CSL305: a dual functional therapeutic antibody targeting complement C2 and FcRn

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Figure S1. SPR sensorgrams of CSL305 and WT mAbs binding to human C2 and activated fragments.

Table S1. Binding of C2 and C2b from different species to CSL305.

Figure S2. SPR sensorgrams of soluble human FcRn/B2M binding to WT and CSL305 mAbs.

Table S2. Binding of cynomolgus monkey and mouse FcRn/B2M to CSL305 under acidic and neutral conditions.

Table S3. Affinity of human Fc gamma receptors to CSL305 by SPR.

Figure S3: Inhibition of complement activity by CSL305 using sera from different species.

Figure S4. The effect of CSL305 on HSA recycling *in vitro*.

Figure S5. Preparation of the CSL305 Fab-C2b complex for crystallization.

Figure S6. Comparison of CSL305-C2b structure with previously generated C2b structure.

Table S4. Epitope determination of parental hu4D8 mAb on human C2 Zymogen by Alanine mutagenesis.

Figure S7. The effect of parental hu4D8 anti-C2 mAb on C1s-mediated cleavage of huC2.

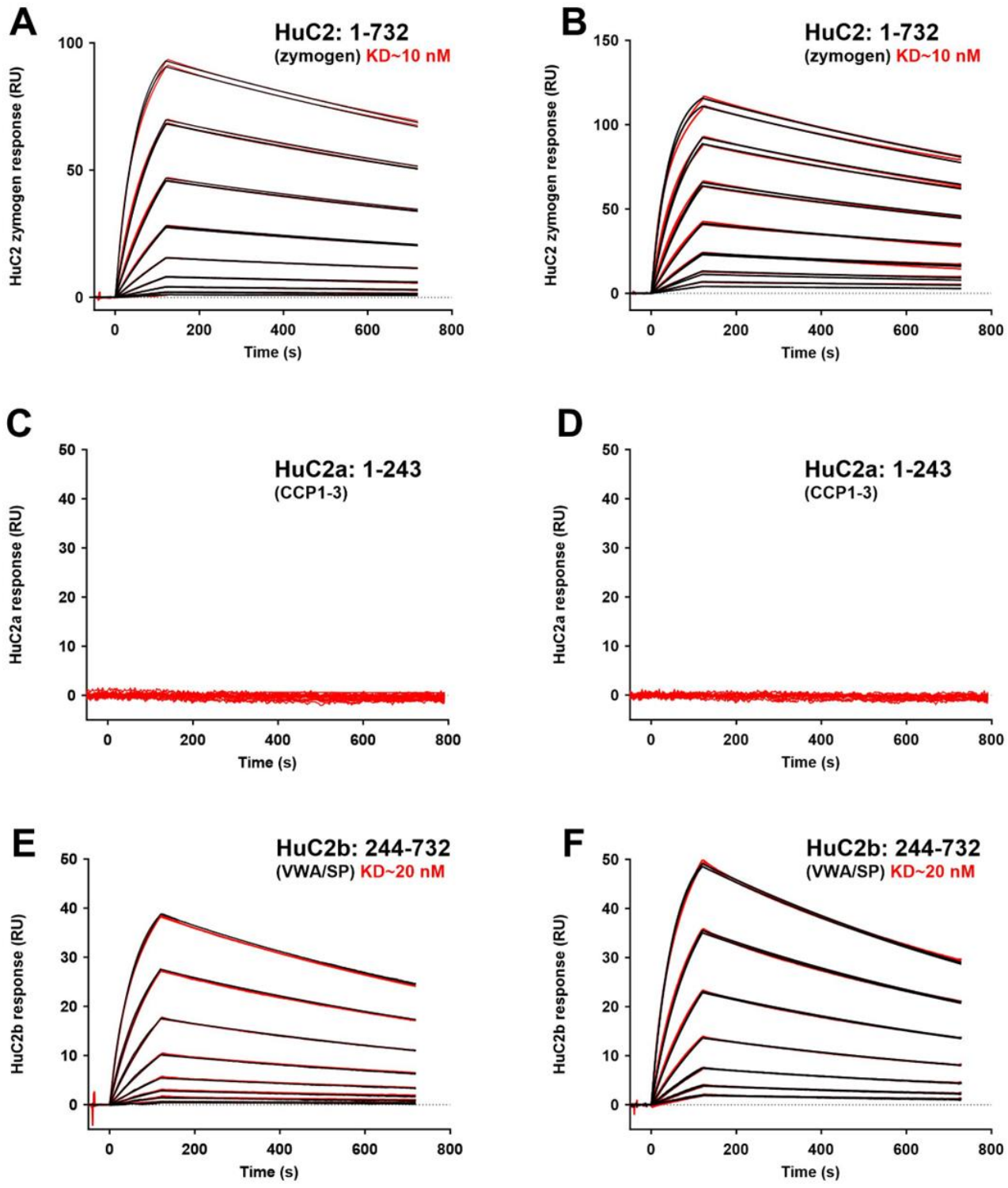


Figure S1. SPR sensorgrams of CSL305 and WT mAbs binding to human C2 and activated fragments. Panels show double-referenced sensorgrams across a range of analyte concentrations of human C2 zymogen binding to (A) CSL305 and (B) WT mAbs; huC2a binding to (C) CSL305 and (D) WT mAbs; huC2b binding to (E) CSL305 and (F) WT mAbs. Each red line represents two overlaid sensorgrams. All data were, where possible, fit to a kinetic 1:1 model (black lines) including a term for mass transport.

