SUPPLEMENTARY INFORMATION (SI) FIGURES AND TABLES

Title: Discovery of a junctional epitope antibody that stabilizes IL-6 and gp80 protein:protein interaction and modulates its downstream signaling

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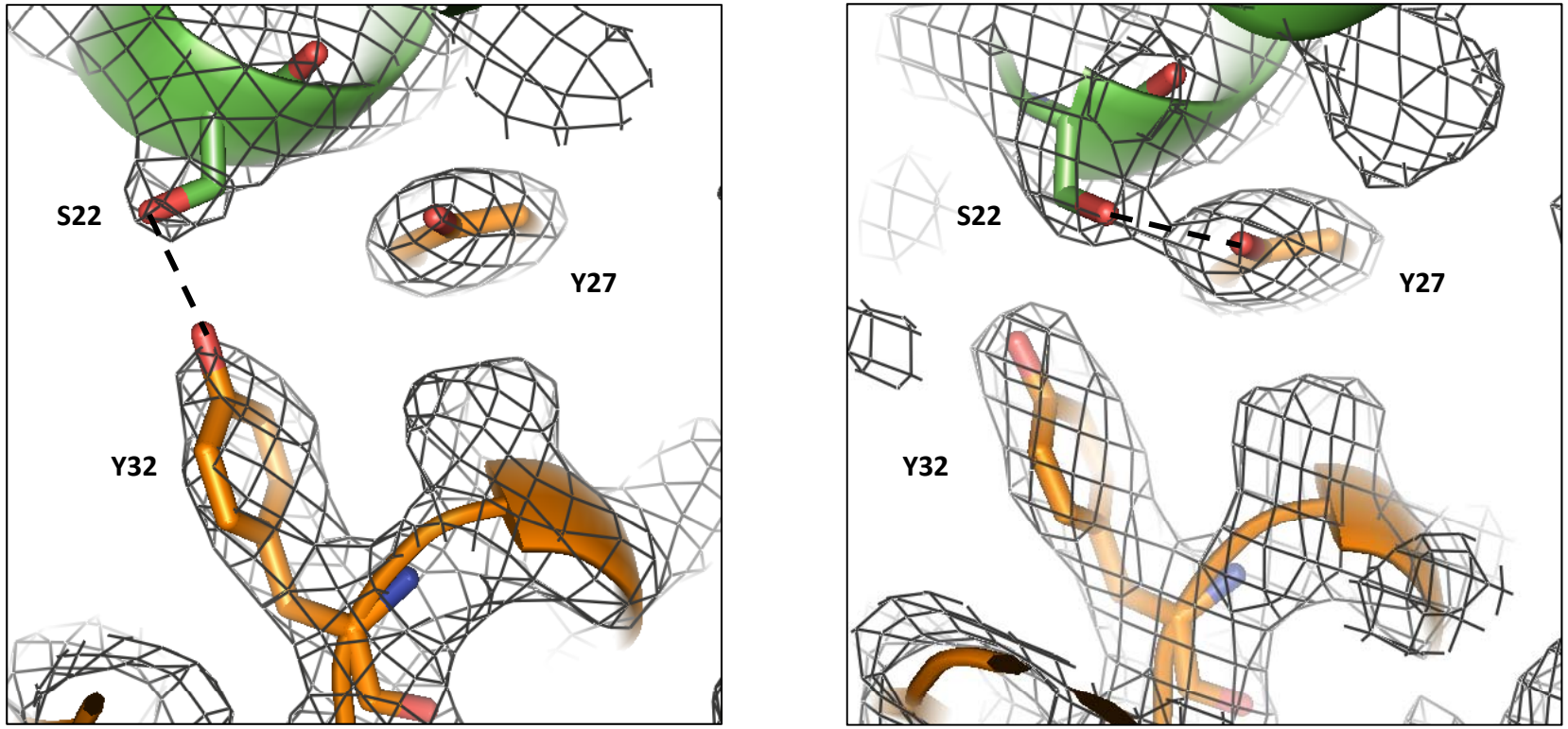
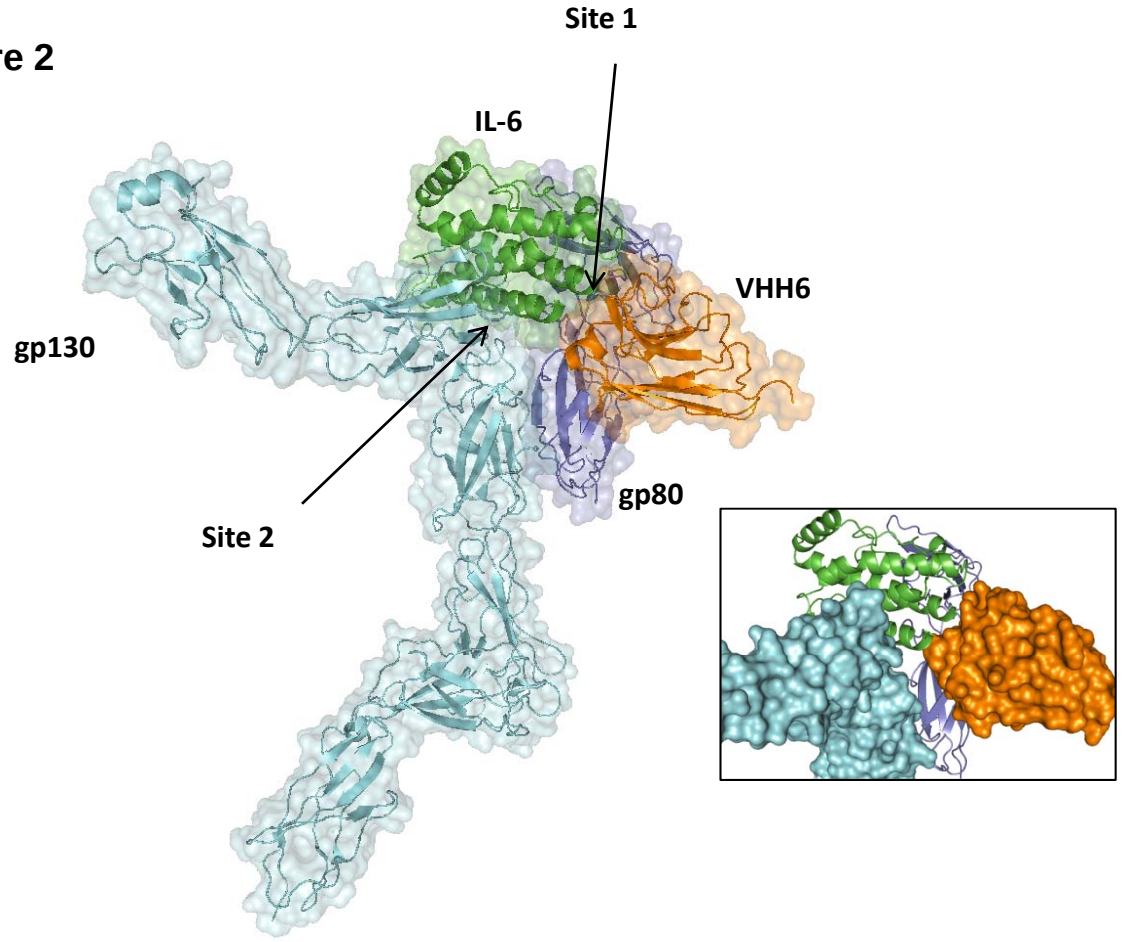
SI Figure 1



