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Supplementary Information for

The Molecular Characterization of Antibody Binding to a Superantigen-like Protein from a Commensal Microbe

Marta T. Borowska[1*], Christoph Drees[2*], Alexander E. Yarawsky[4], Jeffrey J. Bunker[2,5], Andrew B. Herr[3,4,6], Albert Bendelac[2,5], and Erin J. Adams[1,5]

[*]These authors contributed equally to this work.

Correspondence: Erin J. Adams and Albert Bendelac

Email: ejadams@uchicago.edu and abendela@bsd.uchicago.edu

This part of PDF file includes:

Extended methods

Constructs

For mouse Fab clones: variable domain of both heavy and k chains of mouse clones were amplified from plasmids provided by Albert Bendelac. Similarly, both chains of modified IgG constant domain amplified from plasmids gifted by Anthony Kossiakoff were fused with corresponding variable domains by overlap extension PCR. Both chains were cloned with sequence and ligation independent (SLIC) method in a pACgp67a vector with a 3C protease sequence, either basic or acid zippers and a C-terminal 6x histidine tag.

Human Fab clones were obtained and unmodified from Patrick Wilson and were not modified. Full length and truncated Ibp plasmids were obtained from Albert Bendelac. Individual domains were cloned from these plasmids into pET301b with N-terminal 6x histidine tag. For alanine scanning mutagenesis, contacting residues were selected based on PISA analysis of Ibp-VH5 Fab structure. Alanine mutants were introduced by quickchange mutagenesis using most common alanine codons.

Proteins expression and purification

Baculoviruses with all hybrid mouse Fab chains were prepared by transfecting and amplifying into Sf9 cells. Mouse Fabs were expressed in the Hi5 cells by co-infecting with baculoviruses containing both chains and incubating up to 3 days. Fabs were purified from the supernatant using Ni-NTA agarose in buffer A, followed by overnight 4°C cleavage with protease 3C. On the next day, digested sample was passed through a second Ni-NTA subtractive step, where flow-through was collected, concentrated on Amicon 30-kDa MWCO (Millipore) and injected on size exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare).

Human Fabs were transfected into suspension Expi293 cells (ThermoFisher) using Expifectamine and according to manufacturer's protocol. Supernatant was harvested after 5 days, spun down for 15min at 4°C /3,000 xg and incubated overnight with 1mL bed volume (bv) Ni-NTA agarose in buffer A with final concentration (f.c.) 20mM imidazole, pH 7.2. The next day the column was washed with high salt buffer A supplemented with 20 mM imidazole, then eluted in 5mL of buffer A with 350mM imidazole. Samples were further purified by size exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare) in buffer A and single peak fraction was used for binding studies or concentrated for AUC studies using in 30-kDa MWCO Amicon Ultra centrifugal filter (Millipore). Both wild-type and alanine mutants of all Ibp constructs were expressed in the same way. DNA constructs were heat shock transformed into E. coli BL21(DE3)pLysS and single colony was used to inoculate 25mL terrific broth (TB) starter culture supplemented with ampicillin, grown overnight at 37°C and 250 rpm shaking. 1L TB/Amp culture was inoculated with 1% of starter culture, induced

with 1mM IPTG at OD600 and continued to grow for 4hrs/ 37°C with 250rpm. Cells were harvested by centrifugation and resuspended in 50 ml buffer A with high salt (10 mM Hepes pH 7.2, 500 mM NaCl supplemented with 1 mM PMSF and 2 mM DNase I). Cells were lysed by passing three times through a high-pressure microfluidizer (Avastin), soluble fraction was collected by centrifugation for 40 min at 4°C /18,500 xg and 2mL bv Ni-NTA beads, f.c. 20mM imidazole 7.2 were added for overnight incubation. Next day after washing the column with high salt buffer A supplemented with 20 mM imidazole, protein was eluted in buffer A with 10 column volume (c.v.) 350mM imidazole. Typically, the protein was further purified by size exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare) in buffer A. Peak fractions were pooled and concentrated to 5 mg/ml in a 3-kDa or 30-kDa MWCO Amicon Ultra centrifugal filter (Millipore).

Protein concentrations were determined by A280 using a calculated extinction coefficient. Purified IbpA constructs were mixed with 1.2-fold molar excess of smaller component and separated from excess by size exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare) in buffer A. Peak fractions were pooled, concentrated in a 50-kDa MWCO Amicon Ultra centrifugal filter (Millipore), large aggregates were removed by 0.22um spin filter columns, and either used immediately for crystallization or stored at 4°C for up to a month.

PBMC and splenic B cell isolation

Peripheral blood was diluted 1:1 with 1x PBS, overlaid on Ficoll-Paque PLUS density gradient media (GE Healthcare) and centrifuged at room temperature for 30 min and 400 xg with low brakes. Mononuclear cells were harvested from interlayer and washed with PBS before flow cytometry staining. Whole blood was obtained from healthy volunteers with approval from the University of Chicago Institutional Review Board.

For isolation of mouse B cells, single cell suspensions were prepared by grinding spleens from C57BL/6 mice between glass slides. Cell suspension in HBSS, 0.25% BSA were next filtered through 70 um cell strainers (Fisher). Cells were pelleted by 5 min centrifugation at 400 xg, resuspended in 1x RBC lysis buffer (ebioscience) and incubated for 5 min on ice for RBC lysis. To purify untouched mouse B cells, cells were incubated with anti-mouse CD43 (S7) and MACS purified using Streptavidin MicroBeads (Miltenyi) and autoMACS Separator with a purity of ≥90%.

Ibp labeling and lymphocyte flow cytometry staining

Recombinant Ibp protein solutions were buffer exchanged using ZebaTM Spin Desalting Columns (Fisher) and eluted in 1x PBS before labeling with Alexa Fluor 488 and Alexa Fluor 647 using

commercially available Alexa Fluor labeling kits (Fisher). Ibp concentration after labeling was measured by Nanodrop.

