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Billiald, Philippe; Slater, Alexandre; Welin, Martin; Clark, Joanne; Loyau, Stephane ; Pugnière, Martine; Jiacomini , Isabella G.; Rose, Nadia; Lebozec, Kristell; Toledano, Elie; François, Déborah; Watson, Steve; Jandrot-Perrus, Martine

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American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodadvances@hematology.org

Targeting platelet GPVI with glenzocimab: a novel mechanism for inhibition

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Philippe Billiald (INSERM UMR_S1148, France) Alexandre Slater (University of Birmingham, United Kingdom) Martin Welin (SARomics Biostructures, Sweden) Joanne Clark (University of Birmingham, United Kingdom) Loyau Stéphane (INSERM UMR_S1148, France) Martine Pugnière (INSERM, U1194, France) Isabella Jiacomini (Laboratório de Imunoquímica, Universidade Federal do Paraná, Brazil) Nadia Rose (SARomics Biostructures, Sweden) Kristell Lebozec (Acticor Biotech SA, France) Elie Toledano (Acticor Biotech SA, France) Deborah François (Acticor Biotech SA, France) Steve Watson (University of Birmingham, United Kingdom) Martine Jandrot-Perrus (INSERM UMR_S1148, France)

Abstract:
Platelet glycoprotein VI (GPVI) is attracting interest as a potential target for the development of new antiplatelet molecules with a low bleeding risk. GPVI binding to vascular collagen initiates thrombus formation and GPVI interactions with fibrin promote the growth and stability of the thrombus. In the present study we show that glenzocimab, a clinical stage humanized antibody fragment (Fab) with high affinity for GPVI, blocks binding of both ligands through a combination of steric hindrance and structural change. A co-crystal of glenzocimab with an extracellular domain of monomeric GPVI was obtained and its structure determined to a resolution of 1.9 Å. The data revealed that (i) glenzocimab binds to the D2 domain of GPVI; GPVI dimerisation was not observed in the crystal structure because glenzocimab prevented D2 homotypic interactions and the formation of dimers which have a high affinity for collagen and fibrin; (ii) the light variable (VL) domain of the GPVI-bound Fab causes steric hindrance that is predicted to prevent the collagen-related peptide (CRP)/collagen fibers from extending out of their binding site and preclude GPVI clustering and downstream signaling. Glenzocimab did not bind to a truncated GPVI missing loop residues 129-136, thus validating the epitope identified in the crystal structure. Overall, these findings demonstrate that the binding of glenzocimab to the D2 domain of GPVI induces steric hindrance and structural modifications that drive the inhibition of GPVI interactions with its major ligands.

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Targeting platelet GPVI with glenzocimab: a novel mechanism for inhibition

Philippe Billiald [1,2], Alexandre Slater [3], Martin Welin [4], Joanne C. Clark [3], Stephane Loyau [1], Martine Pugnière [5], Isabella G. Jiacomini [6], Nadia Rose [4], Kristell Lebozec [7], Elie Toledano [7], Déborah François [7], Steve P. Watson [3,8], Martine Jandrot-Perrus [1]

Affiliations
[1] LVTS, UMR_S1148 INSERM, Université Paris Cité, Hôpital Bichat, 75018, Paris, France.
[2] Université Paris-Saclay, School of Pharmacy, 91400 Orsay, France.
[3] Institute of Cardiovascular Sciences, Level 1 IBR, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.
[4] SARomics Biostructures, Medicon Village, Scheeletorget 1, 223 63 Lund, Sweden.
[5] IRCM, Institut de Recherche en Cancérologie de Montpellier, INSERM, U1194, Université Montpellier, ICM Institut Régional du Cancer, 34090 Montpellier, France.
[6] Laboratório de Imunoquímica, Departamento de Patologia Básica, Universidade Federal do Paraná, Curitiba 81530, PR, Brazil.
[7] Acticor-Biotech, Hôpital Bichat, 75018 Paris, France.
[8] Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, UK.

Corresponding authors: Philippe Billiald (philippe.billiald@universite-paris-saclay.fr), Martine Jandrot-Perrus (martine.jandrot-perrus@inserm.fr), LVTS, UMR_S1148 INSERM, Université de Paris, Hôpital Bichat, 46 rue Henri Huchard, 75018, Paris, France.

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Key points

- Crystallisation studies map the binding of glenzocimab to the site of dimerisation in the D2 domain of GPVI.
- Glenzocimab inhibits GPVI interactions with CRP, collagen and fibrin by loss of dimerisation, conformational changes and steric hindrance

Abstract

Platelet glycoprotein VI (GPVI) is attracting interest as a potential target for the development of new antiplatelet molecules with a low bleeding risk. GPVI binding to vascular collagen initiates thrombus formation and GPVI interactions with fibrin promote the growth and stability of the thrombus. In the present study we show that glenzocimab, a clinical stage humanized antibody fragment (Fab) with high affinity for GPVI, blocks binding of both ligands through a combination of steric hindrance and structural change. A co-crystal of glenzocimab with an extracellular domain of monomeric GPVI was obtained and its structure determined to a resolution of 1.9 Å. The data revealed that (i) glenzocimab binds to the D2 domain of GPVI; GPVI dimerisation was not observed in the crystal structure because glenzocimab prevented D2 homotypic interactions and the formation of dimers which have a high affinity for collagen and fibrin; (ii) the light variable (VL) domain of the GPVI-bound Fab causes steric hindrance that is predicted to prevent the collagen-related peptide (CRP)/collagen fibers from extending out of their binding site and preclude GPVI clustering and downstream signaling. Glenzocimab did not bind to a truncated GPVI missing loop residues 129-136, thus validating the epitope identified in the crystal structure. Overall, these findings demonstrate that the binding of glenzocimab to the D2 domain of GPVI induces steric hindrance and structural modifications that drive the inhibition of GPVI interactions with its major ligands.

