Targeting platelet GPVI with glenzocimab: a novel mechanism for inhibition
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Supplemental Methods and Data

Supplemental Methods

- GPVI production for crystallography: The construct for human GPVIex comprises extracellular residues 1-183 of UniProt entry Q9HCN6 with the N-terminal tag HHHHHHSSGVDLGTENLYFQS. The construct was subcloned into the pET24a (+) vector and transformed into *Escherichia coli* BL21 DE3 cells. Cultures were grown in LB media to OD_{600} of 0.8 and induced for expression with 0.1 mM isopropyl ß-D-thiogalactopyranoside (IPTG) at 37°C for 4 hours. Refolding and purification were based on the protocol reported by Horii et al with few modifications. [1] Briefly the recombinant GPVI was solubilized from inclusion bodies pellet in a resuspension buffer (6 M guanidine hydrochloride, 20 mM NaH_2PO_4, 10 mM imidazole, 1 mM dithiothreitol, pH 7.3) and purified under denaturing conditions on HisTrap FF column (Cytiva). The sample was refolded by fast dilution in refolding buffer (1 M arginine, 2 mM EDTA, 5 mM reduced L- glutathione, 0.5 mM oxidized L- glutathione, 100 mM Tris-HCl, pH 8.8) under stirring at 4°C for 16h, followed by three step dialysis. Dialysis buffer 1 (0.5 M arginine, 1 mM EDTA, 50 mM Tris, 2.5 mM reduced L-glutathione, 0.25 mM oxidized L-glutathione, pH 8) was followed by dialysis buffer 2 (0.25 M arginine, 0.5 mM EDTA, 50 mM Tris, 1.25 mM reduced L-glutathione, 0.12 mM oxidized L-glutathione, pH 8) and by dialysis buffer 3 (20 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, pH 8).

- Collagen-induced platelet activation: Fab 3J24 has ben obtained by papaïnolysis of the purified IgG 3J24. [2] Citrated whole blood was preincubated for 20 min at room temperature with glenzocimab or the 3J24 Fab 50 $\mu g.mL^{-1}$ before activation was triggered by the addition of Horm collagen 25 $\mu g.mL^{-1}$ (type I fibrils from equine tendon, Takeda Pharm, Zurich, Switzerland) for 15 min at room temperature. FITC-coupled anti P-selectin (Beckman Coulter Villepinte France) was then added and samples fixed in PBS-PFA 2%. Samples were analyzed on an Accuri C6+ flow cytometer using a predefined platelet gate. Data were analysed using PRISM v9. (GraphPad, San Diego, CA).

- Fibrin-induced platelet aggregation: Solubilized fibrin was obtained as described by (ref). Briefly the fibrin clot obtained after incubation of purified human fibrinogen (ERL South Bend IN) with human purified human thrombin was washed with NaCl 0.5M and dissociated in acetic acid 0.02 M as previously reported. [3] After centrifugation, the concentration of solubilized fibrin was determined at 280 nm. Washed human platelets were pre-incubated for 20 min at room temperature with vehicle, eptifibatide 9 µM (Sigma-Aldrich) or glenzocimab 50 $\mu g.mL^{-1}$ before aggregation was initiated by the addition of the fibrin monomer solution (200 $\mu g.mL^{-1}$).

- Surface plasmon resonance (SPR) analysis was performed at 25°C using a BIACORE T200 apparatus (Cytiva, France) at 50 µl.min^{-1} in PBS buffer pH 7.4 containing 0.005% polysorbate. For GPVI-His kinetic measurements, glenzocimab was immobilized covalently (1000 RU) on a CM5S sensor chip using the amine coupling method according to the manufacturer's instructions (Cytiva, France). Different concentrations of GPVI-His (serial dilutions: 3.1 to 400 nM) were injected at 50 µl.min^{-1} for 180s and after a dissociation for 400s, regenerated with a 10s pulse of glycine-HCl pH 1.7. For GPVI-Fc kinetic measurements, anti-human Fc was immobilized using the anti-human Fc kit from Cytiva to capture dimeric GPVI-Fc (500RU). Different concentrations of glenzocimab (serial dilutions: 0.75 to 50 nM) were injected. The kinetic and affinity constants were evaluated from the sensorgrams after double-blank subtraction with T200 Evaluation software 3.0 using a Langmuir 1:1 fitting model.