For flow cytometry staining, human and mouse B cells were first incubated with Zombie dye life/dead stain (BioLegend) and either Human TruStain FcX Fc Receptor Blocking solution (Biolegend) or anti-mouse CD16/CD32 (Fisher). Next, cells were labeled for 20 min at 4°C to 8°C C with Ibp at typically 10 – 100 ng/ml or as indicated in figures or text. After washing with HBSS, 0.25% BSA, cells were incubated with fluorophore conjugated mAbs purchased from Biolegend, eBioscience and BD: anti-mouse CD19 (1D3), anti-human CD19 (HIB1) and antimouse IgM (II/41) for 20 min at 4°C to 8°C. Cells were washed again, resuspended in HBSS, 0.25% BSA and analyzed using a LSR II cytometer (BD).

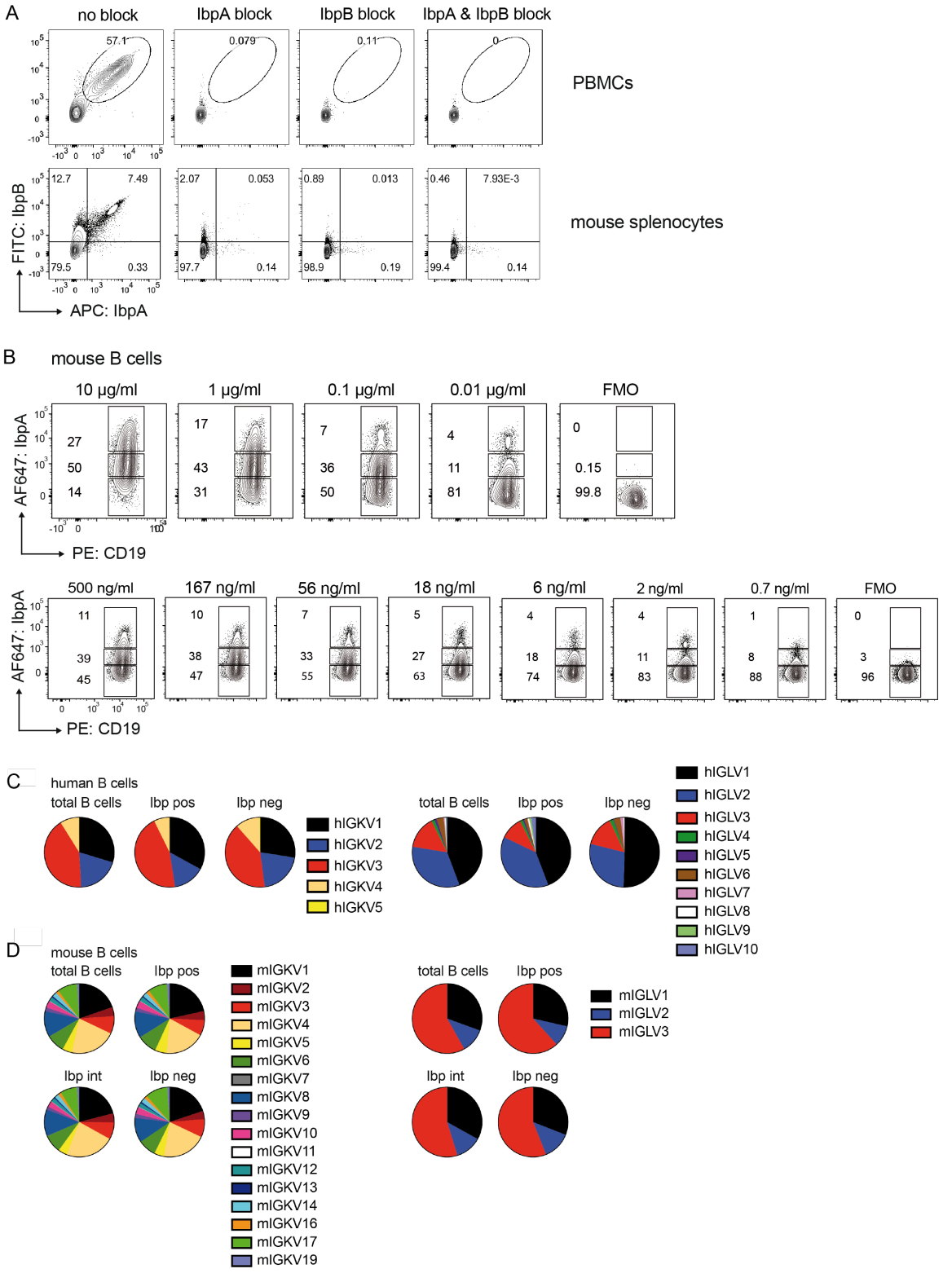
Calcium-flux analysis

Murine splenocytes and PBMCs were resuspended at a concentration of $10x10^6$ – $15x10^6$ cells/ml in HBSS with 6 micromolar Fura Red™ AM (Thermo Fisher) and 0.01% Pluronic F-127 (Thermo Fisher) and incubated for 30 min in a 37° C water bath. After washing with HBSS, splenocytes were stained with anti-mouse CD16/CD32 (Thermo Fisher) and anti-mouse CD19 (1D3) for 5 – 10 min at room temperature. PBMCs were stained with Human FcX Fc Receptor Blocking solution (Biolegend) and anti-human CD19 (clone: HIB1). Cells were equilibrated in a 37°C water bath for at least 10 minutes before stimulation. 10x DAPI was added right before cell acquisition and unstimulated cells were recorded for 20-30 seconds at a threshold rate of 5000 cells per second using a LSR II flow cytometer (BD). Indicated stimuli were prepared at a 10x final concentration in HBSS (final concentration was 10 microgram/ml for all stimuli if not indicated otherwise), added to the cell suspension and samples were recorded for additional 3-5 minutes. Ratiometric calcium flux was calculated with FlowJo (BD) and the Kinetics tool.