Anti-C2 mAb	Description	k_a (1/Ms)	k_d (1/s)	cynoC2 K_D (nM)	N
aghu4D8 (WT)	Affinity-matured wild-type	5.39E+04	5.13E-04	9.5 ± 0.1	4
CSL305	Affinity matured with Fc-YPY motif	5.21E+04	5.30E-04	10.2 ± 0.1	4
		k_a (1/Ms)	k_d (1/s)	cynoC2b K_D (nM)	
aghu4D8 (WT)	Affinity-matured wild-type	3.08E+04	7.46E-04	24.2 ± 0.1	4
CSL305	Affinity matured with Fc-YPY motif	2.95E+04	7.61E-04	25.8 ± 0.3	4
		Mouse, Rat, Rabbit, Dog, Pig, Sheep C2			N
aghu4D8 (WT)	Affinity-matured wild-type	No binding			3
CSL305	Affinity matured with Fc-YPY motif	No binding			3

Table S1. Binding of C2 and C2b from different species to CSL305. Shown are the kinetic rate constants and affinities of CSL305 and WT (aghu4D8) mAbs to C2 zymogen from cynomolgus (cyno) monkey, mouse, rat, rabbit, dog, pig and sheep were assessed. Cyno C2b was also assessed. k_a, association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant. K_D indicated as Mean ± SD (in nM) from multiple measurements. Values were calculated from sensorgram data fit to a 1:1 binding model. Cynomolgus C2 and Rhesus C2 share 100% amino acid identity, based on the GenBank accession numbers XP_005553508 and XP_014991516, respectively, and so are interchangeable.

