Title:
Inhibition of cleavage of human complement component C5 and the R885H C5 variant by two distinct high affinity anti-C5 nanobodies

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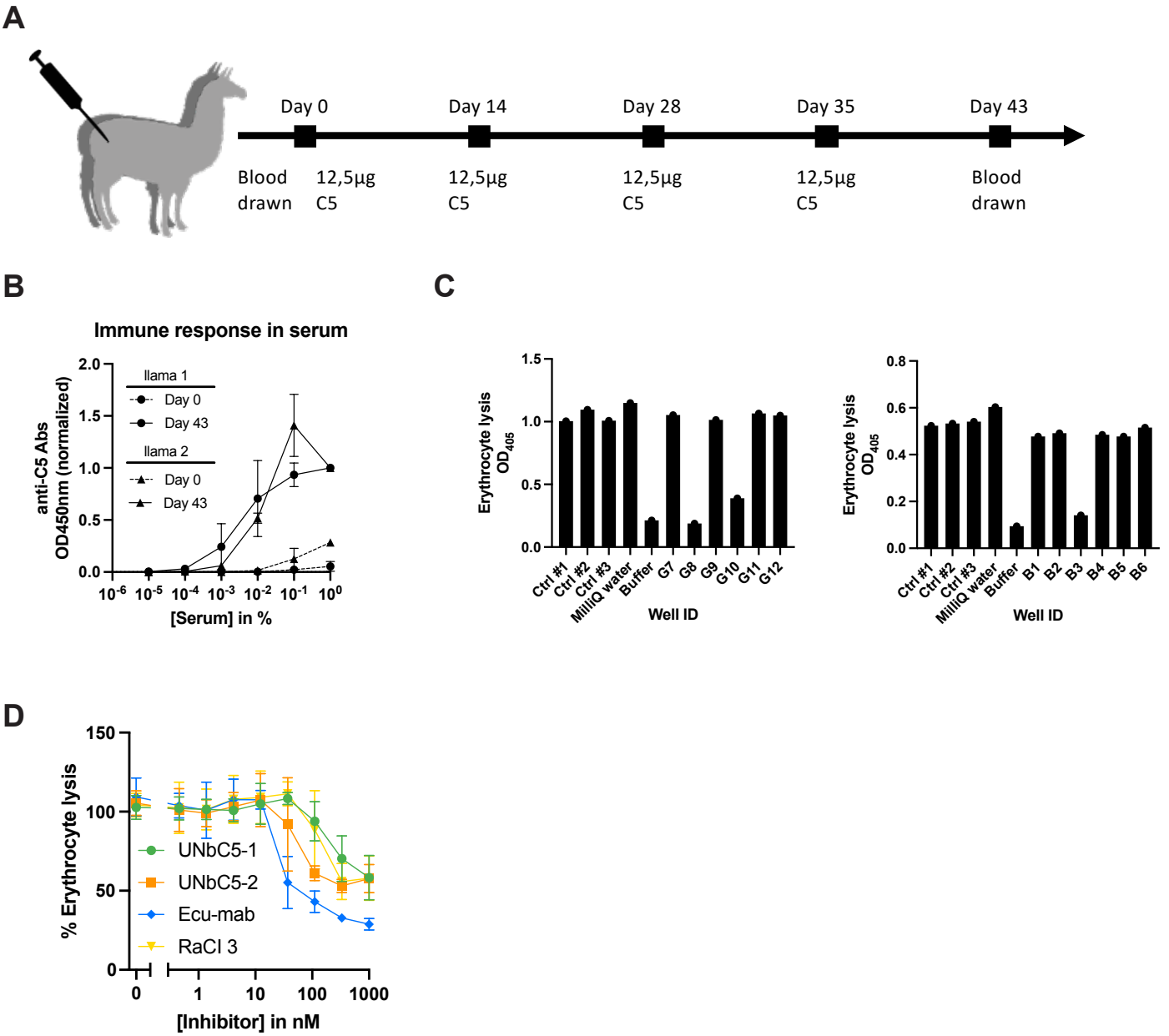
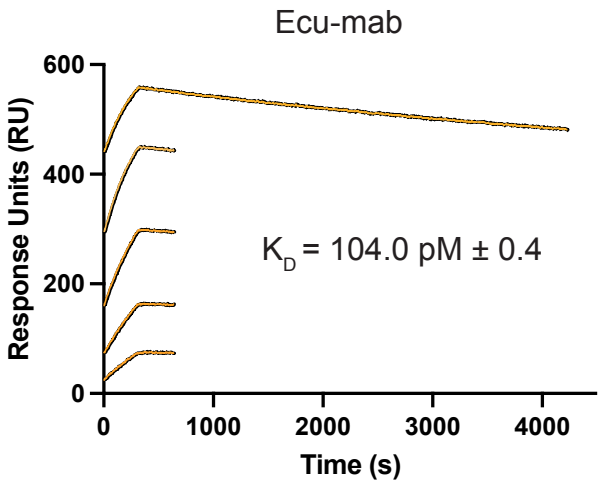
Material included:
- 7 supporting figures