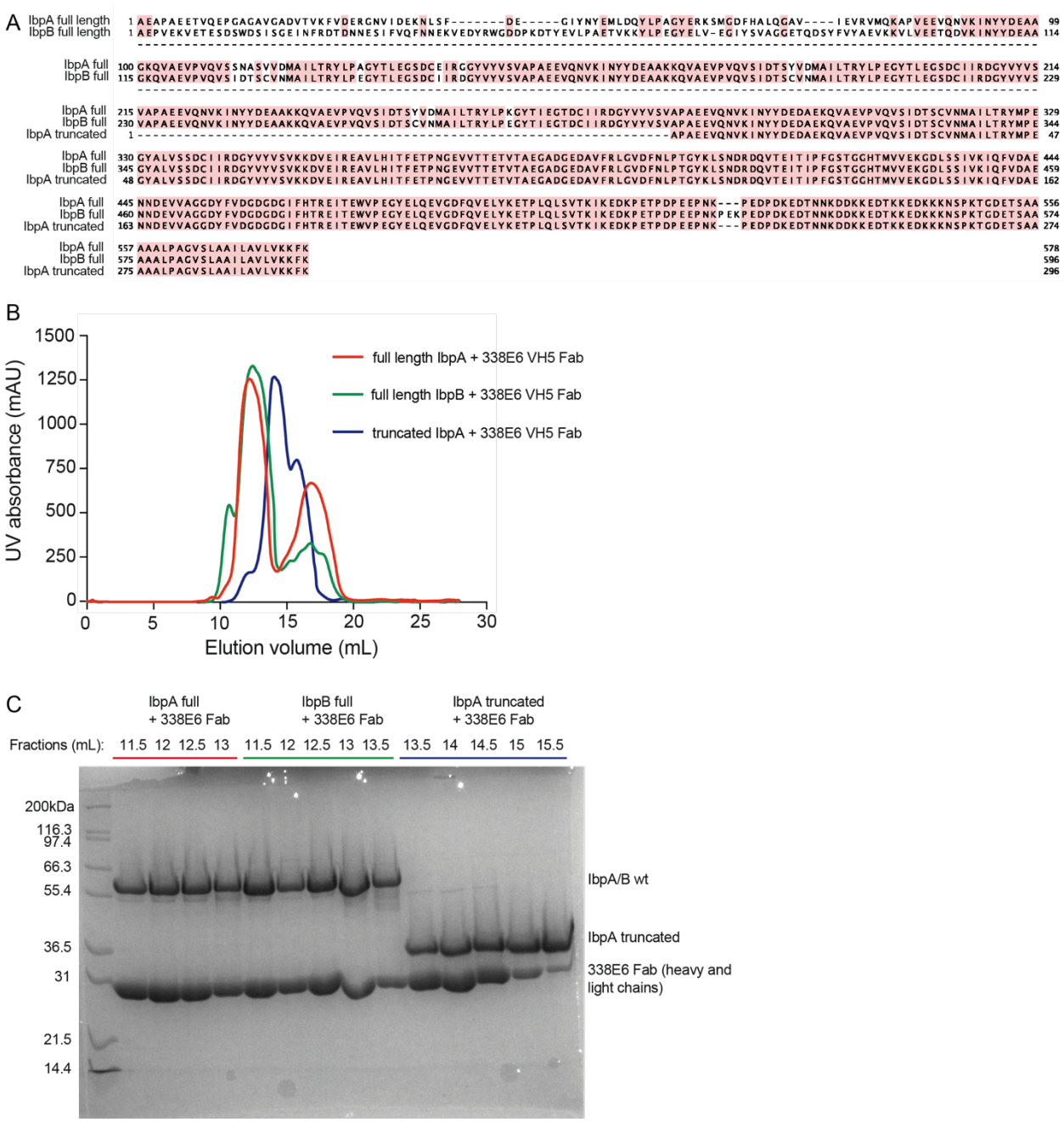
Statistical analysis

Statistical analysis by unpaired student's t test or as indicated in the figure legends was performed using GraphPad Prism 6.