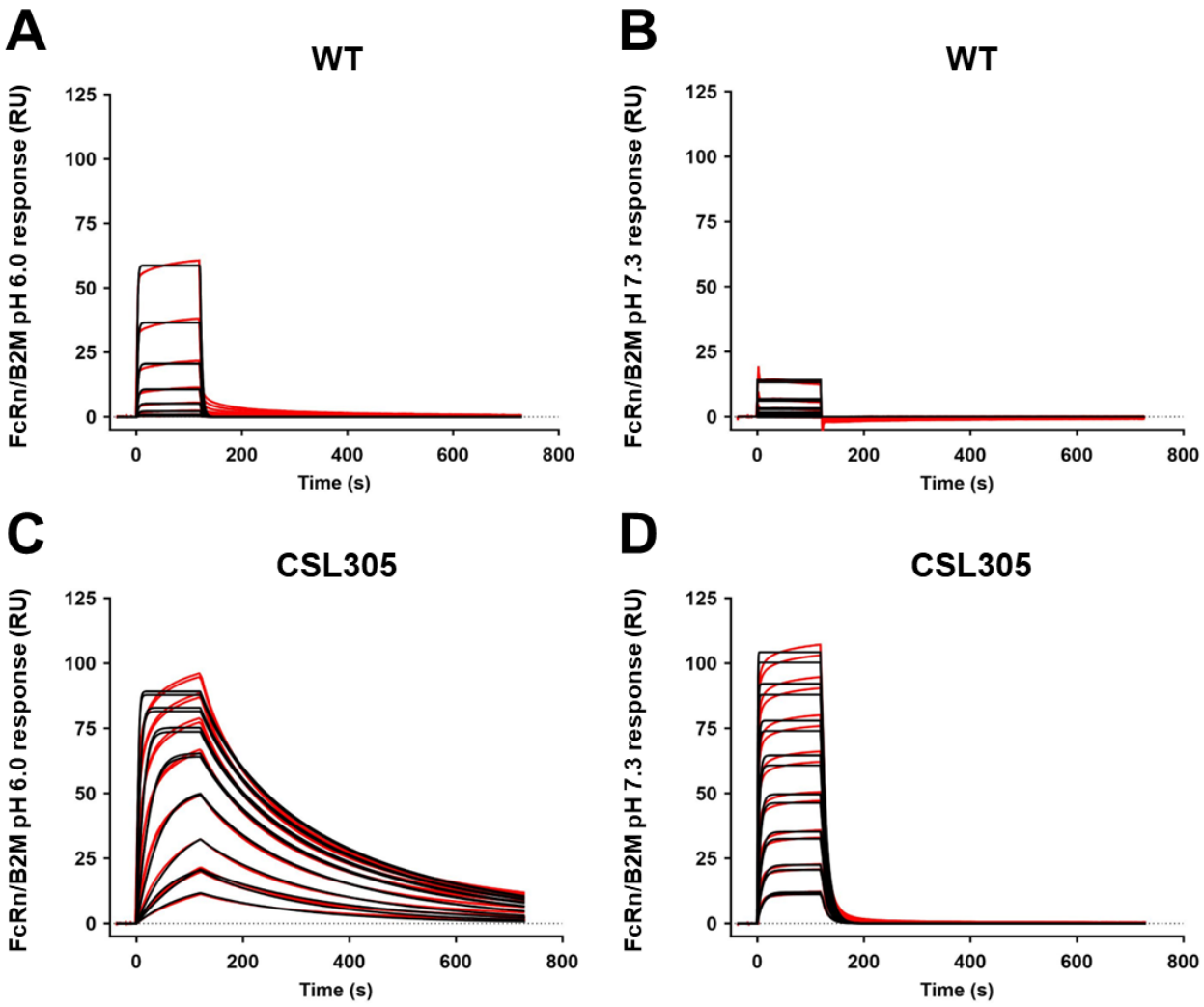


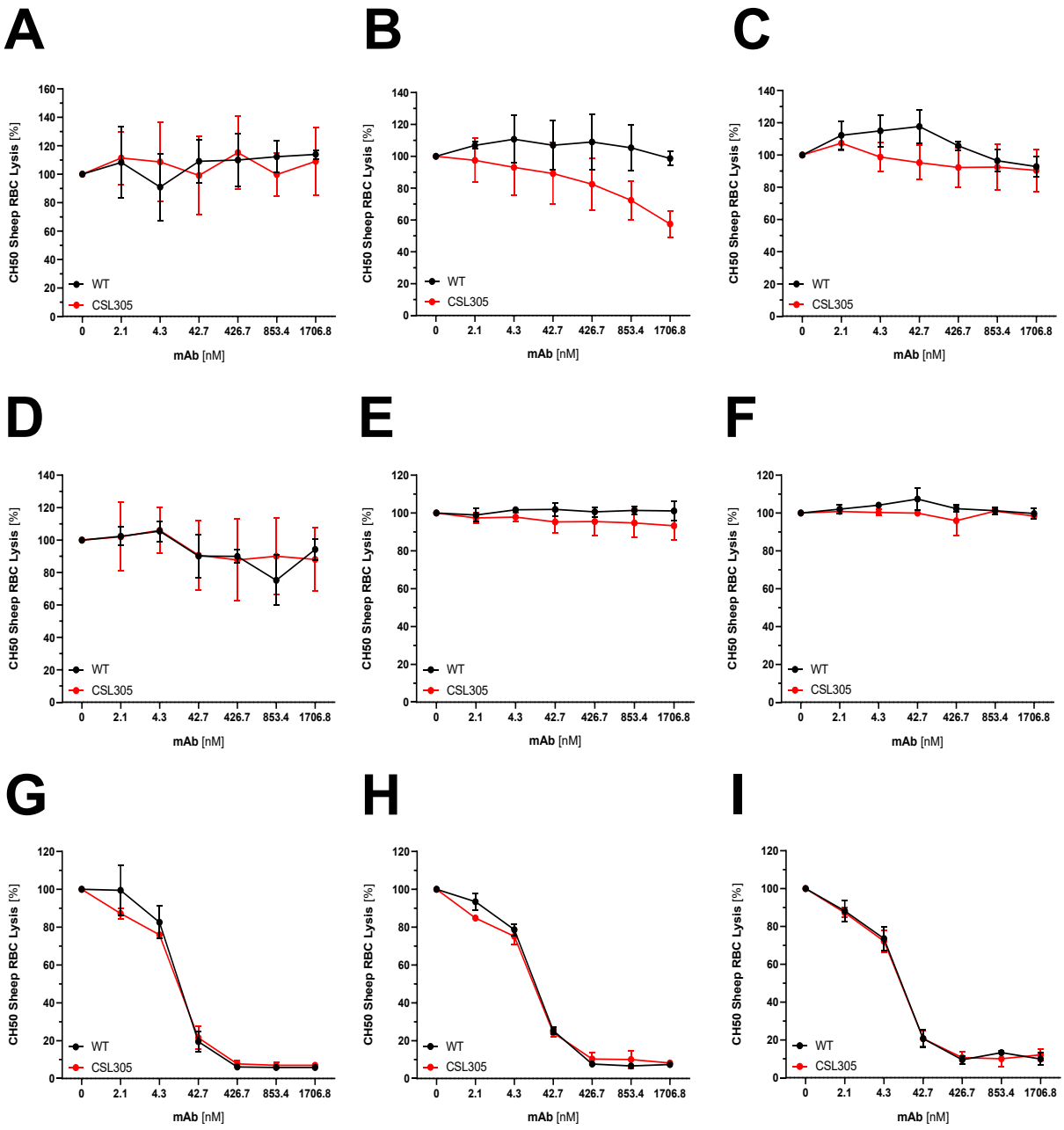
Figure S2. SPR sensorgrams of soluble human FcRn/B2M binding to WT and CSL305 mAbs. Experiments were performed at pH 6.0 (panel A, C) and pH 7.3 (panel B, D). A substantial improvement in binding was observed at pH 6.0 and pH 7.3 for CSL305 (panel C, D), compared to the WT mAb (panel A,B). Each red line represents two overlaid sensorgrams. All data were, where possible, fit to a kinetic 1:1 model (black lines) including a term for mass transport.

Anti-C2 mAb	Cyno FcRn/B2M (pH 6.0)			Cyno FcRn/B2M (pH 7.3)		
	k_a (1/Ms)	k_d (1/s)	K_D (nM)	k_a (1/Ms)	k_d (1/s)	K_D (nM)
WT	-	-	708.3 ± 8.4	-	-	Weak binding
CSL305	4.14-E+05	2.16E-03	5.2 ± 0.3	-	-	1421 ± 55
	Mouse FcRn/B2M (pH 6.0)			Mouse FcRn/B2M (pH 7.3)		
WT	2.64E+04	4.92E-02	1886 ± 352	-	-	Weak binding
CSL305	3.16E+04	5.73E-05	1.8 ± 0.8	-	-	310 ± 21

Table S2. Binding of cynomolgus monkey and mouse FcRn/B2M to CSL305 under acidic and neutral conditions. Alongside CSL305 was analysed the WT (aghu4D8) mAb. Affinities are denoted in nanomolar as the mean ± SD from N=4 measurements. "Weak binding" refers to affinities over 10 uM. Acidic pH: 6.0; Neutral pH: 7.3. k_a, association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant. Values were calculated from sensorgram data fit to a 1:1 binding model.

Anti-C2 mAb	Analyte	k_a (1/Ms)	k_d (1/s)	K_D (μM)	N
CSL305	huCD16 (158F)	-	-	31.3 ± 0.5	3
WT	huCD16 (158F)	-	-	47.2 ± 9.2	6
Control mAb	huCD16 (158F)	-	-	9.3 ± 0.6	4
CSL305	huCD32a	-	-	2.9 ± 0.3	4
WT	huCD32a	-	-	2.5 ± 0.5	4
Control mAb	huCD32a	-	-	1.2 ± 0.3	4
CSL305	huCD32b	-	-	7.0 ± 0.2	3
WT	huCD32b	-	-	3.8 ± 0.3	3
Control mAb	huCD32b	-	-	2.9 ± 1.1	3
CSL305	huCD64	1.66E+06	1.40E-02	8.6 ± 0.5	6
WT	huCD64	2.18E+06	9.89E-03	4.7 ± 0.4	6
Control mAb	huCD64	1.58E+06	3.46E-03	0.6 ± 0.1	4