Supporting Figure 1: Identification of two C5-targeting nanobodies that interfere with complement



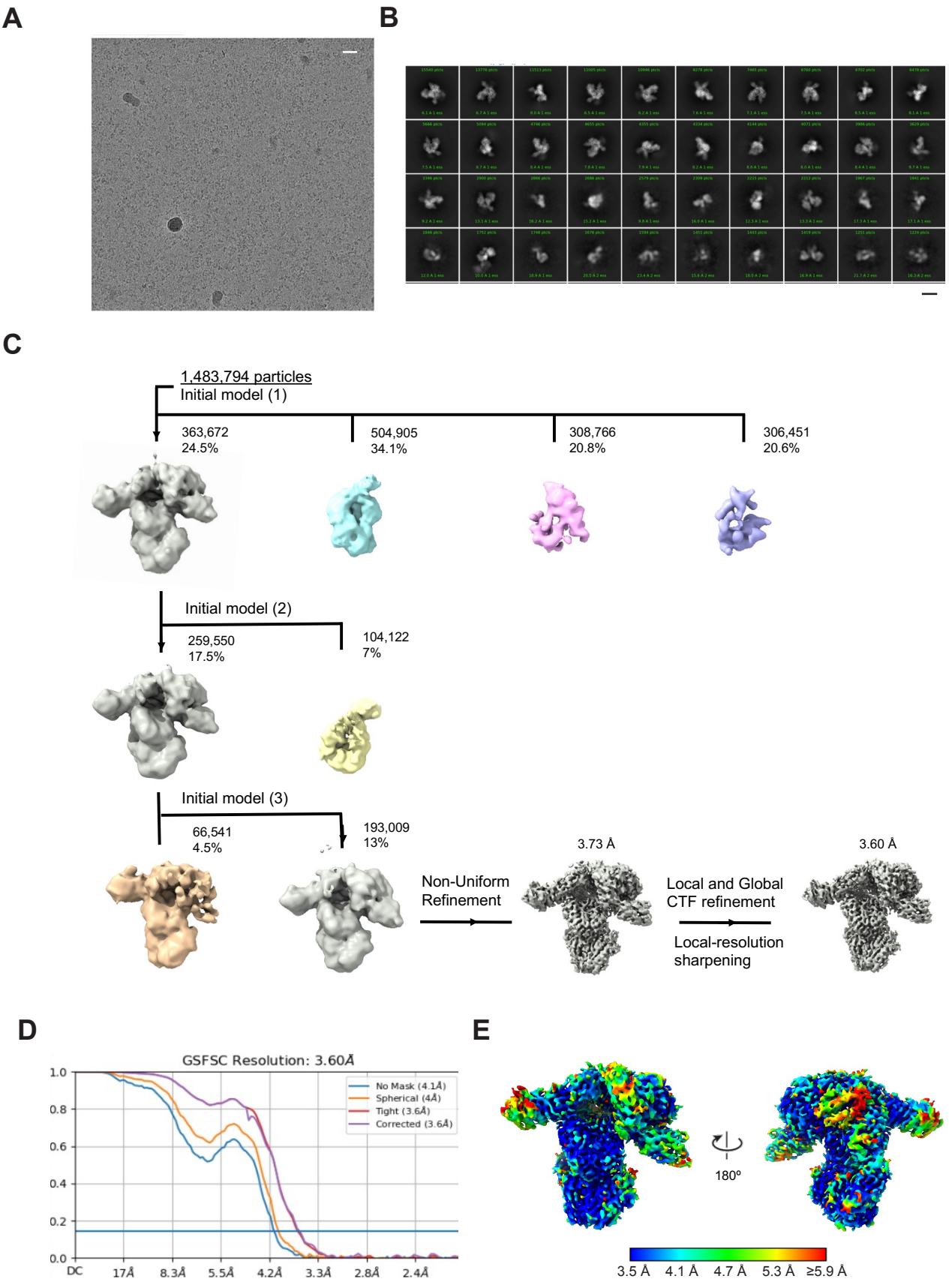
SUPPORTING FIGURE 1: Identification of UNbC5-1 and UNbC5-2. (A) Schematic representation of immunization scheme for two llamas. (B) Immune response against C5 measured in llama serum at day 0 and day 43 using an ELISA. Purified human C5 was coated on microtiter plates. Next, llama serum was added and binding of llama-antibodies was measured using polyclonal rabbit-anti-VHH QE19 antibodies and donkey-anti-rabbit-HRP antibodies, at an OD of 450 nm. Data was normalized against the highest concentration of serum, for both llamas individually. (C) CP hemolysis assay to screen for complement inhibiting nanobodies, in which clones UNbC5-1 (G8) and UNbC5-2 (B3) were identified as potent inhibitors. As controls we included periplasmic fractions of a bacteria expressing an irrelevant nanobody (ctrl #1), expressing an empty vector (ctrl #2), or no bacterial culture (ctrl #3). MilliQ water and buffer were taken along to measure maximum and minimum levels of erythrocyte lysis. (D) AP mediated hemolysis of rabbit erythrocytes incubated with 10% normal human serum and a titration of our nanobodies UNbC5-1 and UNbC5-2 and known complement inhibitors RaCI3 and Ecu-mab. The OD405 values of the supernatants were measured and the % erythrocyte lysis was calculated using a 0% (buffer) and 100% (MilliQ) control sample. Data information: (B-D) Data represent mean ± SD of 1 (C) 2 (B) or 3 (D) individual experiments.

