to the free amine. Note that IBA was added with a tert-butyloxycarbonyl protecting group (IBA-Boc). The IvDde group was removed with 2% hydrazine and Fmoc-EG_4-OH was coupled to the free amine to act as spacer. The amine of Fmoc-EG_4-OH was then deprotected with piperidine and was converted into an ITC moiety (see methods for further details on ITC synthesis). The molecule was then cleaved from the resin using a TFA cleavage cocktail (95/2.5/2.5: TFA/water/TIS) and purified using HPLC.

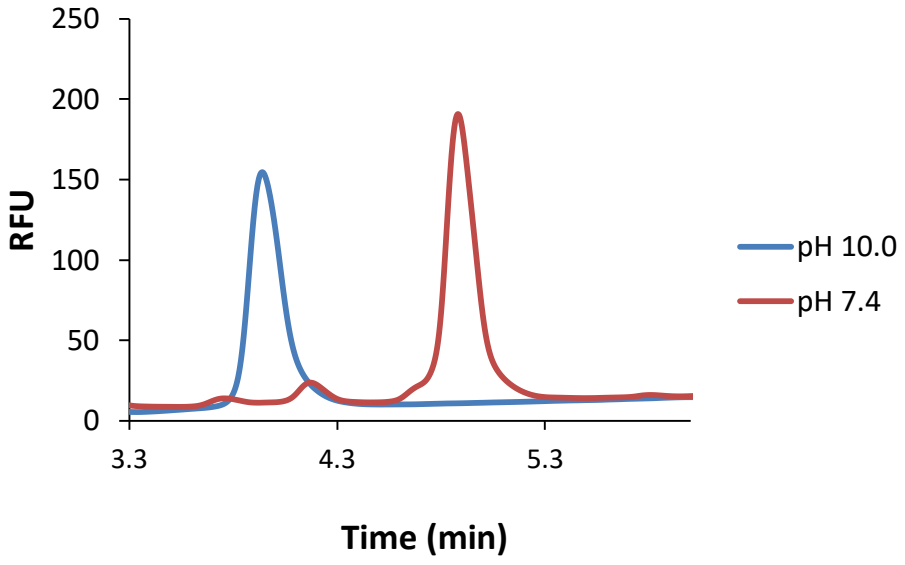


Figure S6. The cHBI molecule demonstrates hydrolysis of the ITC moiety under basic conditions. cHBI$^{Arah2/ep5}$ was incubated in either pH 7.4 (PBS) or 10.0 (bicarbonate buffer) and then injected on the HPLC. Molecules exposed to higher pH levels had a characteristic shift on the HPLC. M/S analysis confirmed the addition of a hydroxyl group, likely to the reactive ITC moiety, confirming the reactivity of these molecules, suggesting that they would be reactive to primary amines.