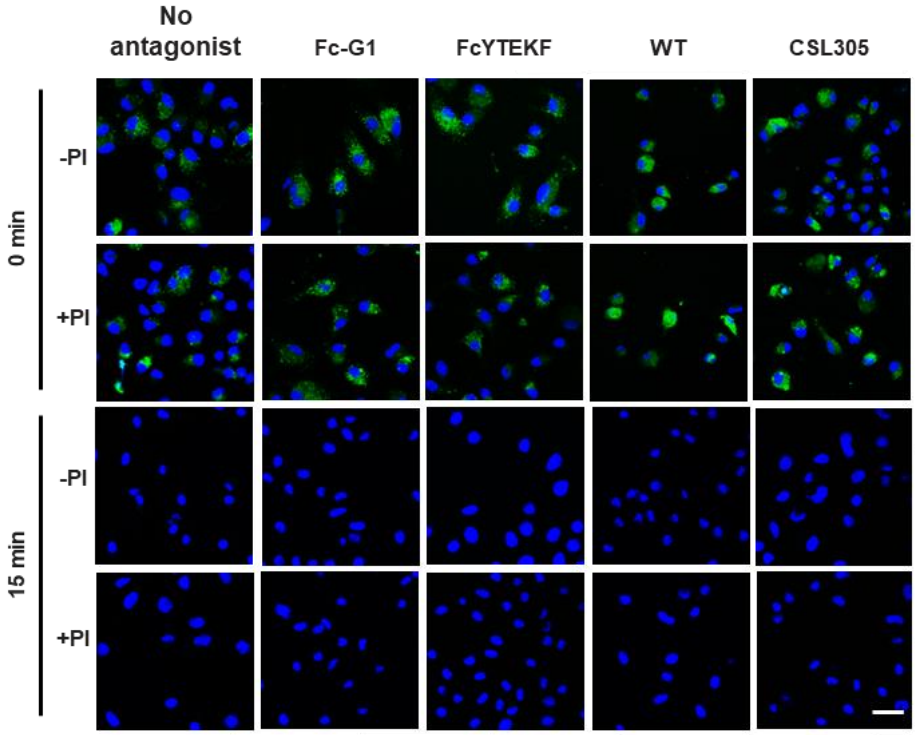
Table S3. Affinity of human Fc gamma receptors to CSL305 by SPR. Shown are the kinetic rate constants and affinities of CSL305, WT (aghu4D8) and a control mAb to huCD16 (158F), huCD32A, huCD32B, and huCD64. k_a, association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant. K_D indicated as Mean ± SD (in nM) from N = 3-6 measurements. Values were calculated from sensorgram data fit to a 1:1 binding model.



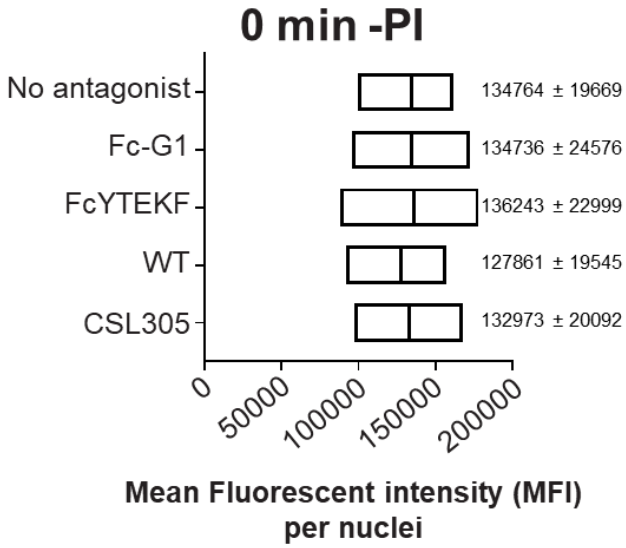
Human CH50 IC_{50}	Cyno CH50 IC_{50}	Rhesus CH50 IC_{50}
13.9 ± 3.7	14.9 ± 2.1	13.9 ± 3.9
16.4 ± 5.5	16.7 ± 0.6	14.1 ± 5.1

Figure S3: Inhibition of complement activity by CSL305 using sera from different species. Diluted Sera from various species: A) C57BL/6 mouse, 1/80 dilution; B) Han Wistar rat, 1/60 dilution; C) NZ white rabbit, 1/15 dilution; D) house pig, 1/16 dilution; E) mini pig, 1/8 dilution; F) dog (Beagle), 1/16 dilution; G) Human A+ blood group, 1/80 dilution; H) cynomolgus monkey, 1/40 dilution; I) Rhesus monkey, 1/40 dilution, were assessed for their complement activity using the classical pathway hemolytic (CH50) assay in the presence of increasing concentrations of CSL305 and WT mAbs (n=3). Where they were able to be calculated, the IC_{50} values (in nM; mean ± SD) for CSL305 (red) and WT (black) mAbs are shown below their respective graph.