- Solid phase binding assays

Solid phase assays were performed based on previously described procedures. [4]

Glenzocimab binding to immobilized GPVI: microtiter plates were coated with GPVI-Fc, WT or D C-C' (2 µg.mL-1) overnight at 4°C. Non-specific sites were saturated with Superblock blocking buffer (Thermo Fisher Scientific Les Ulis France). ACT017 dilutions (0 to 1000 µg.mL-1 in PBS, Superblock 10% and Tween 20 0.1%) were incubated on the coated antigen for 30 min at 37**°**C. Bound Fab were detected by adding anti-human IgG (Fab specific)-peroxidase conjugate (Sigma-Aldrich), using 1-StepTM UltraTMB-ELISA (Thermo Scientific) for developing. The reaction was stopped with H2 SO4 2M (50 µL/well). Absorbance was read at 450 nm. At least 3 washings with 200 µL of the dilution buffer were performed between each intermediate step. Each point was measured in triplicate. Data were analyzed using data Prism 9 software (non-linear regression, binding saturation, one site).

GPVI binding to immobilized glenzocimab : microtiter plates were coated glenzocimab 20 µg.mL-1 in PBS. Non-specific sites were saturated with Superblock blocking buffer. GPVI-Fc dilutions (0 to 2 µg.mL^{-1} in PBS, Superblock 10% and Tween 20 0.1%) were incubated on the coated Fab for 30 min at37**°**C. Bound GPVI-Fc was detected by adding anti-human IgG (Fc specific)-peroxidase conjugate (Jackson ImmunoResearch Europe), using 1-StepTM UltraTMB-ELISA for developing. The reaction was stopped with H2 SO4 2M (50 µL/well). Absorbance was read at 450 nm. At least 3 washings with 200 µL of the dilution buffer were performed between each intermediate step. Each point was measured in triplicate. Data were analyzed using data Prism 9 software (non-linear regression, binding saturation, one site).

Inhibition of GPVI binding to collagen: microtiter plate wells were coated with Horm collagen 20 µg.mL-1 in PBS) at 4**°**C overnight. Non-specific sites were saturated with Superblock blocking buffer. Glenzocimab dilutions (0 to 192 µg.mL^{-1} in PBS, Superblock 10% and Tween 20 0.1%) and GPVI-Fc (4 and 40 µg.mL^{-1}) were mixed in a one-to-one volume ratio and pre-incubated for 10 min at 37**°** C. 100 µL of each mix were loaded in wells, and incubated at 37**°**C for 30 min. The detection and revelation were performed using peroxidase-conjugated AffiniPure F(ab**'**)2 fragment goat anti-human IgG, Fc - specific antibody (Jackson ImmunoResearch Europe) and 1-StepTM Ultra TMB-ELISA. The reaction was stopped with H2SO4 2M. Absorbance was read at 450 nm. At least 5 washings were performed between each intermediate step. Each point was measured in triplicates. Results were expressed as A450nm as a function of ACT017 concentration and the inhibition curve was obtained by nonlinear regression, dose-response inhibition analysis using GraphPad Prism 9
software.

- Fluorescence correlation spectrocopy

HEK293T cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 1% penicillin, 1% streptomycin and 1% glutamine. GPVI-eGFP, CD28-eGFP and CD86-eGFP (A206k) constructs used in FCS experiments were generated as previously described.[5] HEK293T cells were seeded onto acid-cleaned 25 mm coverslips as previously described. [5] The following day, cells were transiently transfected with PEI reagent (Polysciences, Pennsylvania, USA) in serum-free DMEM (phenol red-free) according to manufacturer's instructions (PEI:DNA ratio = 3:1; 3 µl:1 µg) where 100 ng GPVI-eGFP DNA, 500 ng CD28-eGFP DNA and 100 ng CD86-eGFP DNA were used to achieve optimal receptor density. The next day, FCS measurements were made using a Zeiss LSM-880 confocal microscope equipped with gallium arsenide phosphide photon detectors (GaAsP) (Carl Zeiss, Jena, Germany) as described previously.[5] At the start of each experiment, the microscope was aligned and calibrated using Atto-488 dye as described previously.[5] On the day of the experiment, the FCS confocal volume was determined by measurement of the axial and lateral radii. The axial and lateral radii were 2.14$\pm$0.10 µm and 0.23$\pm$0.02 µm respectively and the confocal volume was 0.23$\pm$0.03 µm^3 (0.23 fl). For treatment conditions, Glenzocimab (50 µg/ml) was made in phenol red-free DMEM and added to the cells for 20 min and then imaged. FCS data were analyzed by photon counting histogram (PCH) analysis to determine molecular brightness using Zen 2012 (black edition) software (Carl Zeiss, Jena, Germany) as described previously.[5]