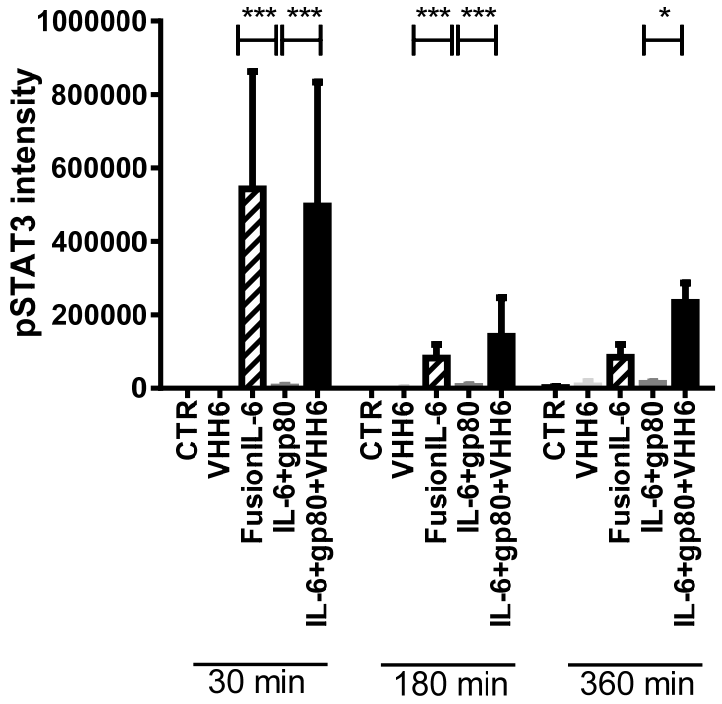
SI Figure 1. Differential hydrogen bonding in the two copies of the crystallographic asymmetric unit. There are two copies of the VHH6–IL-6–gp80 complex in the crystallographic asymmetric unit. In one copy, IL-6 (green) residue Ser22 forms a hydrogen bond with VHH6 (orange) residue Tyr32 (left), and in the second copy, Ser22 forms a hydrogen bond with VHH6 residue Tyr27 (right).