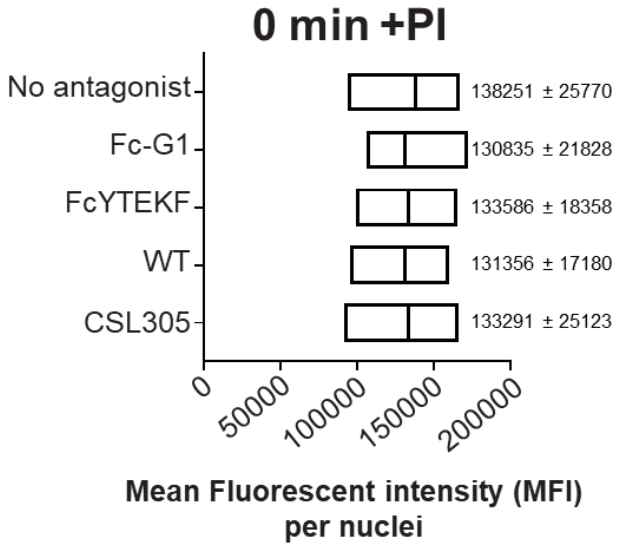
A



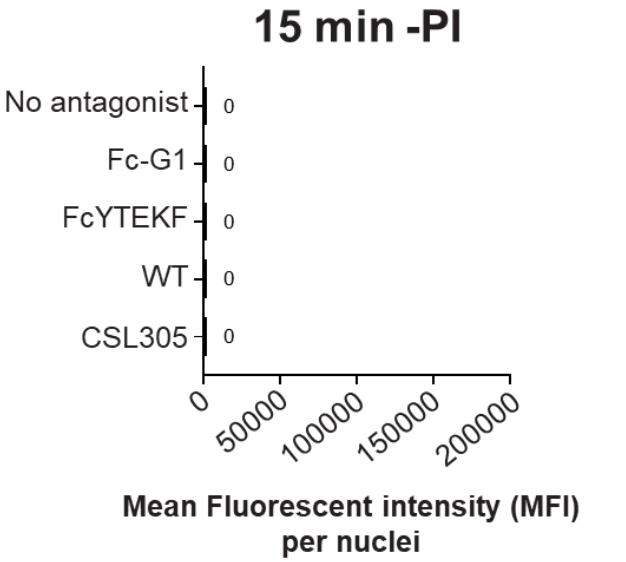
B


0 min -PI
No antagonist
Fc-G1
FcYTEKF
WT
CSL305
134764 ± 19669
134736 ± 24576
136243 ± 22999
127861 ± 19545
132973 ± 20092
0
50000
100000
150000
200000
Mean Fluorescent intensity (MFI) per nuclei

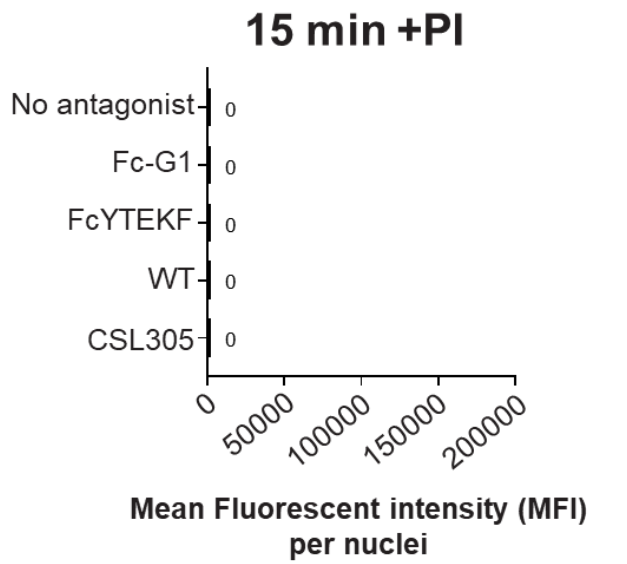
C


0 min +PI
No antagonist
Fc-G1
FcYTEKF
WT
CSL305
138251 ± 25770
130835 ± 21828
133586 ± 18358
131356 ± 17180
133291 ± 25123
0
50000
100000
150000
200000
Mean Fluorescent intensity (MFI) per nuclei

D


15 min -PI
No antagonist
Fc-G1
FcYTEKF
WT
CSL305
0
0
0
0
0
0
50000
100000
150000
200000
Mean Fluorescent intensity (MFI) per nuclei

E


15 min +PI
No antagonist
Fc-G1
FcYTEKF
WT
CSL305
0
0
0
0
0
0
50000
100000
150000
200000
Mean Fluorescent intensity (MFI) per nuclei

F

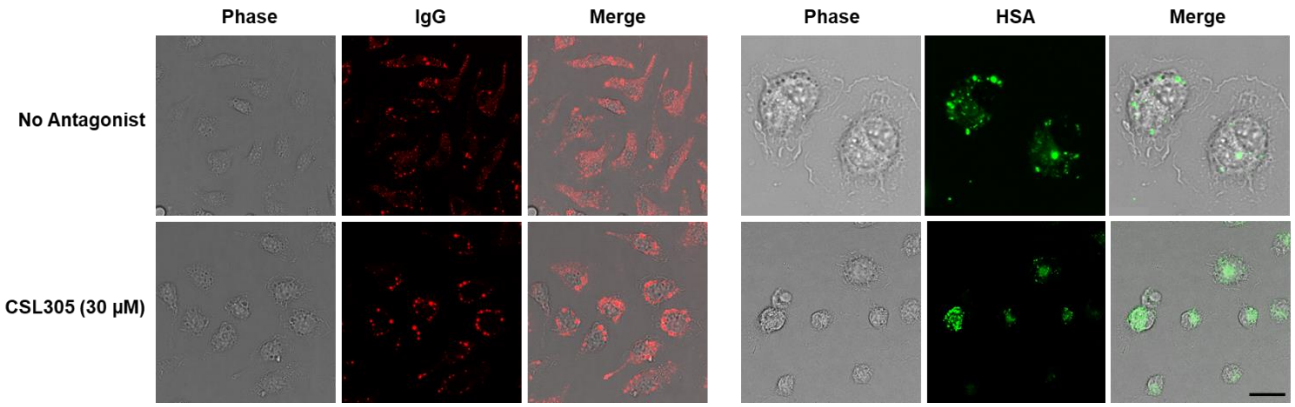


Figure S4. The effect of CSL305 on HSA recycling *in vitro*. Fluorescent intensity per nuclei of BMDM cells following uptake of fluorescently labelled HSA (green; Panel A) using a pulse-chase method. Hoechst staining (blue) represents nuclear staining. Panels show the results from cells that were either left untreated or treated to 30 μM each Fc-G1, FcYTEKF, WT mAb, or CSL305. All conditions used a 15 min pulse to allow for uptake of labelled compound, followed by a 0 min chase (A-C) or a 15 min chase (A, D, E). This was performed in the absence (A, B, D) or presence (A, C, E) or protease inhibitors (PI). Data in panels B-E is displayed as box plots showing the mean with minimum and maximum fluorescent intensities. Next to each box plot are the actual mean ± SD values. Each data point represents the average fluorescent intensity of 20-30 cells from 3 independent experiments. F) Uptake of fluorescently labelled IgG1-AF594 (Red) or HSA-AF488 (Green) in the presence and absence of CSL305. All panels show the results from BMDM cells that were either left untreated or treated with 30 μM of CSL305. All conditions used a 15 min pulse to allow uptake of labelled compound and imaged live on a confocal microscope.