Results are shown as mean ± SD unless otherwise stated and the number of independent experiments is described in Figure legends. Data were analysed using PRISM v8.3.0 (GraphPad, San Diego, CA). For FCS, data sets were first tested for normality using the Shapiro-Wilks test. FCS data were tested by Kruskal-Wallis with Dunn's *post-hoc* test. Significance was set at $P \leq 0.05$.

References

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Supplemental Data

Supplemental Figure 1: Purification of the GPVI/glenzocimab complex

(A) Size exclusion chromatography (SEC) profile of the complex (Superdex 200, HiLoad 26/60). The blue line corresponds to the UV signal at 280 nm. The stars indicate the fractions loaded on the gel (B).

(B) Analysis of the SEC fractions on SDS-PAGE gel under non-reducing conditions. Lane 1: loaded complex on SEC. Lane 2: Novex Sharp pre-stained protein Standard. From lane 3 to 12: analysis of the fractions from the SEC from (a) to (j) indicated with a stars on the chromatogram (A).

Supplemental Figure 2: SPR analysis of the interaction between glenzocimab and recombinant human GPVI.

(A) The CM5S sensor chip was coated with glenzocimab and monomeric GPVI-His was injected at increasing concentrations (0, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 nM). The black curve is the fitting curve with a stoichiometric Langmuir model.

(B) Dimeric GPVI-Fc was captured on anti-human Fc-coated CM5S sensor chips. Glenzocimab was injected at increasing concentrations (0, 0.75, 1.5, 3.12, 6.25, 12.5, 25, 50 nM). The black curve is the fitting curve with a stoichiometric Langmuir model.

Supplemental Figure 3: Expression of GPVI-eGFP,CD86-eGFP and CD28-eGFP on HEK293T cells.

HEK293T cells transfected to express GPVI-eGFP or CD86-eGFP, or CD28-eGFP were obtained as described.[5] The level of receptor expression was asseessed by measuring the average fluorescence

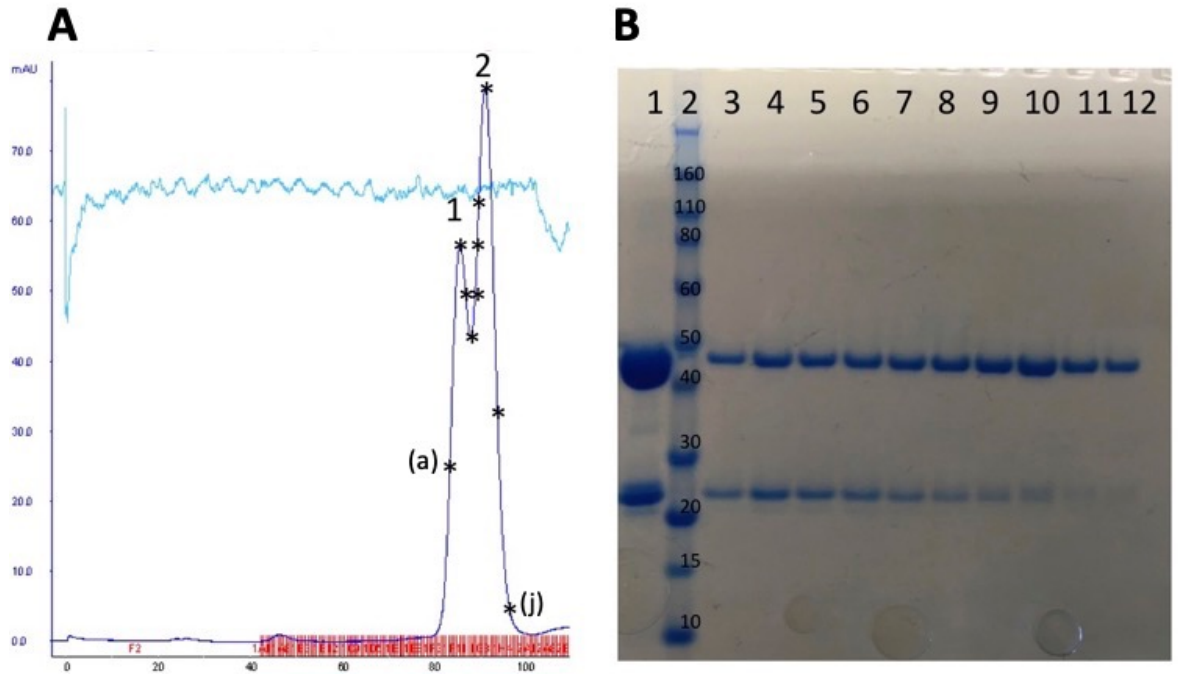
intensity in the cell volume and expressed as count rate. Count rate data shows that the fluorescence intensity of the cells used is similar with no significant difference between the three receptors.