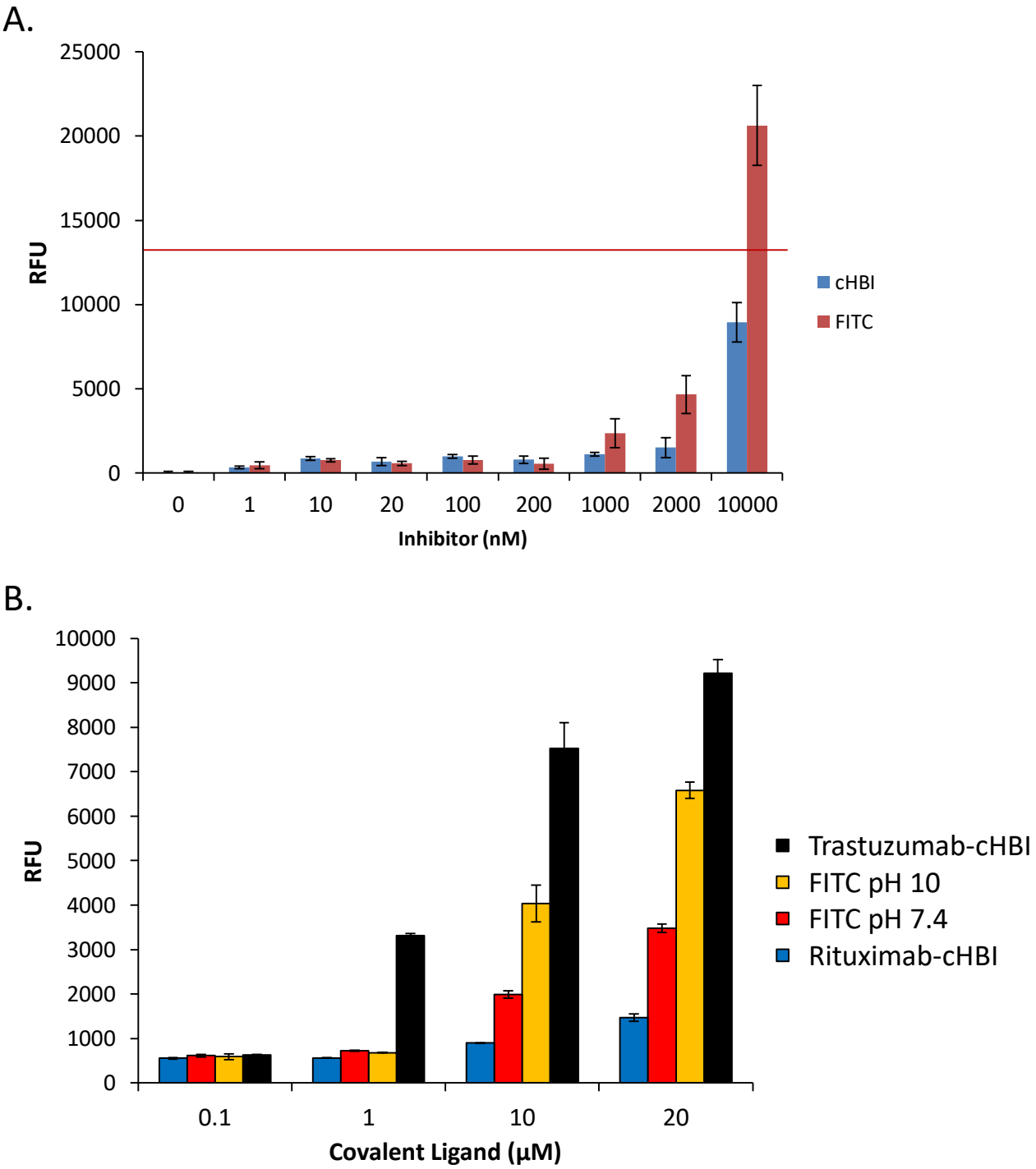


Figure S7. cHBIs do not non-specifically label BSA or off target antibodies. (A) cHBI$^{Ara2/ep5}$ does not label BSA protein. 100 nM of BSA was incubated with varying concentrations of FITC-cHBI$^{Ara2/ep5}$ or FITC for 16 hrs in PBS (pH 7.4) at 37°C, purified with centrifugal filtration (10 kDa filter) to remove unreacted molecule. The amount of conjugation was then assessed using an ELISA for FITC detection. The red line across graph indicates signal from BSA with a known level of FITC conjugation (3.5 FITC conjugations per BSA). (B) cHBIs demonstrate antibody selectivity. Two FITC tagged cHBIs were synthesized using specific pharmecutical antibody targeting peptides, one for Trastuzumab (Trastuzumab-cHBI, peptide: LLGPYELWELSH) and one for Rituximab (Rituxumab-cHBI, peptide: WPRWLEN). These two cHBIs or FITC was incubated with Trastuzumab for 16 hrs in PBS (pH 7.4) at 37°C, purified with centrifugal filtration (10 kDa filter) to remove unreacted molecule. FITC as pH 10 was included as a positive control. Then an ELISA was similarly performed as in part A to determine FITC conjugation.

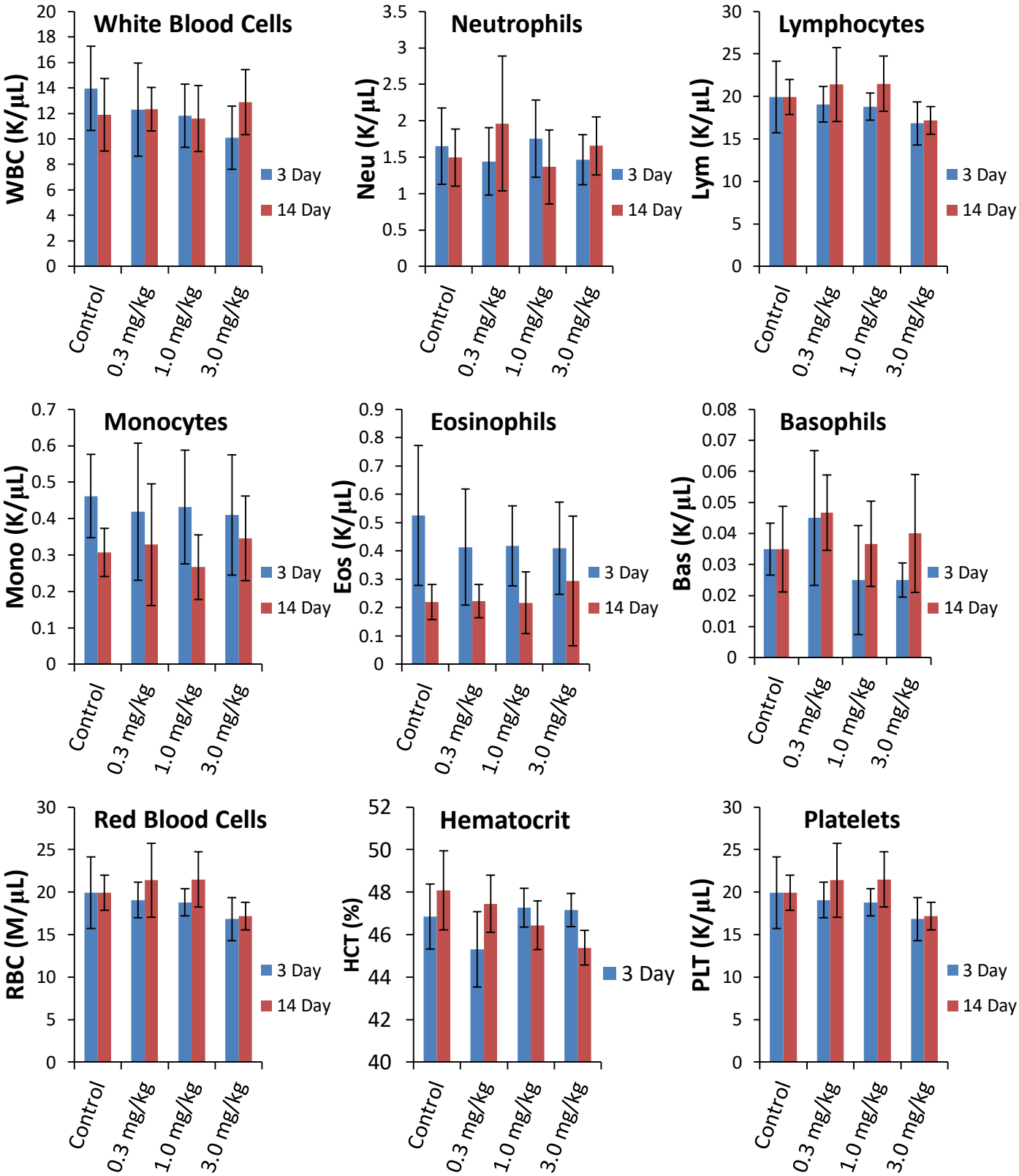


Figure S8. Blood counts for toxicology study of cHBI$^{Arah2:Arah6}$. C57BL/6 mice (5 per group) were i.v. injected with a single dose of 0.3, 1 or 3 mg/kg cHBI$^{Arah2:Arah6}$ and blood counts analyzed after 3 or 14 days in order to assess both acute and chronic toxicity. The data show no statistical difference between control mice and mice injected with any concentration of cHBI$^{Arah2:Arah6}$ at either 3 or 14 days. Error bars indicate ±SEM of five mice per group.