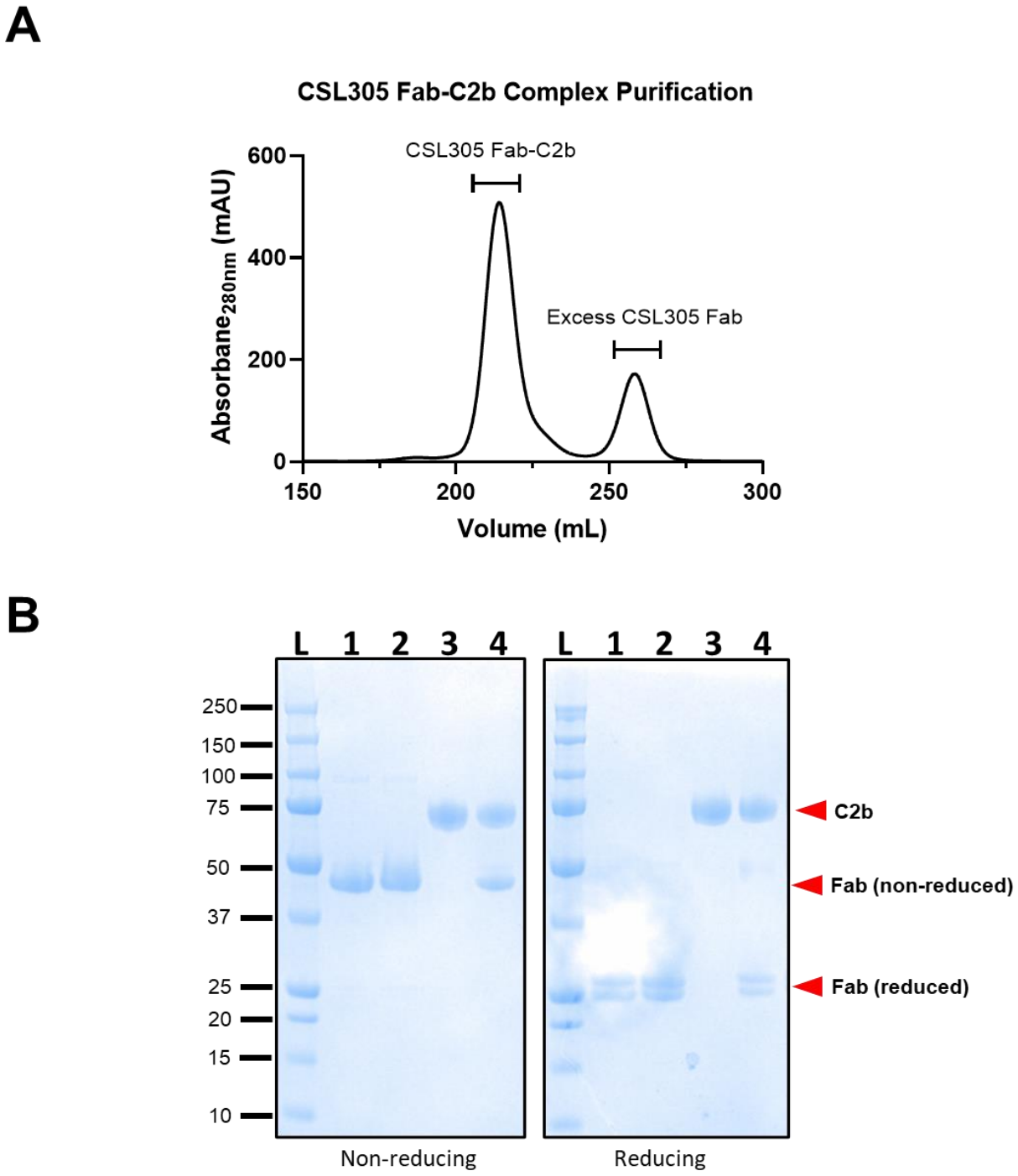


Figure S5. Preparation of the CSL305 Fab-C2b complex for crystallization. (A) Chromatogram showing the purification of the CSL305 Fab-C2b complex using a HiLoad® 26/600 Superdex® 200 pg column. The complex eluted as a relatively uniform peak, clearly separated from the excess CSL305 Fab. (B) Coomassie-stained SDS-PAGE under non-reducing (left) and reducing (right) conditions, including BioRad Precision Plus Marker (L), CSL305 Fab (lane 1), parental hu4D8 Fab (lane 2), C2b (lane 3), and the purified, concentrated CSL305 Fab-C2b complex (lane 4). Molecular weights of the markers are shown on the left.