SI Figure 2



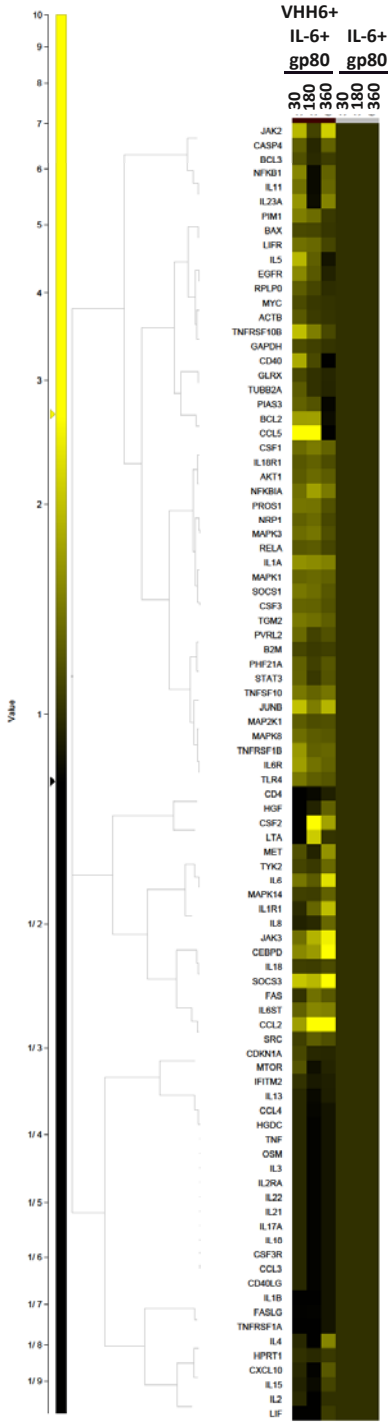
SI Figure 2. Model of crystal structure of VHH6–IL-6–gp80 superimposed with gp130.
Superimposition of IL-6 (green) and gp80 (blue) from the signaling complex, IL-6–gp80–gp130 (PDB code 1P9M), showed low r.m.s.d. values of 1.4±0.2 Å and 1.35±0.05 Å respectively, indicating that VHH6 (orange) holds IL-6 and gp80 together in a form able to bind gp130 (cyan).

SI Figure 3



SI Figure 3. Stabilization of the IL-6–gp80 complex by VHH6 promotes higher and sustained STAT3 phosphorylation signal in HUVECs comparable to FusionIL-6 fusion protein.
HUVECs were treated with FusionIL-6, IL-6+gp80 and VHH6+IL-6+gp80 as described in Materials and Methods. STAT3 phosphorylation signal was quantified at different time points using the “spot total intensity per object” parameter as described in Materials and Methods. Statistical analysis of pSTAT3 signal from three replicates was performed for each time point (statistical significance: *=p≤0.05; **=p≤0.01 and ***=p≤0.001). Unlike IL-6+gp80, in the presence of VHH6 or when FusionIL-6 was added to the culture, pSTAT3 was increased at earlier (30 min) and later time points (180 min and 360 min). y-axis: pSTAT3 fluorescence expressed as spot total intensity per object; x-axis: time (min).