Supporting Figure 2: Determination of the affinity of Ecu-mab for C5



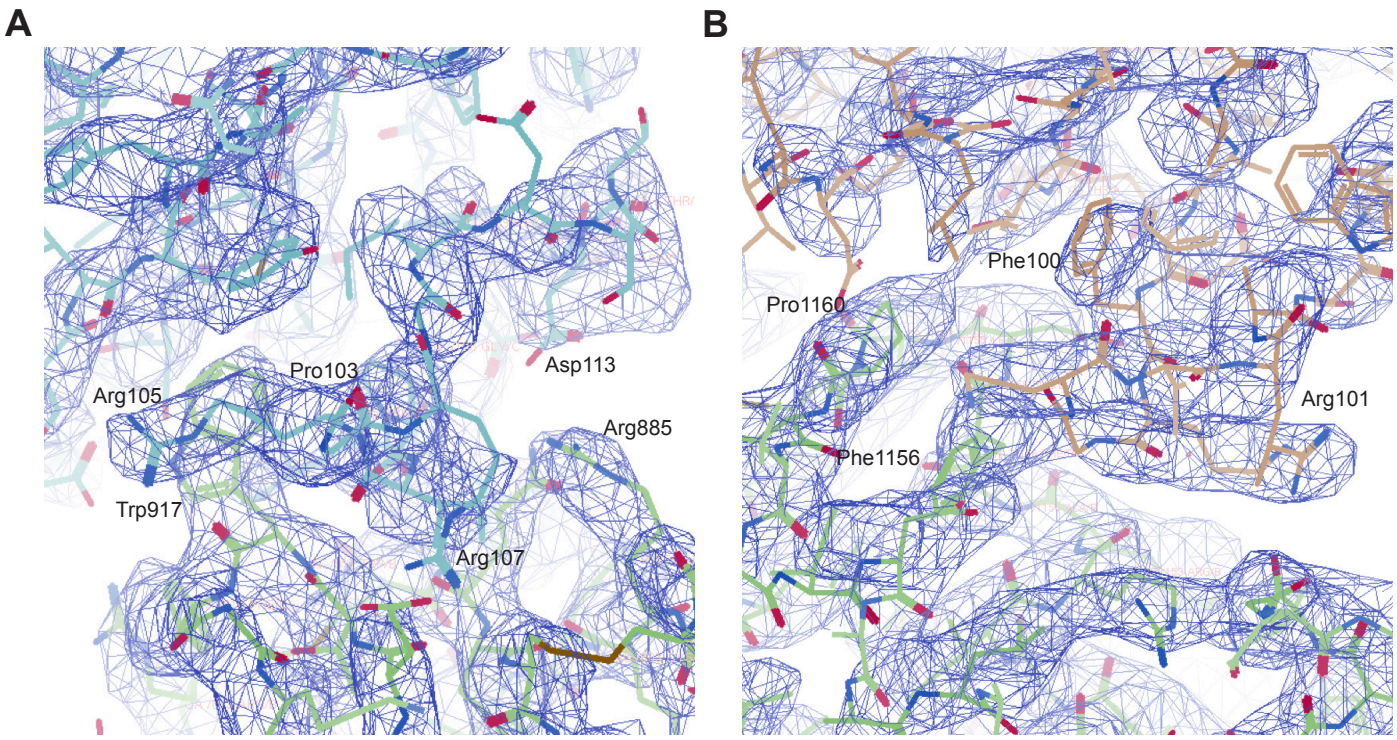
SUPPORTING FIGURE 2: Determination of the affinity of Ecu-mab for C5. SPR curves of Ecu-mab as a ligand and C5 as an analyte at concentrations of 12.5, 6.25, 3.13, 1.56, and 0.78 nM evaluated over 4000 seconds.

Supporting Figure 3: Cryo-EM image processing of C5 in complex with UNbC5-1 and UNbC5-2 datasets