Supplemental Figure 4: Structural comparison between GPVIex/glenzocimab and 7NMU and 2GI7 crystals.

A: Overview of the superimposed structure of GVI in complex with glenzocimab (7R58 green) and swapped GPVI (complex with Nb2, 7NMU grey) on the left or unswapped GPVI (2GI7 grey) on the right. The C-C' loops in 7NMU (red) are engaged in the formation of a swapped domain that is not the case in the presence of glenzocimab (C-C' loop in orange). B: Similarly, when comparing the C-C' loop with unbound GPVI in the non-domain swapped form (2GI7), the binding to glenzocimab results in an upward shift of the loop.

Supplemental Figure 5: GPVI main polymorphisms sites are at a distance to glenzocimab epitope

Overview of the GPVI predicted structure predicted by AlphaFold - identifier AF-Q9HCN6-F1. This structure does not represent O- and N-glycosylation. Indicated in green is the motif formed by amino acids 129-136 important for the binding of glenzocimab on D2. In red are shown the five amino acids that are affected by main GPVI polymorphisms, three being located in the loop connecting D2 to the transmembrane domain (in blue) and two in the intracellular tail.



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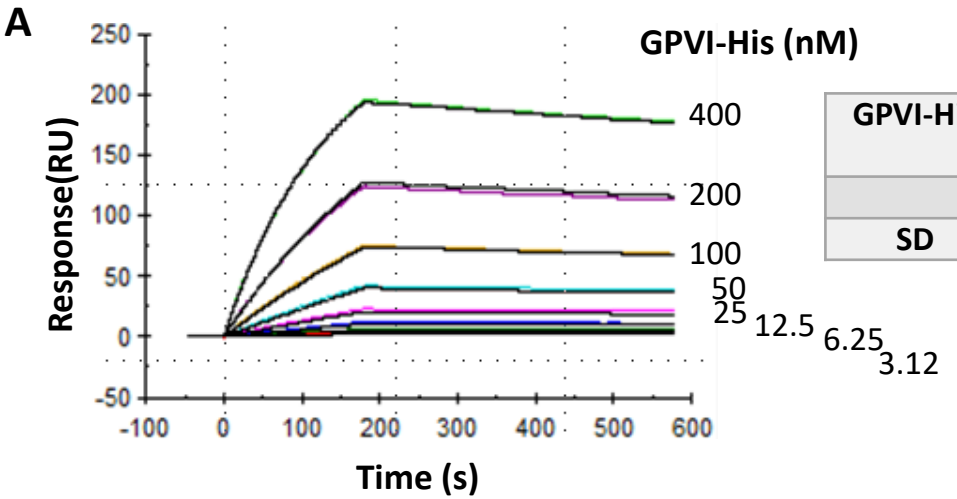
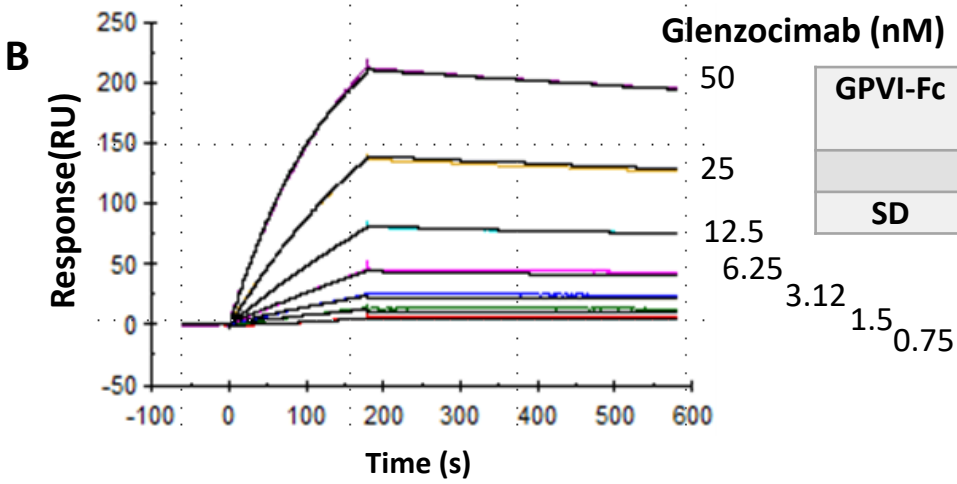