SI Figure 4



SI Figure 4. Transcriptomic analysis of HUVECs treated with VHH6+IL-6+gp80 confirms selective up-regulation of pro-inflammatory genes.
Three different batches of HUVECs were analyzed at 30, 180 and 360 min. VHH6+IL-6+gp80 (sample) were compared to IL-6+gp80 (control). Data were analyzed using the RT^2 Profiler PCR array web based data analysis template v3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis) and changes in gene expression changes were calculated using the $\Delta\Delta C_t$ method with normalization of the raw data to housekeeping genes. A heat map was generated using Genedata's Analyst software. Genes in grey-black are down-regulated, while genes in yellow are up-regulated. Gene regulation for each sample is expressed as fold change compared to control.

SI Table 1

Table 1A: Data collection and refinement statistics (molecular replacement)

	Junctional Epitope Antibody in complex with IL-6 and gp80
Data collection	
Space group	C 1 2 1
Cell dimensions	
***a, b, c* (Å)**	249.03, 67.80, 78.16
α, β, γ (°)	90.00, 104.53, 90.00
Resolution (Å)	47.84-2.70(2.86-2.69)*
R_{meas} **(%)**	7.2(50.1)
$CC_{1/2}$ **(%)**	99.9(91.5)
$I / \sigma I$	20.88(3.97)
Completeness (%)	99.4(96.9)
Redundancy	7.5(7.5)
Refinement	
Resolution (Å)	20.00-2.70
No. reflections	34,870
R_{work}/R_{free}	0.2313/0.2872
No. atoms	
Protein	7590
Water	111
***B*-factors**	
Protein	56.33
Water	43.19
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	1.289

*Values in parentheses are for highest-resolution shell.

SI Table 1B: Single mutants of VHH6

	***k*d (1/s)**
wtVHH6	3.6E-04
N74A	1.5E-04
K113A	2.6E-04
Y32A	3.0E-04
S101A	3.9E-04
Y27A	7.0E-04

SI Table 1C: Double mutants of VHH6

	***k*d (1/s)**
wtVHH6	3.6E-04
Y27A Y32A	4.3E-04
N74A S101A	13.5E-04
K113A S101A	14.3E-04
K113S S101A	16.4E-04

SI Table 1. Additional data supporting the junctional epitope nature of VHH6.

(A) Data collection and refinement statistics (molecular replacement). To assess the contributions of the side chains to binding, alanine-scanning was carried out. The dissociation rate of single **(B)** and double mutants **(C)** were assessed using SPR.

SI Table 2: Peptides identified and quantified in IL-6 and gp80 by HDX-MS

SI Table 2A: Peptide identified in IL-6

Start Residue	End Residue	Sequence	2 timepoints with p<0.01	4 timepoints with p<0.01
0	11	APVPPGEDSKDV		
3	23	PPGEDSKDVAAPHRQPLTSSE		
21	28	SSERIDKQ	*	
21	31	SSERIDKQIRY	*	
22	31	SERIDKQIRY	*	
24	33	RIDKQIRYIL	*	*
25	36	IDKQIRYILDGI	*	*
29	39	IRYILDGISAL		
38	49	ALRKETCNKSNM	#	
39	49	LRKETCNKSNM	#	
40	49	RKETCNKSNM	#	
40	51	RKETCNKSNMCE		
41	61	KETCNKSNMCESSKEALAENN	#	
42	56	ETCNKSNMCESSKEA		
52	75	SSKEALAENNLNLPKMAEKDGCFQ		
53	63	SKEALAENNLN		
53	75	SKEALAENNLNLPKMAEKDGCFQ		
55	65	EALAENNLNLP		
58	67	AENNLNLPKM		
58	74	AENNLNLPKMAEKDGCF	*	*
59	80	ENNLNLPKMAEKDGCFQSGFNE	*	
60	67	NNLNLPKM		
60	70	NNLNLPKMAEK		
60	82	NNLNLPKMAEKDGCFQSGFNEET	*	
68	74	AEKDGCF	*	*
70	81	KDGCFQSGFNEE	*	
75	84	QSGFNEETCL		
85	92	VKIITGLL		
85	93	VKIITGLLE		
87	93	IITGLLE		
98	108	LEYLQNRFESS		
99	109	EYLQNRFESSE		
99	110	EYLQNRFESSEE		
99	112	EYLQNRFESSEEQA		