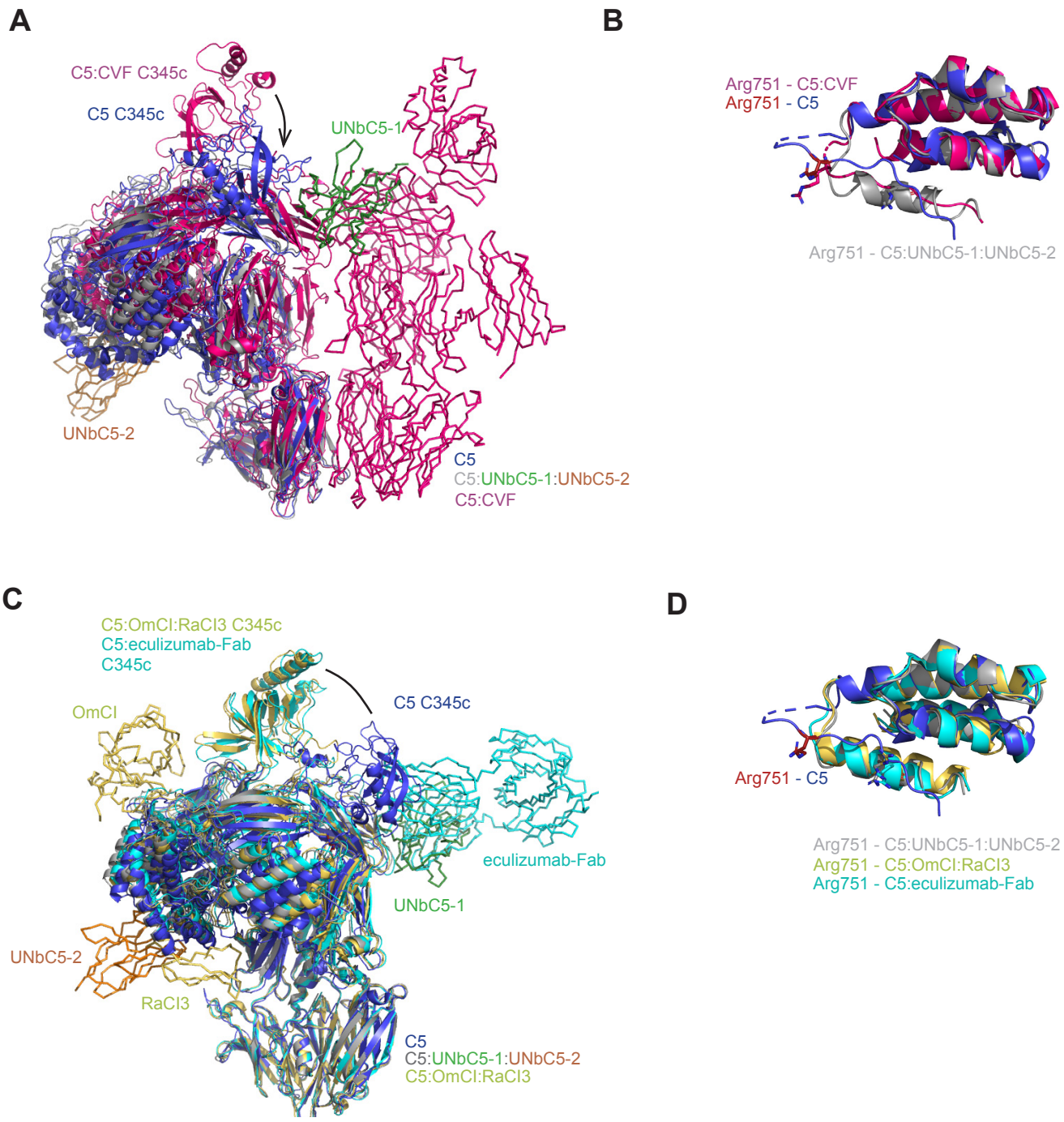
SUPPORTING FIGURE 3: Cryo-EM image processing of C5 in complex with UNbC5-1 and UNbC5-2 datasets. (A) Micrograph of C5:UNbC5-1:UNbC5-2 particles in vitreous ice. The scale bar length is 200 Å. (B) Selected 2D-class averages of the complex generated in CryoSPARC. The scale bar length is 100 Å. (C) Three rounds of 3D Initial model classification and subsequent refinement strategy for the C5:UNbC5-1:UNbC5-2 reconstruction. (D) Fourier-shell correlation plots for the gold-standard refined C5:UNbC5-1:UNbC5-2 reconstruction, computed from unmasked (blue), with a spherical mask (orange), tight mask (red), and corrected (purple). (E) C5:UNbC5-1:UNbC5-2 complex density map colored by local resolution (blue = high, red = low), computed in CryoSPARC.