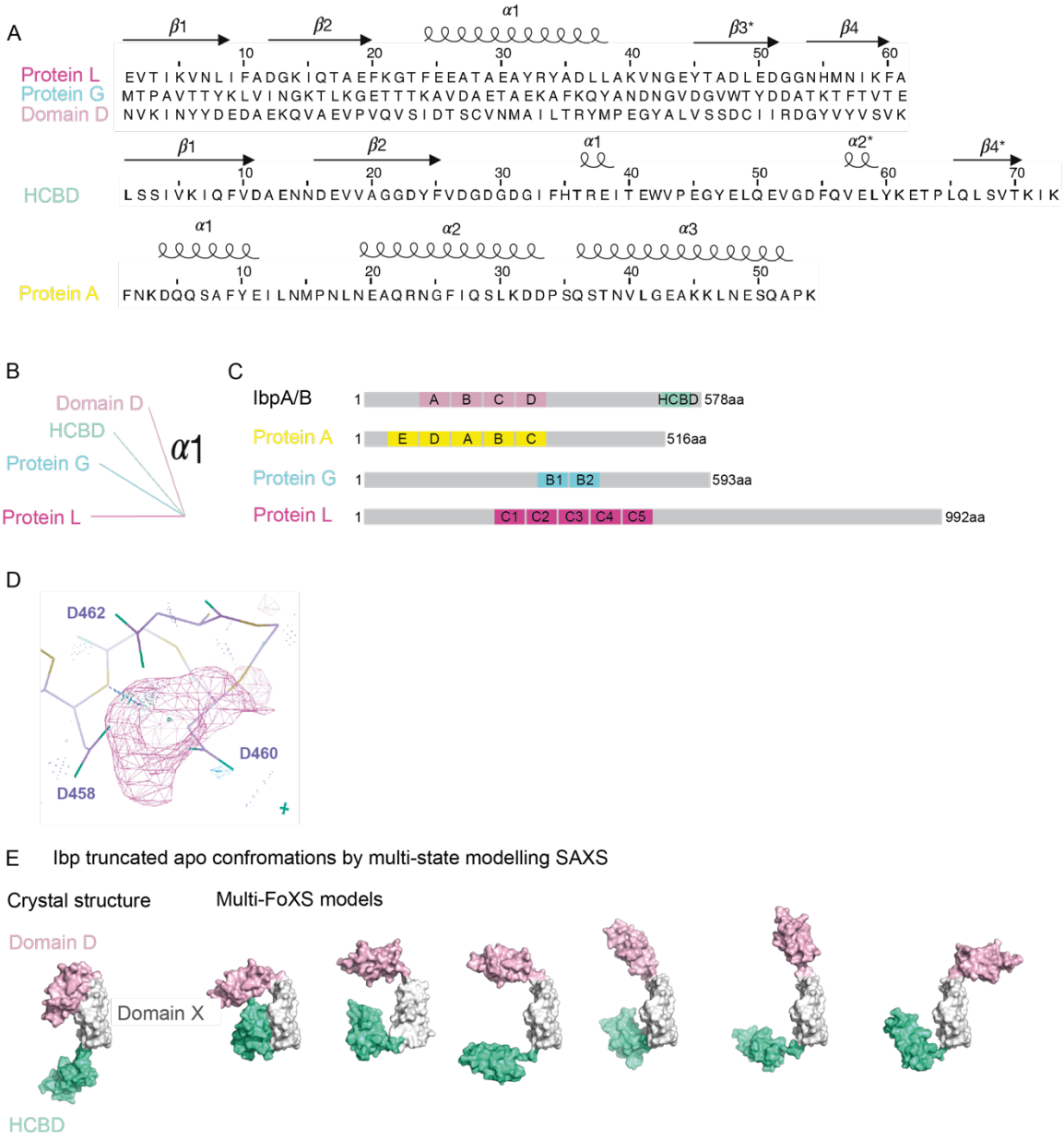
Supplementary Figures and Tables



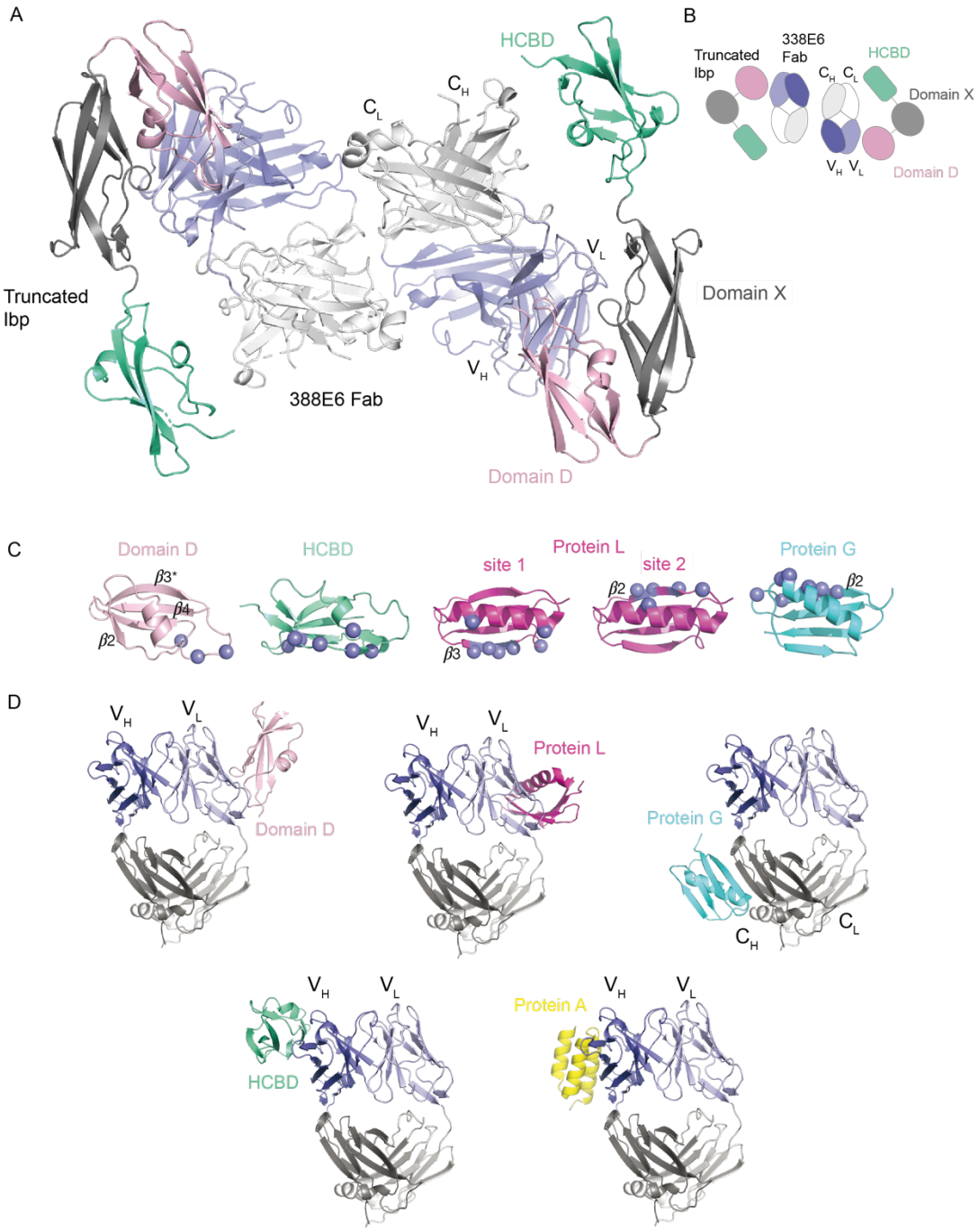
Supplement Figure 1. (A) Blocking studies using 15ug/mL of block either IbpA, IbpB or both, followed by staining with 60ng/mL of IbpA and IbpB. (B) Representative contour plots show IbpA titrations using wild-type mouse B cells. Pre-gates were drawn on CD19pos splenocytes. (C) Immunoglobulin light chain lambda (left) and kappa (right) repertoire of sorted human B cells. (D) Immunoglobulin light chain lambda (left) and kappa (right) repertoire of sorted mouse B cells.