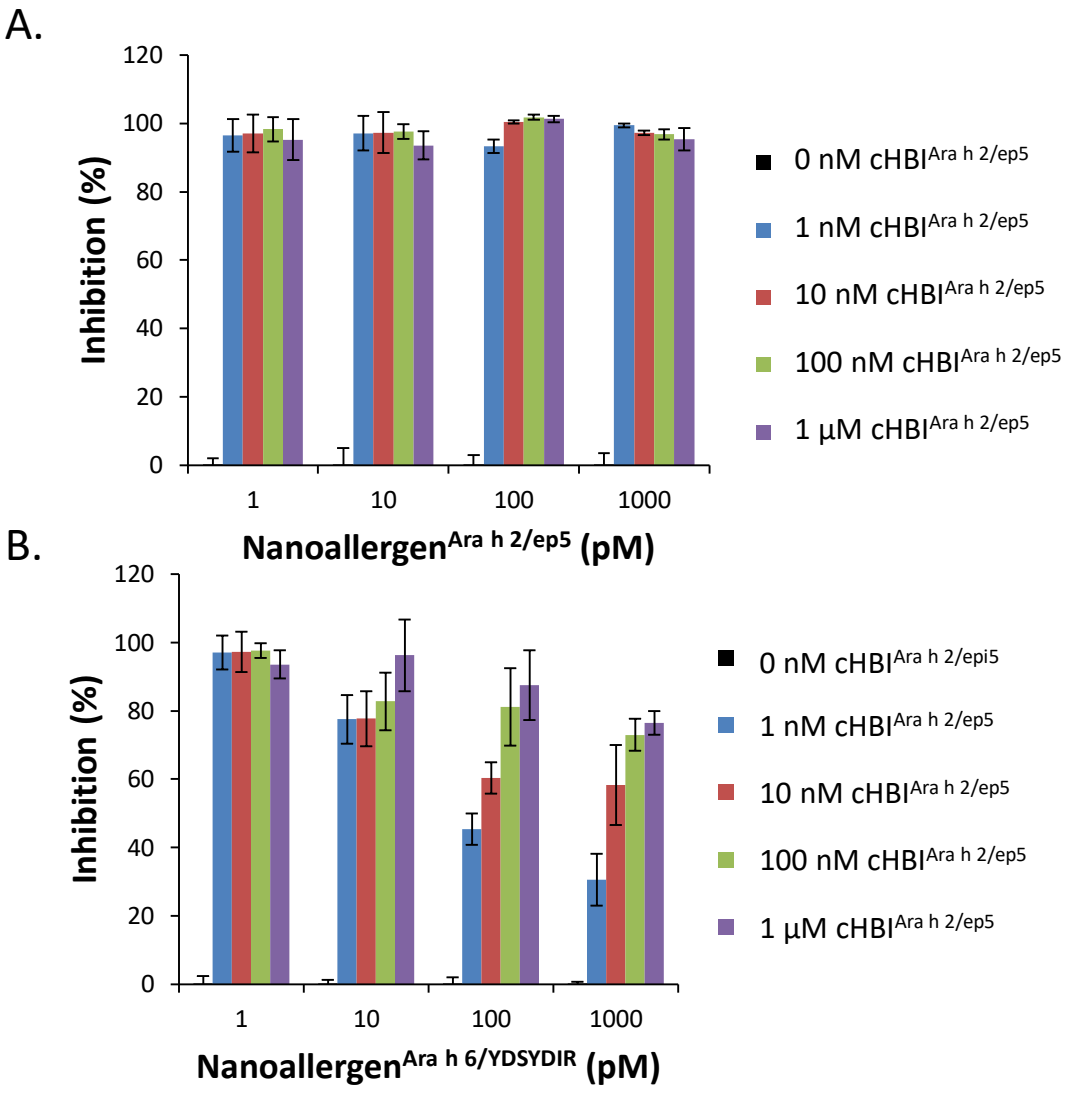


Figure S9. RBL-SX38 cells were primed with serum-4 and then was incubated with cHBI$^{Arah2/ep5}$.Cellular degranulation was triggered with (A) nanoallergen$^{Arah2/ep5}$ (nanoallergens loaded with 2% Ara h 2 epitope 5) or with (B) a nanoallergen presenting a cross-reactive epitope from Ara h 6 (EQYDSYDIRSTRSS) identified in our cross-reactivity analysis, nanoallergen$^{Arah6/YDIRSTRSS}$, and percent inhibition was calculated. Error bars indicate ±SD of triplicate experiments.