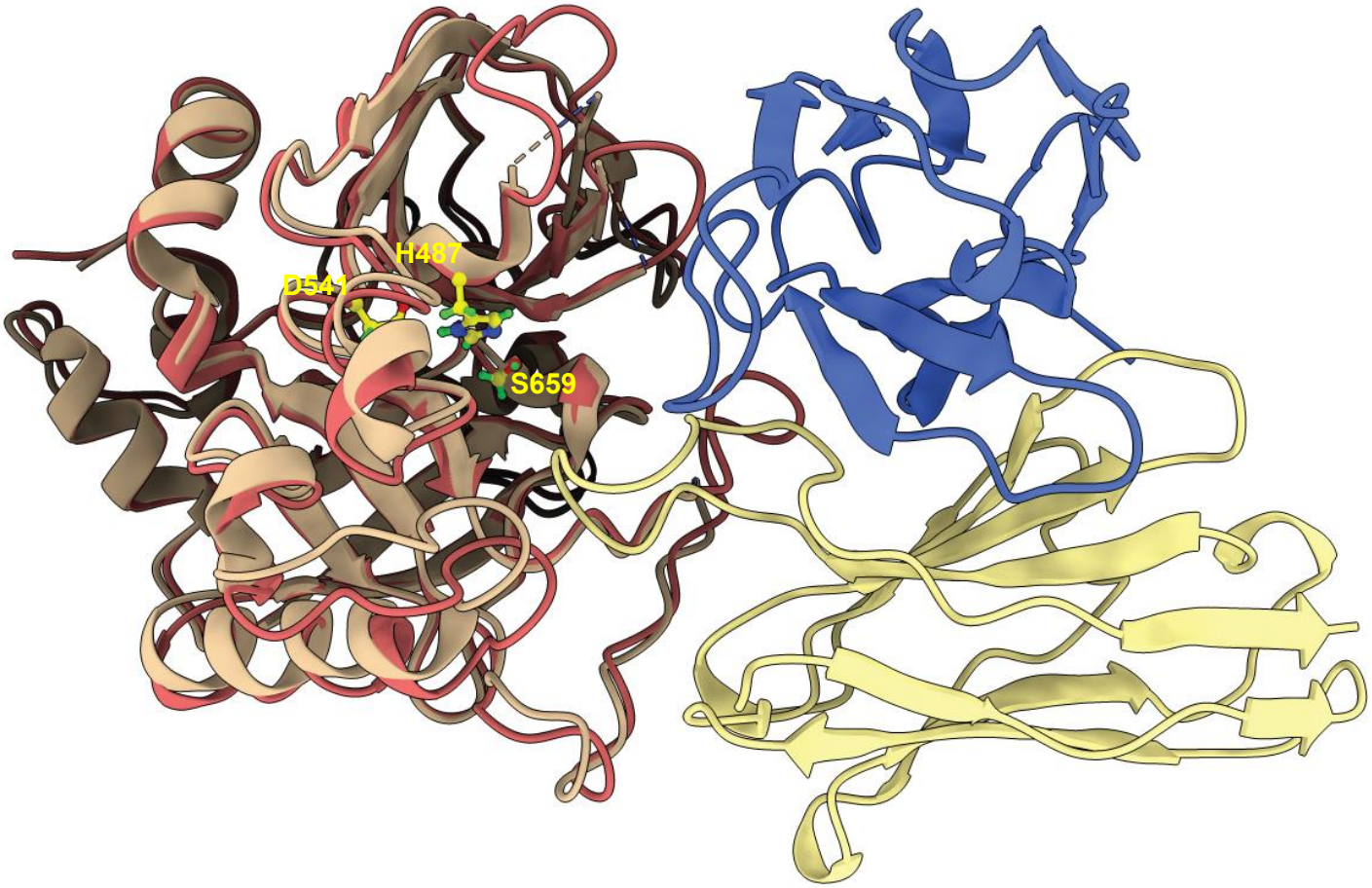


Figure S6. Comparison of CSL305-C2b structure with previously generated C2b structure. Overlay of CSL305-Fab in complex with C2b (yellow: CSL305 variable heavy chain; blue: CSL305 variable light chain; red: C2b serine protease domain) with apo C2b (brown; PDB 2ODP; reference: https://doi.org/10.1016/j.jmb.2006.12.039). The catalytic triad of C2b (H487, D541, S659) is also shown with side chains.

Human C2 Variant (Zymogen)	Affinity to hu4D8 (nM)	N	CSL305 contact residue
Wild type	9.8 +/- 0.2	21	
T463A	8.7	1	
K465A	13.6	1	
P466A	24.7	1	
K467A	40.9	1	YES
Q469A	24.4	1	YES
E470A	6.8	1	
T471A	21.1	1	
R473A	51.3	3	
H487A (Catalytic)	18.1	1	YES
R490A	77.5	1	YES
D491A	18.5	1	
R499A	19.6	3	
G510A	12.1	1	
K511A	13.0	1	
E512A	16.6	1	
K530A	11.8	1	
E536A	144.4	1	YES
Y538A	no binding	3	YES
D541A (Catalytic)	no binding	3	
L600A	41.2	3	
N601A	41.6	2	
E625A	803.8	1	YES
T627A	203.0	1	YES
M628A	783.1	1	YES
P630A	30.8	1	
K656A	645.7	1	YES
G657A	27.5	3	
E666A	14.3	2	
R668A	11.5	1	
F669A	9.4	1	
L681A	no binding	3	YES
Y682A	192.1	1	YES
N683A	175.7	1	YES
P684A	9.5	1	
L686A	577.7	1	YES
G687A	77.0	1	YES
S688A	15.4	1	YES
A689S	35.2	1	YES
D690A	30.7	1	YES
K691A	91.6	1	
S693A	6.2	1	YES
R694A	234.5	1	
K695A	no binding	2	YES
A697S	7.1	1	
P698A	1.0	1	YES
R699A	9.9	1	
S700A	18.6	1	
K701A	112.8	1	YES
V702A	15.8	1	
P703A	74.6	1	
P704A	8.6	1	