99	120	EYLQNRFESSEEQARAVQMSTK		
100	110	YLQNRFESSEE		
100	112	YLQNRFESSEEQA		
104	114	RFESSEEQARA		
106	112	ESSEEQA		
106	116	ESSEEQARAVQ		
109	116	EEQARAVQ		
115	122	VQMSTKVL		
115	122	VQMSTKVL		
120	126	KVLIQFL		
123	135	IQFLQKKAKNLDA		
126	133	LQKKAKNL	*	
126	135	LQKKAKNLDA	*	
126	144	LQKKAKNLDAITTPDPTTN	*	
126	145	LQKKAKNLDAITTPDPTTNA		
126	147	LQKKAKNLDAITTPDPTTNASL	*	
129	143	KAKNLDAITTPDPTT		
136	147	ITTPDPTTNASL	*	*
148	155	LTKLQAQN		
148	155	LTKLQAQN		
148	158	LTKLQAQNQWL		
148	160	LTKLQAQNQWLQD		
152	158	QAQNQWL		
159	165	QDMTTHL	*	
159	167	QDMTTHLIL	*	
161	167	MTTHLIL	*	
166	172	ILRSFKE	*	*
166	173	ILRSFKEF	*	*
166	174	ILRSFKEFL	*	
168	174	RSFKEFL	*	*
168	178	RSFKEFLQSSL	*	*

SI Table 2B: SI Table 2A: Peptide identified in gp80

Start Residue	End Residue	Sequence	2 timepoints with p<0.01	4 timepoints with p<0.01
66	86	HENLYFQGLAPRRCPAQEVAR		
73	82	GLAPRRCPAQ		
73	83	GLAPRRCPAQE	*	
73	85	GLAPRRCPAQEVA	*	
78	96	RCPAQEVARGAGAGDVPPE		
81	102	AQEVARGAGAGDVPPEEPQLSC	*	
82	95	QEVARGAGAGDVPP		
83	94	EVARGAGAGDVP	*	
84	100	VARGAGAGDVPPEEPQL	*	
86	100	RGAGAGDVPPEEPQL	*	
92	110	DVPPEEPQLSCFRKSPLSN	*	
94	100	PPEEPQL		
103	111	FRKSPLSNV		
105	123	KSPLSNVVCEWGPRSTPSL		
111	123	VVCEWGPRSTPSL		
112	123	VCEWGPRSTPSL		
115	129	WGPRSTPSLTTKAVL		
124	130	TTKAVLL		
130	141	LVRKFQNSPAED		
131	141	VRKFQNSPAED		
135	145	QNSPAEDFQEP		
139	158	AEDFQEPCQYSQESQKFSCQ		
141	147	DFQEPCQ		
146	164	CQYSQESQKFSCQLAVPEG		
147	164	QYSQESQKFSCQLAVPEG		
148	158	YSQESQKFSCQ		
152	158	SQKFSCQ		
157	170	CQLAVPEGDSSFYI		
159	168	LAVPEGDSSF	*	
162	168	PEGDSSF	*	
174	183	CVASSVGSKF		
176	183	ASSVGSKF		
195	205	LQPDPPANITV	*	
201	210	ANITVTAVAR		
206	216	TAVARNPRWLS	*	
207	214	AVARNPRW		
208	214	VARNPRW		
208	217	VARNPRWLSV	*	