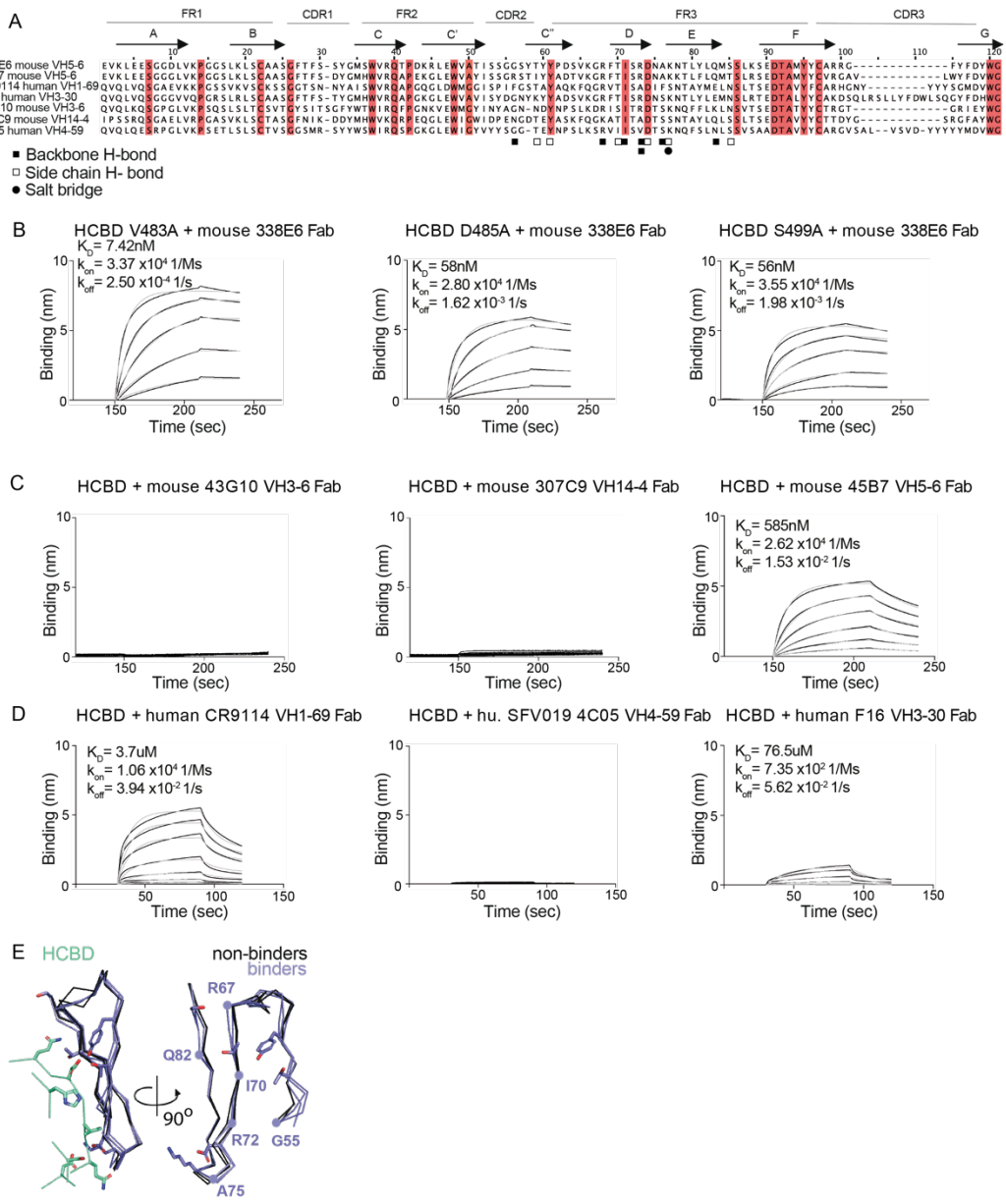
Supplement Figure 2. (A) Amino acid sequence alignment for full length IbpA, B and truncated IbpA. Red highlighting marks conserved residues. (B) Size exclusion chromatography profile of full length IbpA, B or truncated IbpA in complex with mouse 338E6 VH5 Fab. (C) Reduced SDS-PAGE size exclusion fractions of full length IbpA, B and truncated IbpA complexes with mouse 338E6 VH5 Fab.