GPVI-His	ka (1/Ms)	kd (1/s)	KD (M) ±SE	Rmax (RU)	Chi² (RU²)
	183.00 10^2	2.31 10^{-4}	12.6 10^{-9}	269	1.45
SD	0.12 10^2	0.008 10^{-4}	0.04 10^{-9}		



GPVI-Fc	ka (1/Ms)	kd (1/s)	KD (M) ±SE	Rmax (RU)	Chi² (RU²)
	1500 10^2	1.97 10^{-4}	1.31 10^{-9}	288.5	2.78
SD	1.00 10^2	0.0083 10^{-4}	0.0057 10^{-9}		

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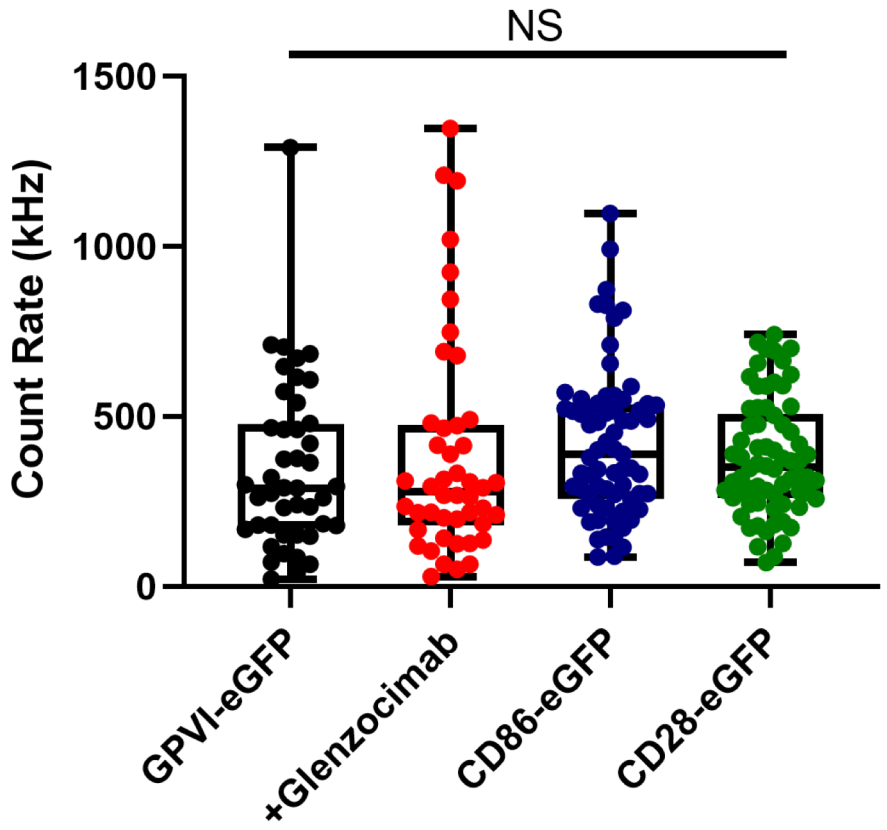