208	218	VARNPRWLSVT	*	
218	229	TWQDPHSWNSSF	*	
221	229	DPHSWNSSF		
230	236	YRLRFEL		
235	245	ELRYRAERSKT		
237	245	RYRAERSKT		
243	256	SKTFTTWMVKDLQH	*	*
250	262	MVKDLQHHAVIHD	*	
250	266	MVKDLQHHAVIHDAWSG	*	*
250	273	MVKDLQHHAVIHDAWSGLRHVVQL	*	
265	273	SGLRHVVQL		
267	273	LRHVVQL		
268	275	RHVVQLRA		
274	283	RAQEEFGQGE	*	*
274	284	RAQEEFGQGEW	*	*
274	286	RAQEEFGQGEWSE	*	*
277	295	EEFGQGEWSEWSPEAMGTP		
280	296	GQGEWSEWSPEAMGTPW		
284	291	WSEWSPEA	*	
284	298	WSEWSPEAMGTPWTE		
284	303	WSEWSPEAMGTPWTESRSPP		
285	291	SEWSPEA		
285	298	SEWSPEAMGTPWTE		
285	303	SEWSPEAMGTPWTESRSPP		
287	298	WSPEAMGTPWTE		
289	303	PEAMGTPWTESRSPP		
292	298	MGTPWTE		

SI Table 2: Additional data supporting HDX-MS.

List of peptides identified in IL-6 **(A)** and gp80 **(B)** and quantified by HDX-MS. Asterisk (*) indicates deuterium uptake increasing the presence of VHH6; hash (#) indicates deuterium uptake decreasing in the presence of VHH6.

SI Table 3: SPR analysis

IL-6 immobilized	***k*d (1/s)**	**SE (*k*d)**	**KD (M)**	**SE (KD)**
gp80	0.046	5.40E-05	3.42E-08	7.20E-09
gp80+VHH6 (2 μM)	1.94E-04	1.90E-07	NA	

gp80 immobilized	***k*d (1/s)**	**SE (*k*d)**	**KD (M)**	**SE (KD)**
IL-6	0.048	4.70E-05	1.54E-08	1.50E-09
IL-6+VHH6 (2 μM)	2.10E-04	2.80E-07	NA	

VHH6 immobilized	***k*d (1/s)**	**SE (*k*d)**	**KD (M)**
IL-6+gp80 (2 μM)	3.09E-04	4.40E-07	NA
gp80+IL-6 (2 μM)	3.75E-04	6.00E-07	NA

gp130 immobilized	***k*a (1/Ms)**	***k*d (1/s)**
gp80 [IL-6 (2 μM)]	3.6 10^5	0.037
gp80 [IL-6 (2 μM)+VHH6 (2 μM)]	4.7 10^5	0.029
IL-6 [gp80 (2 μM)]	3.6 10^5	0.035
IL-6 [gp80 (2 μM) + VHH6 (2 μM)]	4.4 10^5	0.036

SI Table 3: Additional SPR data analysis.

During SPR studies binding of IL-6, gp80 and VHH6 were individually tested in a concentration series (0-250 nM, as two-fold serial dilution). When proteins were tested in combinations (IL-6 and gp80, IL-6 and VHH6, gp80 and VHH6, gp80 and IL-6 and VHH6) one of the proteins was titrated in a concentration series (0-250 nM), while the other was kept constant at an excess concentration of 2 μM (in brackets) to ensure complex formation. A total of four proteins were immobilized on the chip, from top to bottom: IL-6, gp80, VHH6 and gp130.

SI Table 4: In-house constructs

Construct	Expression Host	Promoter	Affinity Tag	Expressed Protein
pNAFL-8His-fusionIL-6(gp80D123)	CHO	CMV	8His	gp80(L20-S320)(C211A, C277A)-IL-6(V30-M212)
pNAFL-gp80D123-hscFc	CHO	CMV	Single chain human IgG Fc fragment	gp80(M1-P322)(C211A, C277A)-hscFc
pNAFL-8His-gp80V4	CHO	CMV	8His	gp80V4
pTrx-6His-hIL-6	*E.coli*	T7	Thioredoxin-6His	Trx-6His-IL-6(A28-M212)
pTrx-6His-IL-6(S21)	*E.coli*	T7	Thioredoxin-6His	Trx-6His-IL-6(S49-M212)
pIMMs-6His-VHH6	Expi-HEK	CMV	6His	VHH6-6His

SI Table 4: In-house designed constructs.

All constructs listed in the table were designed in-house to support the immunization campaign, antibody screening, junctional antibody characterization and biophysics analysis.