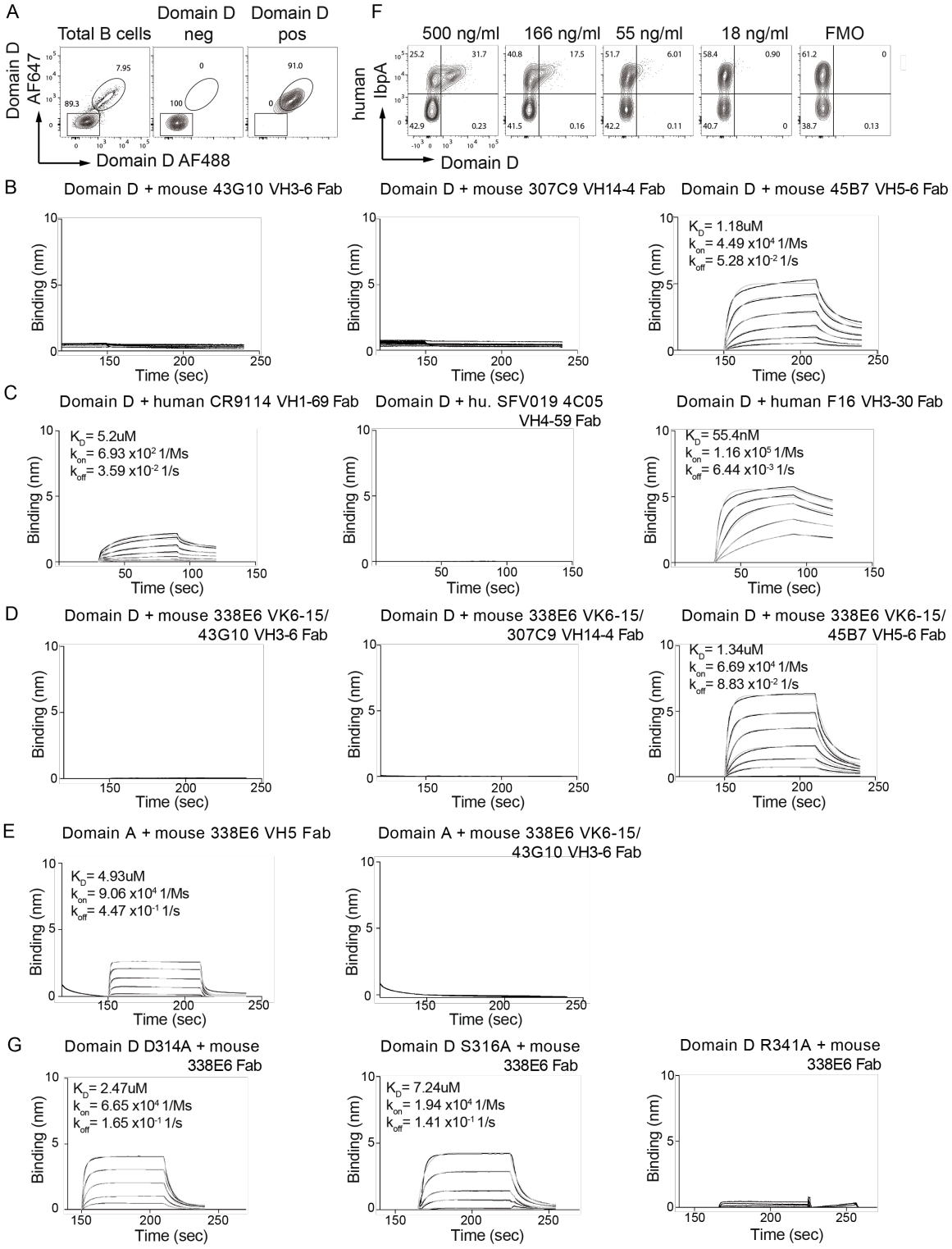
Supplement Figure 3. (A) Amino acid sequence alignment and secondary structure annotations of individual repeat domains from Protein L (magenta), Protein G (cyan) and Domain D (lightpink), HCBD (greenteal) and from Protein A (yellow). (B) Schematic of relative positioning of alpha helices in Domain D, HCBD, Protein G and L. (C) Cartoon representation of repeat regions in full length sequence of superantigens Protein A, G and L in comparison with IbpA/B. (D) Metal ion binding site by a DGDGDG motif in HCBD. (E) IbpA truncated apo conformations determined by multi-state modelling by SAXS.



Supplement Figure 4. (A) Asymmetric unit of the complex crystal structure. (B) Cartoon representation of asymmetric unit. (C) Structures and binding interface of superantigen-like folds of Ibp Domain D and HCBD in comparison with Protein L and G. Spheres represent C_a of interacting residues. (D) Crystal structures show docking sites and superantigens bound to Fab fragments: IbpA Domain D (top left), Protein L (top middle), Protein G (top right), IbpA HCBD (bottom left) and Protein A (bottom right).

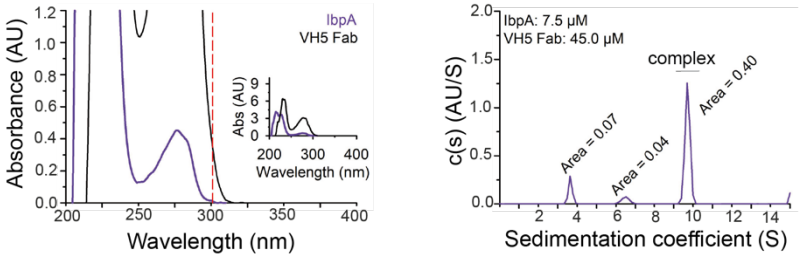


Supplement Figure 5. (A) Sequence alignment of analyzed mouse and human VH germlines and comparison between interacting residues from mouse 338E6 VH5 Fab from structure. Secondary structures, loop regions and interacting residues are marked accordingly. Participating residues are marked according to the legend below. Red highlighting marks conserved residues. (B) Example binding plots and affinities to 338E6 Fab of alanine mutants compared with wild type HCBD. (C) Example binding plots between wild-type HCBD and different mouse VH germlines. (D) Example binding plots between wild-type HCBD and different human VH germlines. (E) Ribbon representation of selected structures of binding (slate) and non-binding (black) VH families to HCBD (greenteal); highlighted are interacting residues: backbone (slate circles and names of residues) and side chains (right).