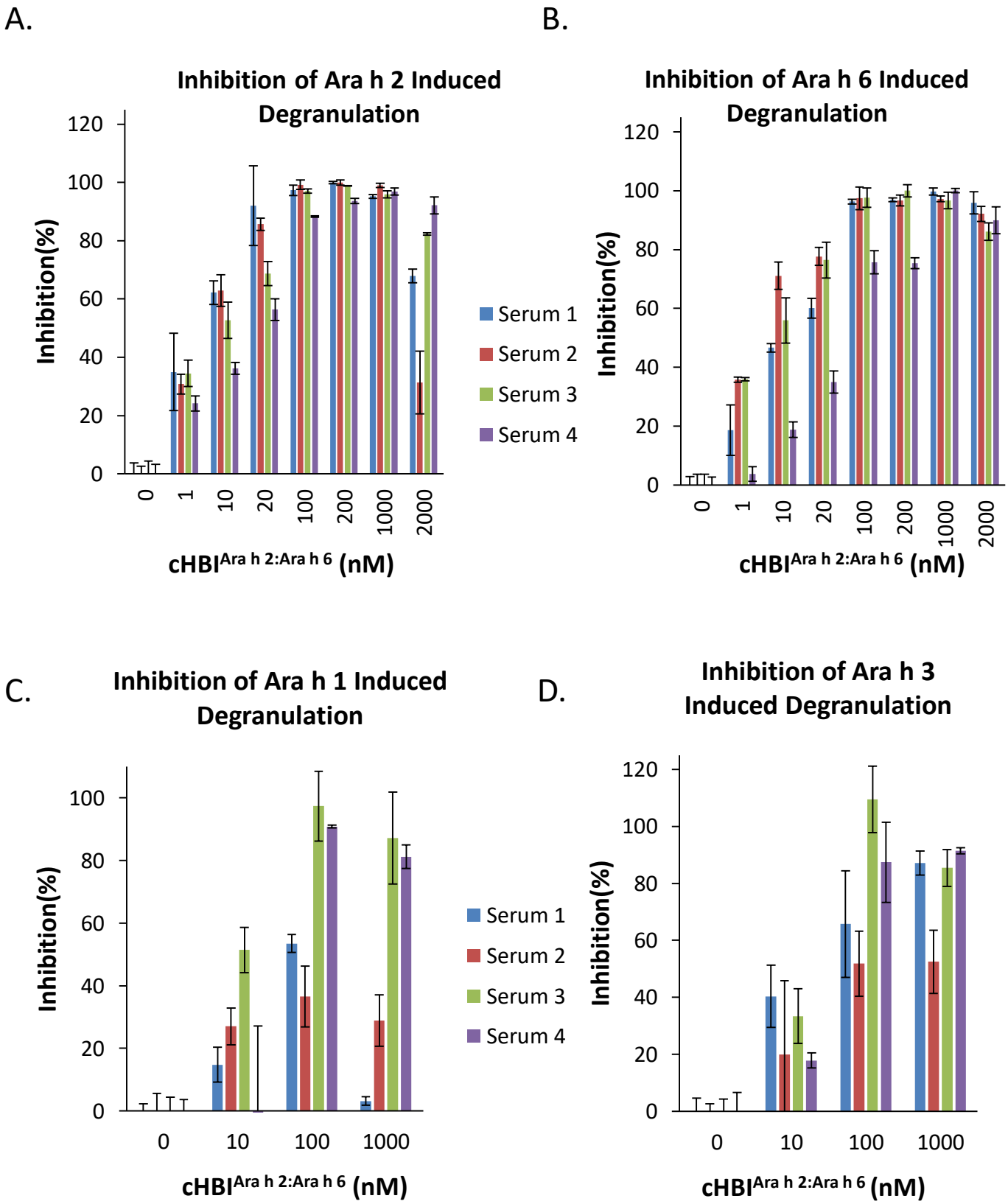


Figure S10. cHBI$^{Arah2:Arah6}$ inhibits degranulation induced by Ara h 1, Ara h 2, Ara h 3 or Ara h 6. RBL-SX38 cells were primed with 10% patient serum, washed and incubated with cHBIs at varying concentrations overnight. Degranulation was triggered with (A) 10 nM Ara h 2, (B) 10 nM Ara h 6, (C) 100 nM Ara h 1 or (D) 100 nM Ara h 3 and percent inhibition was calculated by comparing to PBS control (no inhibitor). Error bars indicate ±SD of triplicate experiments.

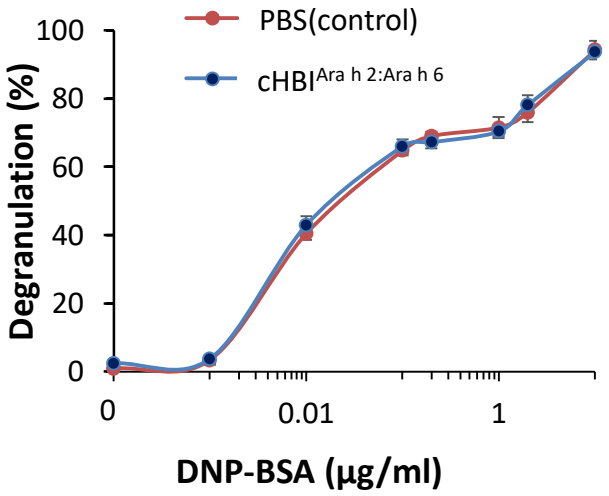


Figure S11. cHBI$^{Arah2:Arah6}$ inhibition does not occur via a non-specific mechanism. RBL-2H3 cells primed with anti-DNP IgE were incubated with or without cHBI$^{Arah2:Arah6}$, and washed. Degranulation was triggered by DNP-BSA and observed via beta-hexosaminidase assay. Since cHBI$^{Arah2:Arah6}$ was not designed for DNP, as expected, cHBI$^{Arah2:Arah6}$ did not inhibit degranulation induced by DNP-BSA. Data represent mean ±SD of triplicate experiments