Supporting Figure 4: C5:nanobody interfaces



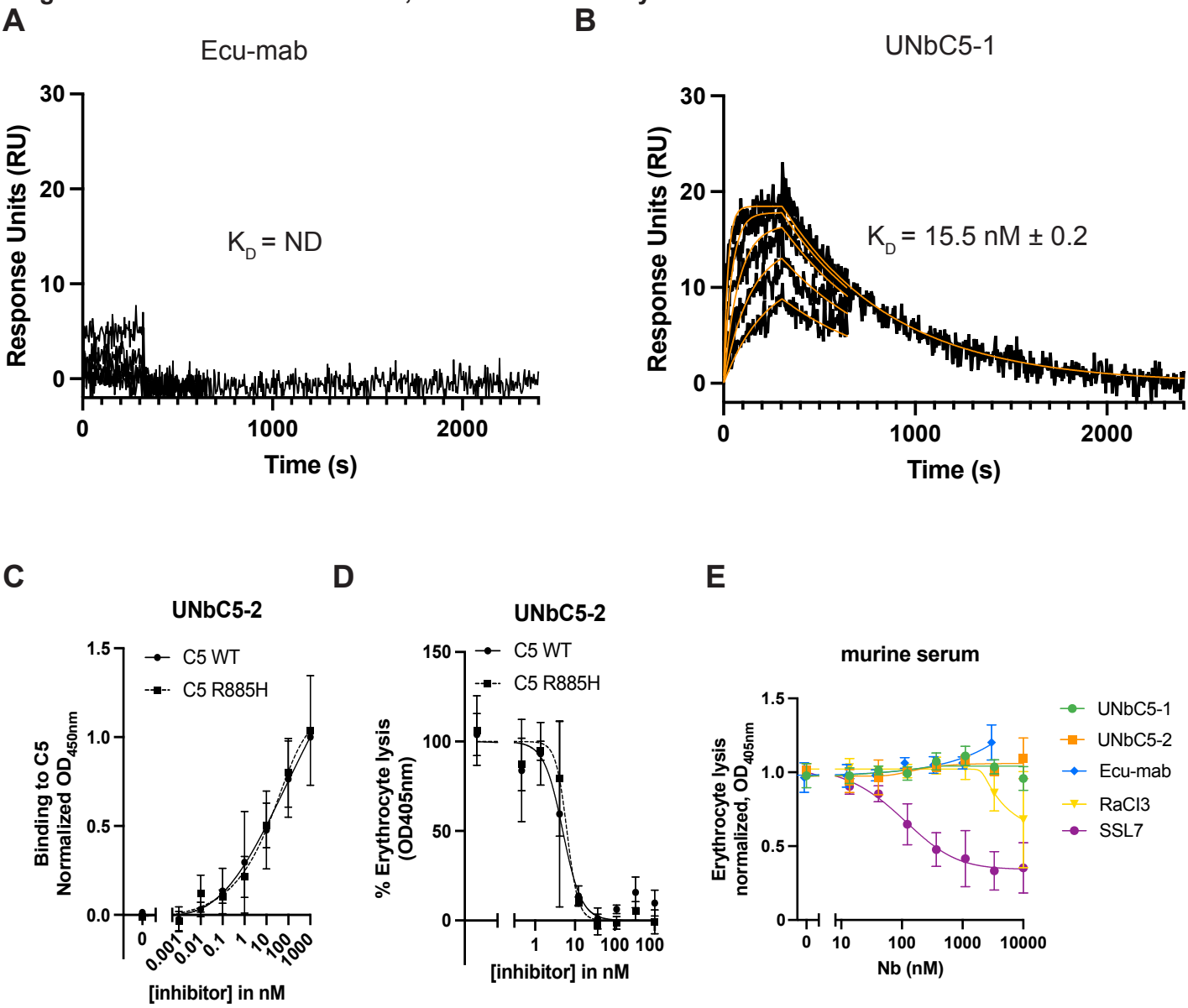
SUPPLEMENTARY FIGURE 4: Interfaces of C5 and UNbC5-1 or UNbC5-2. (A-B) Cryo-EM density maps depicting the interface of C5 (green) and UNbC5-1 (cyan) (A) or C5 (green) and UNbC5-2 (orange) (B) in detail. Densities are depicted delineated in dark blue and important residues of the interfaces are indicated. Figures were made in Coot (61).

Supporting Figure 5: Comparison of the C5:UNbC5-1:UNbC5-2 structure with different C5 structures



SUPPORTING FIGURE 5: Structural comparission of C5:UNbC5-1:UNbC5-2 with C5 and C5 inhibitors eculizumab-Fab, OmCI-RaCI3, CVF and CVF with a small inhibitory molecule. (A) Cartoon representation of native C5 (dark blue, PDB 3CU7), C5 (grey) in complex with UNbC5-1 (green) and UNbC5-2 (orange), and C5 bound to CVF (pink, PDB 3PVM). Nanobodies and CVF are depicted in ribbon. Black arrow indicates a different orientation of the C345c domain. (B) Alignment of C5a domains with Arg751 (from native C5 shown in red) shown in stick representation. Colors of the structures correspond with panel A. (C) Cartoon representation of native C5, C5 in complex with our nanobodies (both similarly depicted as in panel A), C5 in complex with OmCI and RaCI3 (yellow, PDB 5HCC), and C5 in complex with eculizumab-Fab (cyan, PDB 5I5K). Inhibitors are depicted in ribbon. (D) Alignment of C5a domains with Arg751 (from native C5 shown in red) shown in stick representation. Colors of the structures correspond with panel C.