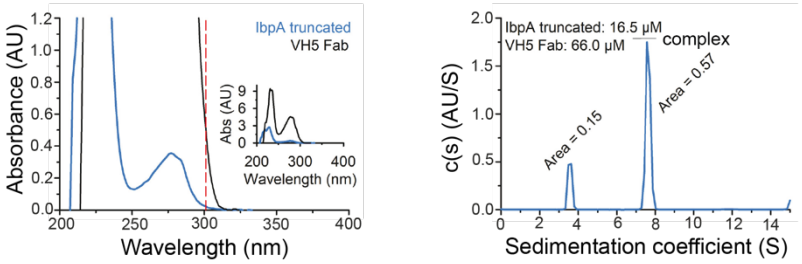

A
Total B cells
Domain D neg
Domain D pos
Domain D AF647
Domain D AF488
F
500 ng/ml
166 ng/ml
55 ng/ml
18 ng/ml
FMO
human IbpA
Domain D
B
Domain D + mouse 43G10 VH3-6 Fab
Domain D + mouse 307C9 VH14-4 Fab
Domain D + mouse 45B7 VH5-6 Fab
K_D= 1.18uM
k_on= 4.49 x10^4 1/Ms
k_off= 5.28 x10^-2 1/s
C
Domain D + human CR9114 VH1-69 Fab
K_D= 5.2uM
k_on= 6.93 x10^2 1/Ms
k_off= 3.59 x10^-2 1/s
Domain D + hu. SFV019 4C05 VH4-59 Fab
Domain D + human F16 VH3-30 Fab
K_D= 55.4nM
k_on= 1.16 x10^5 1/Ms
k_off= 6.44 x10^-3 1/s
D
Domain D + mouse 338E6 VK6-15/ 43G10 VH3-6 Fab
Domain D + mouse 338E6 VK6-15/ 307C9 VH14-4 Fab
Domain D + mouse 338E6 VK6-15/ 45B7 VH5-6 Fab
K_D= 1.34uM
k_on= 6.69 x10^4 1/Ms
k_off= 8.83 x10^-2 1/s
E
Domain A + mouse 338E6 VH5 Fab
K_D= 4.93uM
k_on= 9.06 x10^4 1/Ms
k_off= 4.47 x10^-1 1/s
Domain A + mouse 338E6 VK6-15/ 43G10 VH3-6 Fab
G
Domain D D314A + mouse 338E6 Fab
K_D= 2.47uM
k_on= 6.65 x10^4 1/Ms
k_off= 1.65 x10^-1 1/s
Domain D S316A + mouse 338E6 Fab
K_D= 7.24uM
k_on= 1.94 x10^4 1/Ms
k_off= 1.41 x10^-1 1/s
Domain D R341A + mouse 338E6 Fab
Binding (nm)
Time (sec)

Supplement Figure 6. (A) Flow cytometry of sorted Domain D negative and positive B cells used for heavy chain repertoire analysis. (B) Example binding plots between wild-type Domain D and different mouse VH germlines. (C) Example binding plots between wild-type Domain D and different human VH germlines. (D) Example binding plots between wild-type Domain D and mouse fixed VK6-15 and swapped VH germlines. (E) Example binding plots between wild-type Domain A and mouse 338E6 VH5 Fab or hybrid Fab (338E6 Vk6-15 paired with 43G10 VH3 chain). (F) Human PBMCs were stained with IbpA-AF647 at 166ng/ml and Domain D-AF488 titrated as indicated above representative contour plots. (G) BLI plots of binding between three different alanine substitutions in Domain D interface and mouse 338E6 VH5 Fab.

A IbpA full + mouse 338E6 VH5 Fab complex



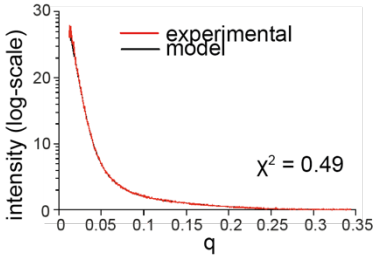
B IbpA trunc. + mouse 338E6 VH5 Fab complex



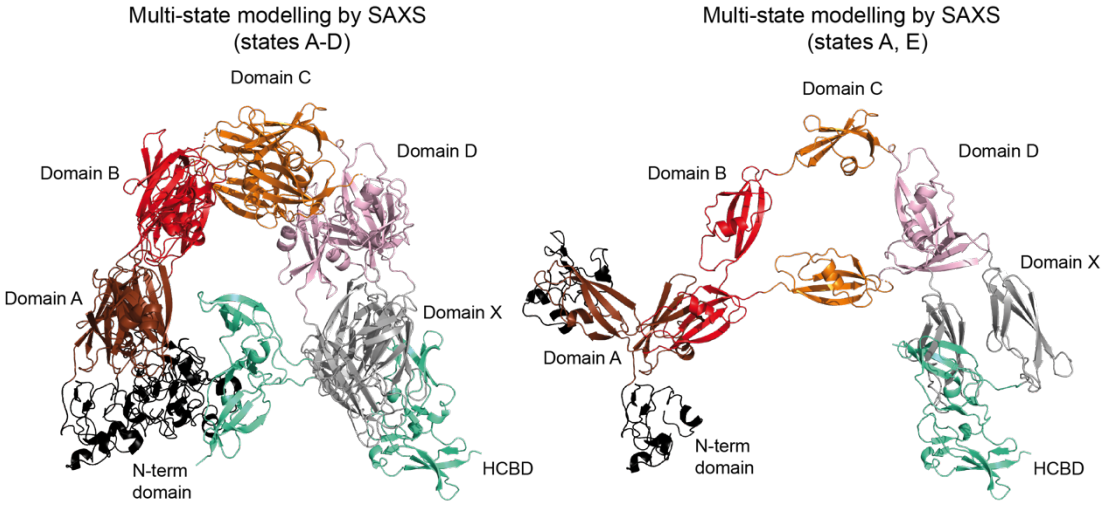
C Experimental molecular weight calculations from AUC

Protein/ Complex	$S_{20,w}$	f/f_0	MW_{exp} (kDa)	MW_{seq} (kDa)
IbpA full wt	2.81	2.13	67.0	61.3
IbpA trunc. wt	2.11	1.81	33.8	30.5
338E6 Fab	3.57	1.42	50.4	50.6
IbpA full+338E6 Fab	9.0	1.9	~343	314.3
IbpA trunc.+338E6 Fab	7.2	1.58	~190	182.3
F16 Fab	3.6	1.32	47.2	50.2
IbpA full + F16 Fab	N/A	N/A		
IbpA trunc.+ F16 Fab	4.0	1.32	78.4	80.7