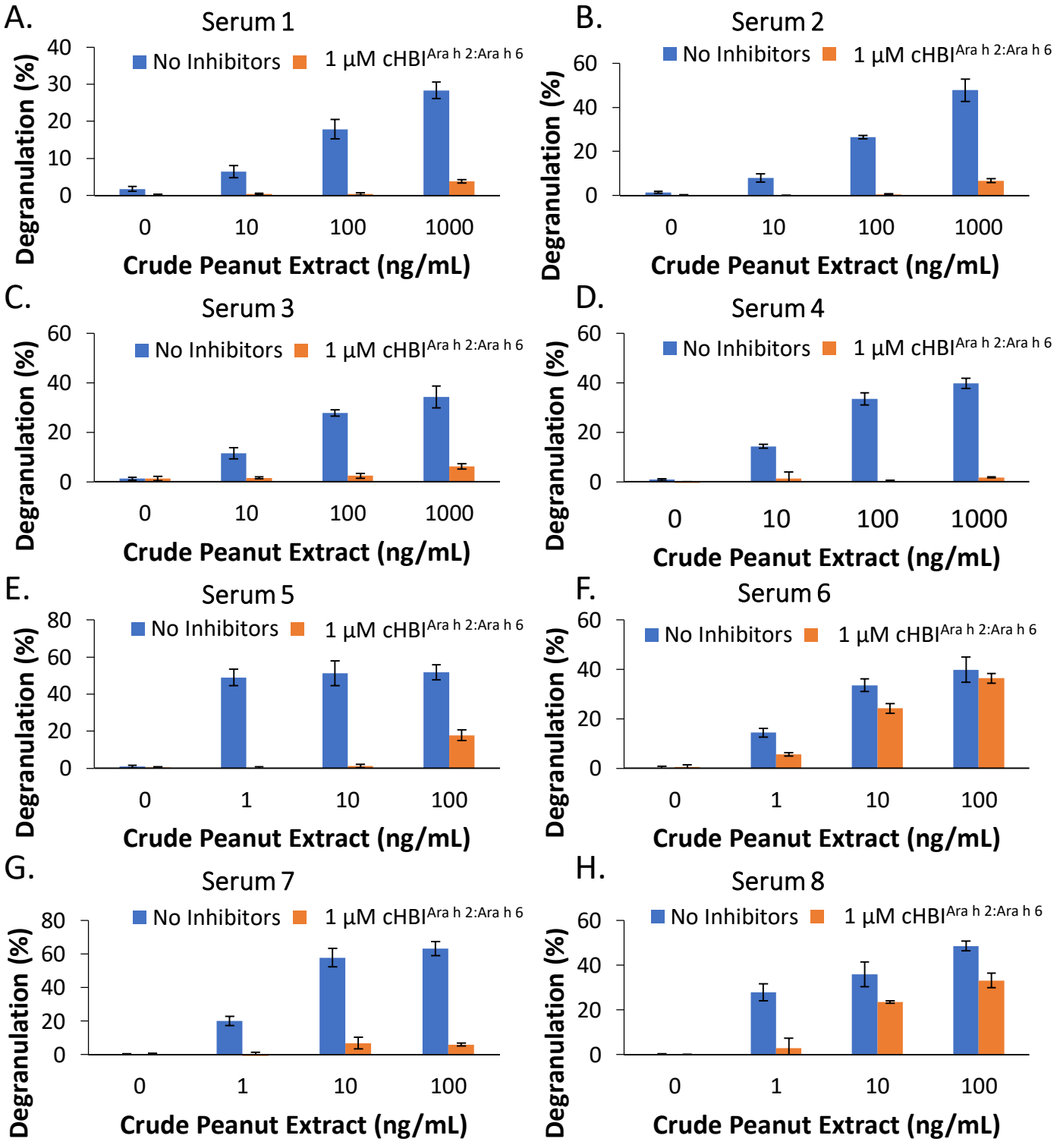


Figure S12. cHBI$^{Arah2:Arah6}$ inhibits cellular degranulation from RBL-SX38 cells primed with patient serum. RBL-SX38 cells were primed with each of the first 8 patient sera (serum 1-8), washed and incubated with 1 µM of cHBI$^{Arah2:Arah6}$ for 16 hours or without inhibitors. Cells were then washed and challenged with varying concentrations between 0-1000 ng/mL of crude-peanut-extract. Results presented in A to H are degranulation inhibition experiments for serum 1 to 8 respectively.