Table S4. Epitope determination of parental hu4D8 mAb on human C2 Zymogen by Alanine mutagenesis. Individual point mutations of the listed human C2 amino acids to alanine were made (based on mature amino acid sequence) as purified recombinant proteins using human C2 zymogen as a template, and the mutants subjected to SPR analysis against hu4D8. Affinities in nM are listed. Light brown shadings denote a 3-10 drop in mAb affinity compared to wild-type human C2; the next gradations of brown shading denote a 10-50-fold then a 50-500-fold drop in affinity to hu4D8; the darkest brown shading denotes alanine mutants showing no binding to mAb. The P698A mutant shaded blue showed a > 10-fold increase in affinity. All shaded residues are therefore suggested to play some part of the 4D8-C2 epitope. Residues within the epitope that are divergent from mouse C2 are: R490, E625, T627, M628, Y682, N683, L686, K701; those divergent from dog/rat/rabbit: E625, T627, M628, L686, G687, K691, R694, K695, P703. No effect on binding to hu4D8 compared to wild type human C2 zymogen was determined for an additional 94 human C2 point mutants as measured by SPR: R223A, K224A, N248A, D249A, F250A, L251A, I252A, K254A, S258A, L259A, D262A, M296A, T297A, I300A, E304A, N305A, A306S, N307A, Y308A, K309A, H311A, E347A, E355A, N360A, N383A, D384A, G394A, K395A, D397A, V398A, D399A, W400A, N404A, G407A, S408A, K410A, D411A, G412A, E413A, R414A, H415A, A416S, F417A, I418A, L419A, D421A, T422A, K423A, A424S, H426A, Q427A, V428A, E430A, H431A, M432A, L433A, D434A, V435A, S436A, K437A, V445A, G446A, N447A, S449A, KA450S, N451A, A452S, S453A, E456A, H461A, S468A, G492A, N493A, D494A, H495A, S496A, L497A, N501A, K506A, S507A, Q508A, W509A, L514A, E516A, P522A, F527A, N531A, G533A, L535A, Q549A, K550A, H556A, M566A, Q576A, R581A, N585A, K590A, H596A, S603A, N606A, M611A, V613A, A619S, E620A, V621A, Q624A, K626A, N631A, R636A, Q649A, E650A, E652A, S659A(catalytic), N692A, R696A, H709A, R714A, M715A, Q721A, H722A, G724A, D725A.

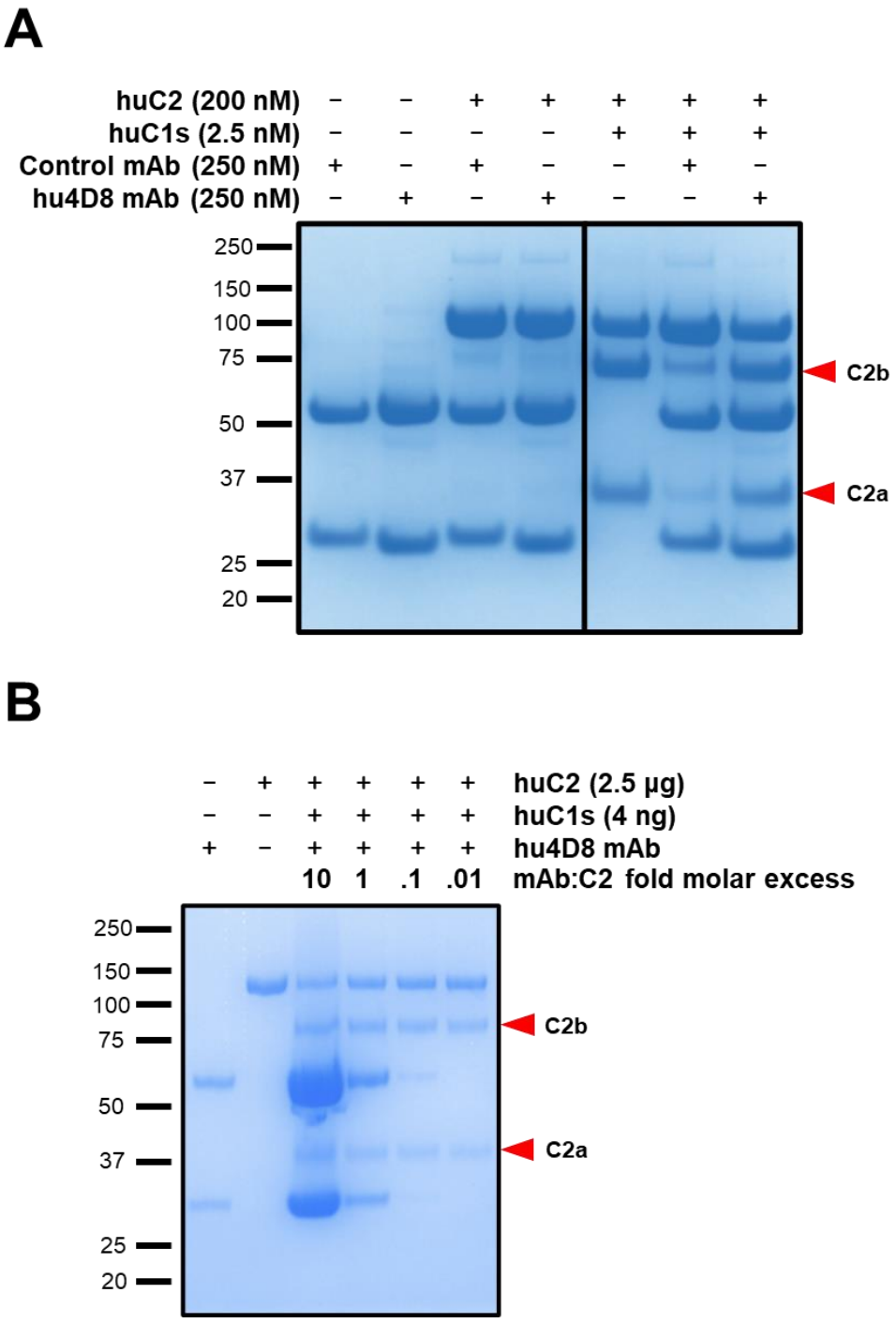


Figure S7. The effect of parental hu4D8 anti-C2 mAb on C1s-mediated cleavage of huC2. (A) hu4D8 or a positive control anti-C2 mAb were pre-incubated with huC2, before huC1s was added as appropriate (all at the listed concentrations), then the mixture analysed by non-reducing sodium dodecylsulphate polyacrylamide gel electrophoresis followed by Coomassie blue staining for the presence of the cleaved huC2 fragments, C2a and C2b (red arrows). (B) The experiment repeated with different concentrations of hu4D8 and fixed concentrations of huC2 and huC1s (as indicated). Molecular weight protein markers were used, with weights in kilodaltons indicated to the left of each gel image.