Supporting Figure 6: Affinity determination for UNbC5-1 and Ecu-mab for C5 R885H, UNbC5-2 binding to and inhibition of C5 R885H, and cross-reactivity with murine C5



F

UNbC5-1

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     848      857  881      891  912      922
hC5  YRTSGMQFCV   SKCVRQKVEGS  FSLETWFGKEI
mC5  YMTSGTKFCV   SRCVFQRIEGS  FSLETSFGKDI
sim. * *** :***   *:** *::*** ***** ***:*
     852      861  885      895  916      926
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UNbC5-2

```
     1089     1099 1153         1168 1192     1202
hC5  VNKYVEQNQNS  RKAFDICPLVKIDTAL  AYALSLGDKTH
mC5  VAKYVKQDENS  RKAVDICPTMKIHTAL  AYALSLGDRTH
sim. * ***:*::** ***.**** :**.***  ********:**
     1093     1103 1157         1172 1196     1206
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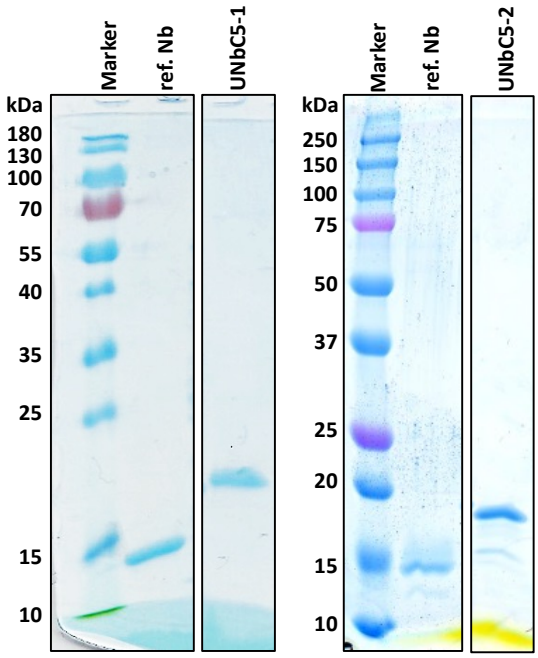
SUPPORTING FIGURE 6: UNbC5-2 binding to and inhibition of C5 R885H. (A-B) SPR curves with Ecu-mab (A) and UNbC5-1 (B) as a ligand and C5 R885H as an analyte at concentrations of 400, 200, 100, 50, and 25 nM evaluated over 2400 seconds. (C) Binding of UNbC5-2 to C5 WT and C5 R885H, using C5 WT or C5 R885H coated microtiter plates, incubated with increasing concentrations of UNbC5-2. Binding was assessed with a monoclonal anti-human-kappa antibody and an HRP-labeled secondary antibodies, at OD450. (D) CP mediated hemolysis of antibody-coated sheep erythrocytes incubated with 2.5% C5 depleted human serum, repleted with physiological concentrations of C5 WT or C5 R885H and a titration of UNbC5-2. The OD405 values of the supernatants were measured, and the % erythrocyte lysis was calculated using a 0% (buffer) and 100% (MilliQ) control sample. (E) AP mediated hemolysis of rabbit erythrocytes incubated with 10% murine serum and diferent concentrations of C5 inhibitors. The OD405 values of the supernatants were measured and data were normalized to the condition without inhibitor. (F) sequence alignment of human and murine C5 to compare nanobody binding interfaces in both C5 molecules. Colored amino acids (green for UNbC5-1 and orange for UNbC5-2) indicate amino acids frmo C5 involved in the binding interfaces. Annotation (sim.): conserved amino acid (*), amino acid with strong similar properties (:), amino acid with weakly similar properties (.). Data represent mean ± SD of 2 (A-B) and 3 (C-E) individual experiments. (C-E) Curves were fitted.

Supporting Figure 7: UNbC5-1 and UNbC5-2 on SDS-PAGE



SUPPORTING FIGURE 7: UNbC5-1 and UNbC5-2 on SDS-PAGE. SDS-PAGE to check for purity, size and concentration, UNbC5-1 and UNbC5-2. For both nanobodies and the reference nanobody (ref. Nb) 1 µg was loaded.