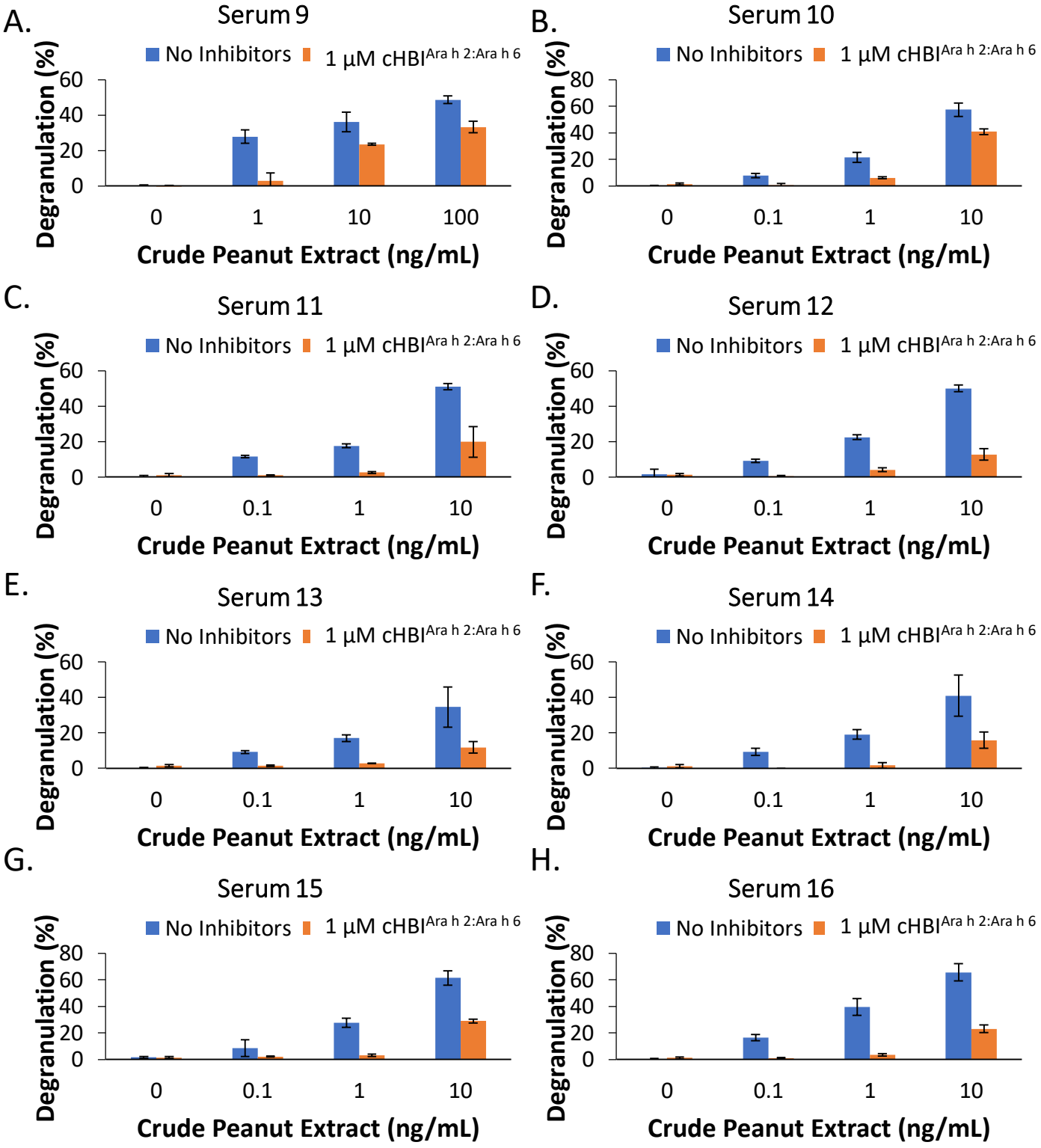


Figure S13. cHBI$^{Arah2:Arah6}$ inhibits cellular degranulation from RBL-SX38 cells primed with patient serum. RBL-SX38 cells were primed with each of remaining 8 patient sera (serum 9-16), washed and incubated with 1 µM of cHBI $^{Arah2:Arah6}$ for 16 hours or without inhibitors. Cells were then washed and challenged with varying concentrations between 0-100 ng/mL of crude-peanut-extract. A-H represents degranulation inhibition for serum 9-16 respectively.