D IbpA full length apo



E Ibp truncated apo confromations by multi-state modelling SAXS



Supplement Figure 7. (A) Absorbance spectrum of full length IbpA and mouse 338E6 VH5 Fab at the concentrations loaded in the AUC experiment (left). The inset shows the full range of absorbance along the Y-axis. A red dashed line marks the wavelength (301 nm) used in the AUC experiment shown (right). (B) Absorbance spectrum of truncated IbpA and mouse 338E6 VH5 Fab at the concentrations loaded in the AUC experiment (left). The inset shows the full range of absorbance along the Y-axis. A red dashed line marks the wavelength (301 nm) used in the AUC experiment shown (right). (C) Table of experimental molecular weight calculations of IbpA constructs apo and in complex with mouse 338E6 VH5 Fab from AUC. (D) Experimental fit of SAXS data of full length IbpA apo analyzed by multi-state modelling with MultiFoXS and plotted by I(q). (E) Apo models of full-length IbpA based on SAXS data; states A-D are in similar conformations (left), distinct state E in comparison to state A (right).

Supplement Table 1. Data collection and refinement statistics of truncated IbpA + mouse 338E6 VH5 Fab complex.

	338E6 VH5 Fab + IbpA 283-578aa
Wavelength energy (keV)	12.68
Wavelength range (Å)	0.98
Resolution range	76.59 - 2.102 (2.177 - 2.102)
Space group	P 1 21 1
Unit cell	85.2401 102.191 118.28 90 102.05 90
Total reflections	3855863 (373296)
Unique reflections	115284 (11391)
Multiplicity	33.4 (32.5)
Completeness (%)	99.10 (98.16)
Mean I/sigma(I)	21.03 (1.12)
Wilson B-factor	34.94
R-merge	0.6793 (3.98)
R-meas	0.6898 (4.046)
R-pim	0.1178 (0.7122)
CC1/2	0.971 (0.307)
CC*	0.993 (0.686)
Reflections used in refinement	114342 (11300)
Reflections used for R-free	1997 (198)
R-work	0.2297 (0.3517)
R-free	0.2658 (0.3975)
CC(work)	0.840 (0.394)
CC(free)	0.821 (0.352)
Number of non-hydrogen atoms	9999
macromolecules	9724
ligands	2
solvent	273
Protein residues	1265
RMS(bonds)	0.008
RMS(angles)	1.04
Ramachandran favored (%)	95.58
Ramachandran allowed (%)	4.02
Ramachandran outliers (%)	0.4
Rotamer outliers (%)	5.83
Clashscore	7.33
Average B-factor	48.71
macromolecules	48.97
ligands	41.6
solvent	39.36
Number of TLS groups	1

(Statistics for the highest-resolution shell are shown in parentheses.)

Supplement Table 2. Contact residues between individual HCBD, Domain D and Domain X of truncated IbpA and mouse 338E6 VH5 Fab

Contact residues between IbpA truncated (HCBD, Domain D and middle Domain X) and 338E6 Fab

338E6 Fab Heavy chain	HCBD	Contact
Ile51	Val483, Gly484	vdW
Gly55 O	Val483 N	H***
Gly55	His466, Val483	vdW
Thr58 OG1	Asp485 OD2	H***
Thr58	Asp485	vdW
Tyr60 OH	Asp485 OD2	H***
Tyr60	Asp485	vdW
Gly66	Ile464	vdW
Arg67 O	Gln487 NE2	H***
Arg67	Gln487	vdW
Phe68	Gln487	vdW
Thr69 OG1	Gln487 NE2	H***
Thr69	Ile464, Asp485, Gln487	vdW
Ile70 O	Asp485 N	H***
Ile70	Asp485	vdW
Ser71	Val483, Asp485, Phe486	vdW
Arg72 N	Val483 O	H***
Arg72 O	Glu482 CA	H*
	Val483 N	H***
Arg72	Glu482, Val483	vdW
Asp73 OD1	Ser499 OG	H*
OD2	Ser499 OG	H***
Asp73	Glu482, Ser499	vdW
Asn74	Gln481, Glu482	vdW
Ala75 N	Gln481 OE1	H*
Ala75	Ala443, Gln481	vdW
Lys76 NZ	Asp447 OD2	SB
Lys76 NZ	Asp447 OD2	H***
Lys76	Asp447	vdW
Gln82 O	Gln487 NE2	H*
Gln82	Gln487, Leu490	vdW
Ser84 OG	Gln487 OE1	H***
Ser84	Gln487	vdW

338E6 Fab Light chain	Domain D	Contact
Ser7	Asp342	vdW
Met11	Ser316	vdW
Ser12	Asp314	vdW
Val19	Ser316	vdW
Ser20 N	Ser316 OG	H***
Ser20	Ser316, Ile339	vdW
Thr22	Ile340	vdW
Asp70	Arg341	vdW
Phe71 O	Arg341 NH1	H*
Phe71	Arg341	vdW
Thr72 OG1	Arg341 NH1	H***
	NH2	H***
Thr72	Ile339, Arg341	vdW
Lys107 NZ	Asp314 OD2	SB
Lys107 CD	Asp314 OD2	H*
Lys107 CE	Asp314 OD2	H*
Lys107	Asp314	vdW

338E6 Fab Light chain	middle Domain X	Contact
Asp60	Asp408	vdW
Ser76	Thr411	vdW
Asn77	Leu403	vdW

vdW: van der Waal contact (distance < 4 Å);
H: hydrogen bond (distance < 3.4 Å, *** and * denote high and low possibilities for bond formation);
SB: salt bridge (distance > 3.4 and < 4.5 Å, polar interaction between oppositely charged atoms).