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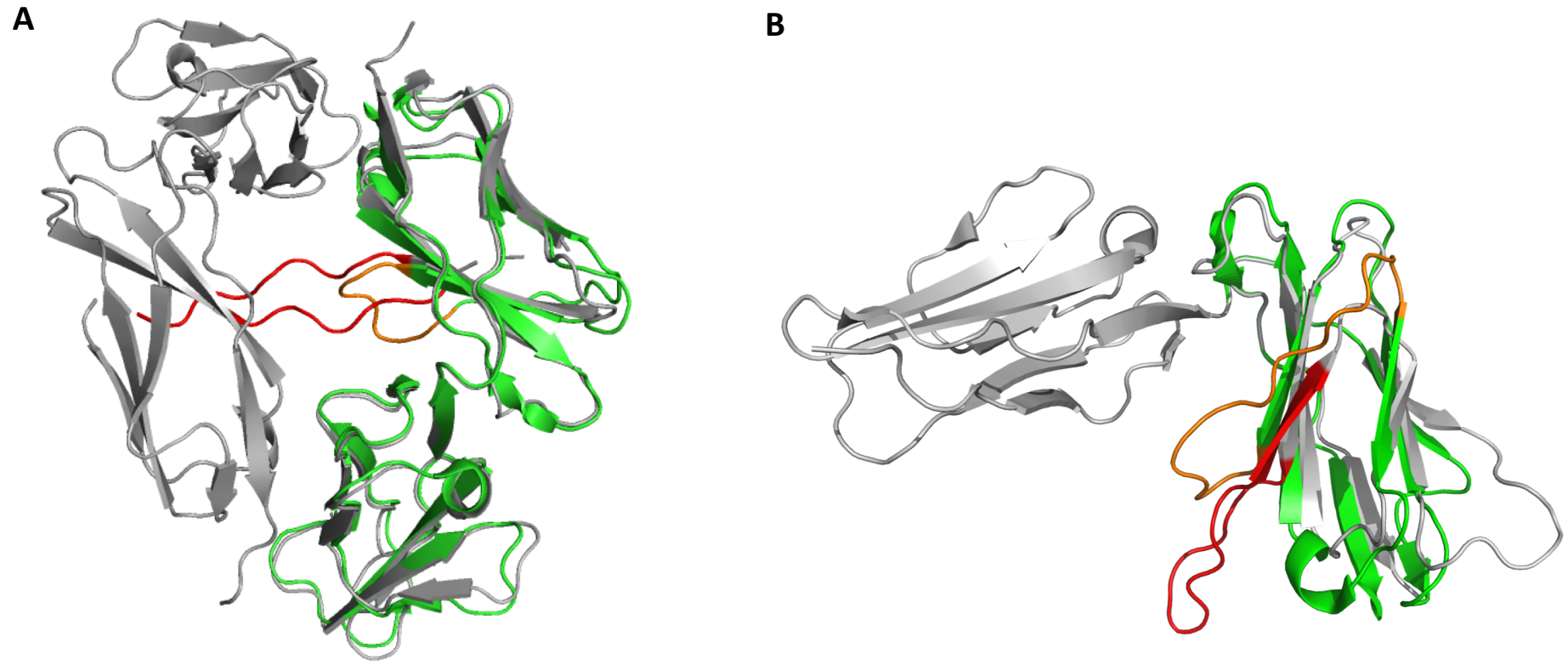

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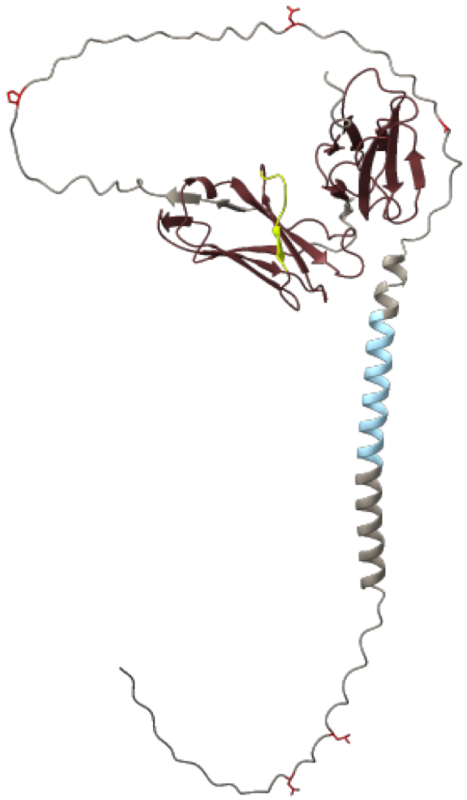

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