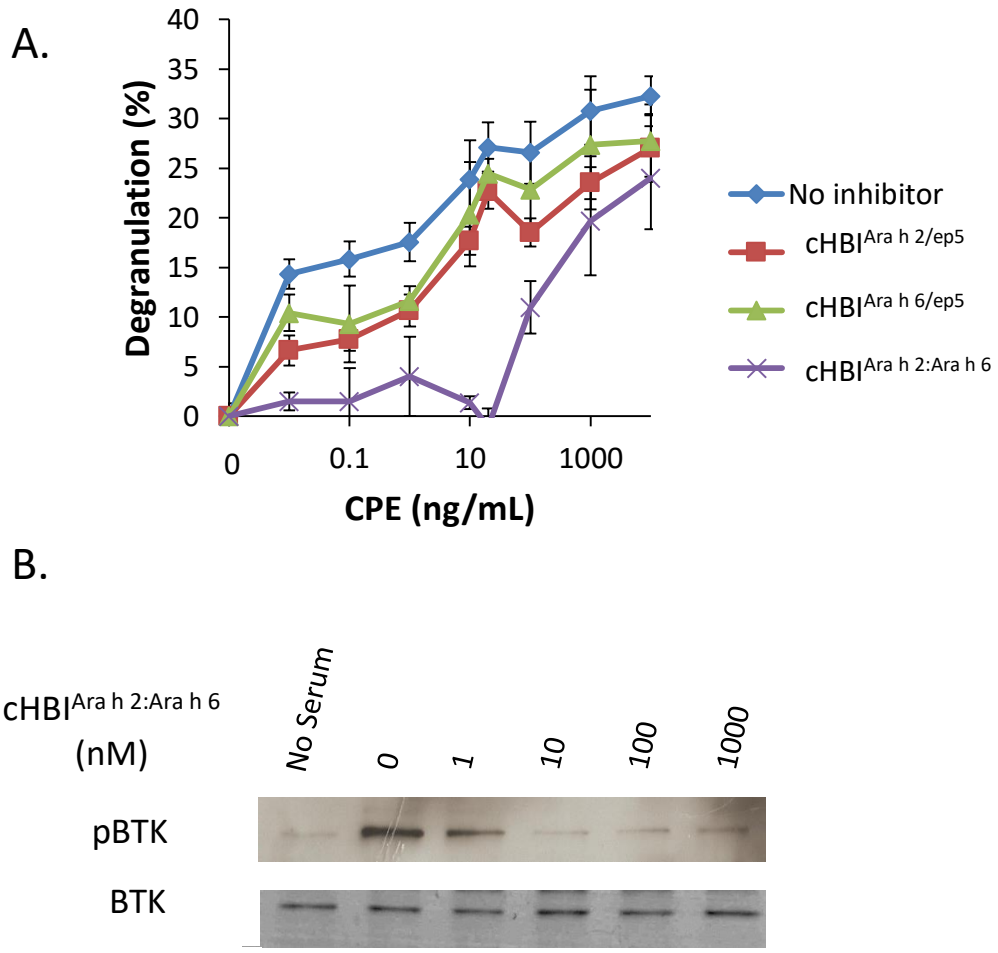


Figure S14. Both cHBI formulations in cHBI$^{Arah2:Arah6}$ are required for inhibition of degranulation triggered by crude-peanut-extract, and cHBI$^{Arah2:Arah6}$ inhibits intracellular activation. (A) RBL-SX38 cells primed with serum-4 were inhibited with 1 µM of either cHBI $^{Arah2/ep5}$ or cHBI $^{Arah6/ep5}$ or cHBI$^{Arah2:Arah6}$; next washed and then challenged with 100 ng/mL of crude-peanut-extract. (B) RBL-SX38 cells primed with serum-4 were given various concentrations of cHBI$^{Arah2:Arah6}$ and then challenged with 100 ng/mL of crude-peanut-extract and then the level of pBTK activity was observed with western blot. Error bars indicate ±SD of triplicate experiments.

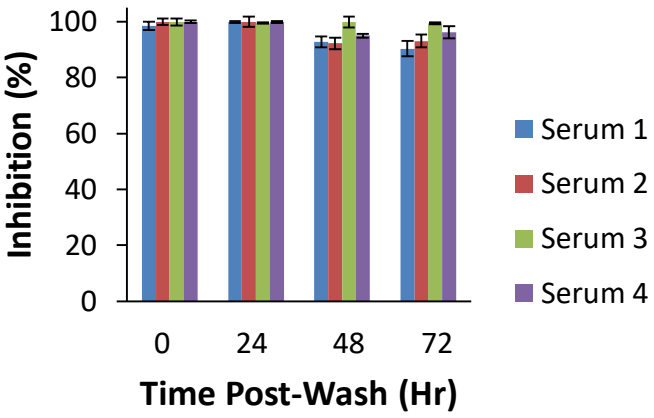


Figure S15. cHBI$^{Arah2:Arah6}$ inhibit peanut extract induced degranulation in an irreversible fashion. RBL-SX38 cells were similarly primed with serum 1,2,3 or 4 and incubated with 1 µM of cHBI $^{Arah2:Arah6}$ for 16 hours. 3A. After washing to remove unbound cHBI but prior to allergen challenge, cells were incubated in cell culture media for 24-72 hours and then challenged with 100 ng/mL of crude-peanut-extract. Degranulation inhibition was retained for a period of at least 72 hours. Error bars indicate ±SD of triplicate experiments.

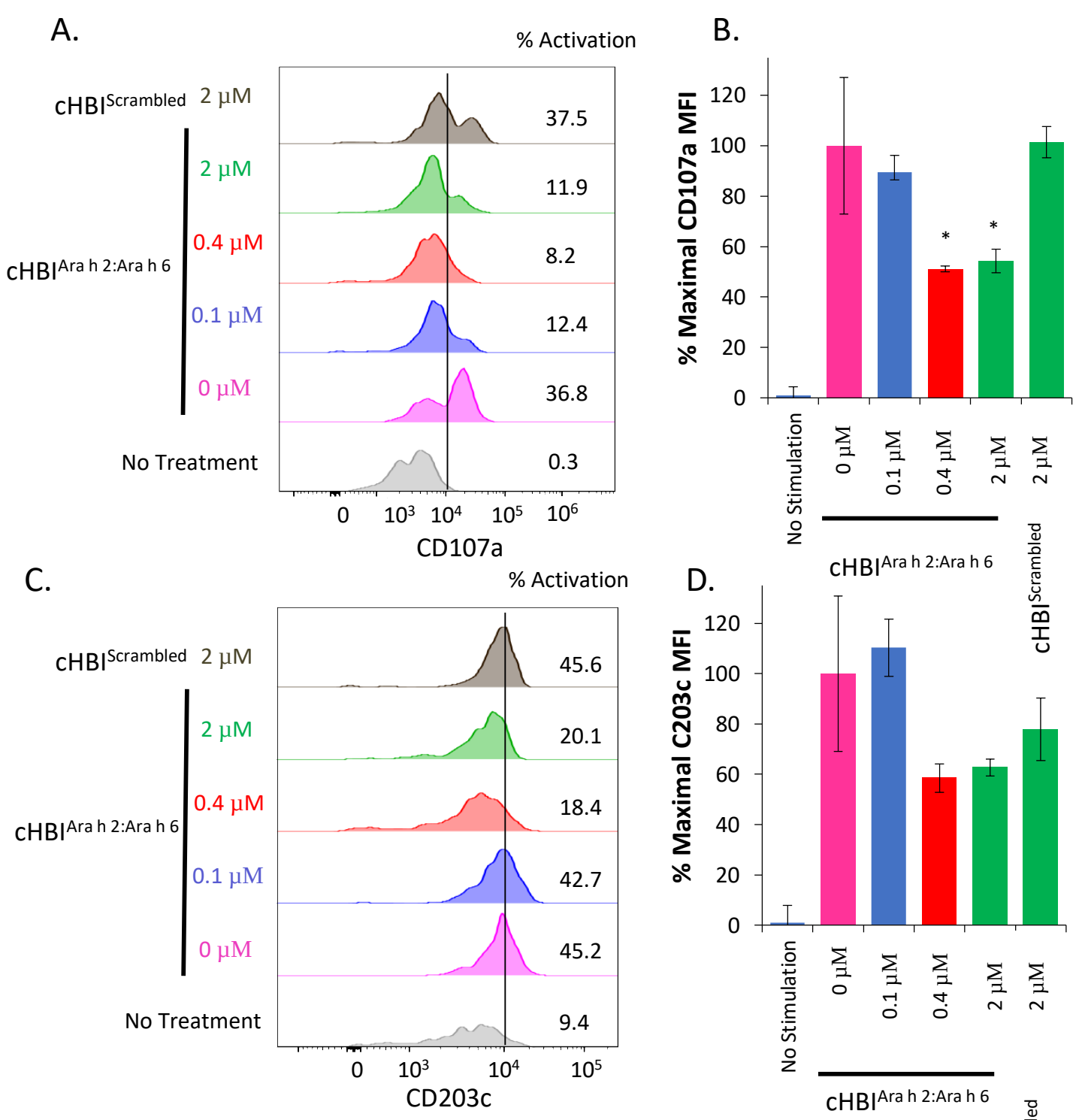


Figure S16. BAT test for CD107a and CD203c (A)Basophil activation tests (BAT) for CD107a expression. Whole blood was taken from a volunteer with a history of peanut allergies, mixed 1:1 with RPMI media with cHBI$^{Arah2:Arah6}$ at either 0.1 µM (in blue), 0.4 µM (in red) or 2 (in green) µM for 4 hours, challenged with 100 ng/mL of crude-peanut-extract, stained for the basophil expression marker CD107a and then analyzed for upregulation of CD107a in basophils using flow cytometry. Flow cytometry plots are shown with fluorescence intensity on x axis and number of basophils on y axis. A control where blood was incubated without crude-peanut-extractor inhibitor (No Treatment) or where blood was given no inhibitor (0 µM) were used. As an additional control, blood was incubated with 2 µM of a 1:1 mixture of cHBIs presenting scrambled sequences of Ara h 2 epitope 5 and Ara h 6 epitope 5 respectively (cHBI^{Scrambled}, shown in dark grey). Basophils were considered activated if their CD107a fluorescence intensity was greater than the threshold of $1x10^4$ (black line). Percent activated basophils are indicated on the right side of the flow cytometry plot. (B) Percent MFI (mean fluorescence intensity) of flow cytometry plots from part A are shown. (C)Basophil activation tests (BAT) for CD203c expression. BAT was performed in a similar fashion as A and analyzed for upregulation of CD203c. Basophils were considered activated if their CD203c fluorescence intensity was greater than the threshold of $1x10^4$ (black line). Percent activated basophils are indicated on the right side of the flow cytometry plot. (D) Percent MFI (mean fluorescence intensity) of flow cytometry plots from part C are shown. Error bars indicated ±SD of triplicate experiments. * $p<0.05$

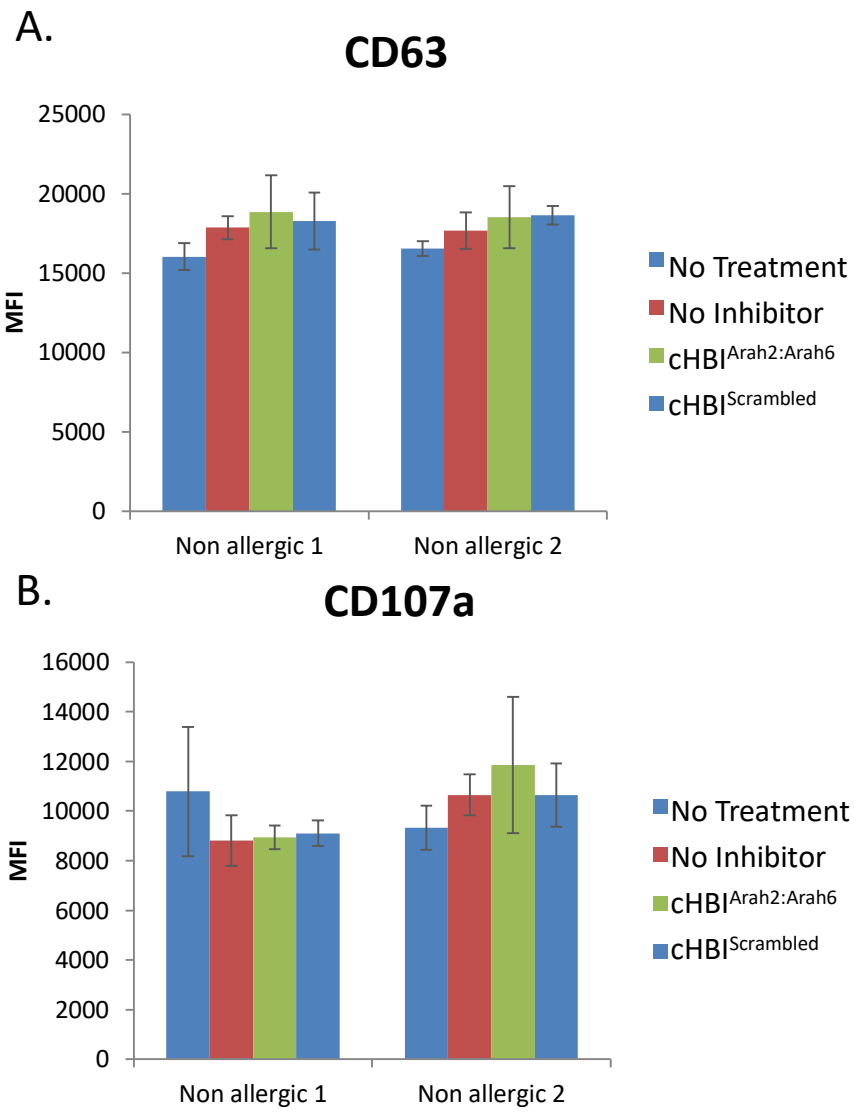


Figure S17. BAT assay of unallergic patients. Whole blood taken from two unallergic patients were incubated with 1 μM cHBI$^{Arah2:Arah6}$ or cHBI^{Scrambled} and then challenged with 100 ng/mL peanut-extract. Levels of surface activation markers (A) CD63 expression (B) CD107a expression are shown. Error bars indicate SD of technical triplicates.