Introduction

The finely tuned formation of a platelet plug at sites of vascular injury ensures hemostasis by preventing excessive blood loss. By contrast, uncontrolled platelet activation causes thrombotic events and acute ischemic events such as myocardial infarction or stroke. Moreover, platelets and immune cells act jointly in injured tissues, leading to thromboinflammation that contributes to cell death and organ dysfunction.[1] Antiplatelet drugs are largely used for the treatment and prevention of arterial thrombosis, including coronary artery diseases and ischemic stroke. However, despite the considerable progress achieved in antiplatelet therapies, standard antiplatelet drugs, aspirin and P2Y12 antagonists have non-optimal efficacy and there is a major drawback that they increase the risk of bleeding which can even be fatal.[2] Thus, safe and efficient antiplatelet therapy remains an important unmet medical need.

Platelet glycoprotein VI (GPVI) is of interest because of its potential as a target for the development of new antiplatelet molecules involving a lower risk of bleeding.[3] GPVI expression is restricted to megakaryocytes and platelets, and it is predicted that GPVI antagonists will not have off-target effects.[4] GPVI is expressed at a moderate level of 4000-6000 copies per platelet which is favorable for pharmacological saturation.

GPVI was initially recognized as a receptor for collagen. Indeed, GPVI clustering by type I or type III collagen triggers platelet degranulation, aggregation and exposure of procoagulant phospholipids at the platelet surface.[5,6] Collagen is present in large quantities in atherosclerotic plaques and several studies have demonstrated that GPVI is a crucial factor in atherothrombosis.[7-10] The extracellular domain of GPVI consists of two Ig-like domains D1 and D2.[4] The crystal structures of the GPVI exodomain, with and without triple helical (GPO)n-containing collagen-related-peptide (CRP), have previously been solved revealing a dimer interface at D2 and the site of CRP binding at D1.[11,12] A major advance in our understanding of the role of GPVI was the discovery that it binds to fibrin(ogen). [13-15] Interactions between GPVI and fibrin and fibrinogen endow it with an important role in the growth and stability of a thrombus.[16]

GPVI-deficient animals have been shown to be protected in various models of thrombosis, such as lethal thromboembolism and atherothrombosis, myocardial ischemia reperfusion injury and cerebral ischemia.[17,18] However, GPVI is not strictly required for physiological hemostasis and subjects with a GPVI deficiency exhibit only a mild bleeding diathesis or even

no bleeding at all.[19,20] GPVI is therefore considered to have promise as a safe antithrombotic target.[3,21]

Glenzocimab, formerly designated ACT017, is a humanized Fab fragment derived from the mouse monoclonal antibody 9O12.[22,23] Glenzocimab recapitulates the properties of the 9O12 Fab with the advantage of being administrable to human. It retains nanomolar affinity for GPVI and inhibits GPVI interactions with collagen and fibrin(ogen) *in vitro*; *in vivo*, it protects humanized GPVI mouse models and non-human primates from thrombosis.[15,23-25] In a first-in-human phase I trial, the safety outcome was favorable, and pharmacokinetic and pharmacodynamic data demonstrated the reversible binding of glenzocimab to platelets and inhibitory effect.[26,27] A Phase II clinical trial, ACTIMIS (NCT03803007), with glenzocimab in addition to thrombolysis or thrombectomy for patients with acute ischemic stroke has been completed confirming the safety. Furthermore, despite the study being underpowered for efficacy, it provided promising results with a lower frequency of hemorrhagic transformation, a lower percentage of patients with poor outcome and a reduction in the number of death in patients treated with glenzocimab compared to placebo. [28]

Here, our objective was to determine the mechanism of action of glenzocimab at the molecular level. For this purpose, we have analyzed the crystallographic structure of the binary GPVI/glenzocimab immunocomplex. We identified the area of interaction between the Fab and GPVI, which corresponds to a site that does not overlap with the collagen/CRP binding site and we show that the Fab hinders the homotypic interactions between GPVI at the cell surface. It creates steric hindrance blocking interactions with ligands and thus prevents subsequent platelet activation.

Materials and Methods

Recombinant proteins

For affinity determination, the extracellular domain of GPVI residues 1- 249 was expressed as either a monomer with a N-terminal His_6-Tag (GPVI-His) or as a dimer fused with the Fc (GPVI-Fc) domain of human IgG_1 as previously reported.[23,29,30]

For crystallography, the non-glycosylated human GPVI extracellular domain residues 1-183 (GPVIex) was produced by heterologous expression in *Escherichia coli*. Briefly the protein was extracted from inclusion bodies, purified by affinity chromatography and refolded as previously reported [11] with some modifications described in Supplementary Methods.

The GPVI variant, GPVI-Fc ΔC-C' truncated of the hinge region ($G^{129}DPAPYKN^{136}$) was produced as previously described.[31]

Glenzocimab is a clinical grade $IgG_{1,k}$ humanized Fab fragment (ACT017) derived from the monoclonal mouse antibody 9O12. Glenzocimab was produced in CHO cells using the GPEx® protein expression technology (Catalent, US) and was obtained after Protein L capture followed by ion exchange chromatography and polishing steps (Supplemental Figure 1). The product was concentrated at 10 $g.L^{-1}$ in citric acid 20 mM, 130 mM NaCl, pH 5.0. [23]

Functional assays were performed mostly as previously described and details are reported in Supplemental Methods.

Collagen-induced platelet activation was assessed by P-selectin exposure at the surface of platelets by flow cytometry.

Surface plasmon resonance (SPR) analysis was performed with a BIACORE T200 apparatus (Cytiva, France). Briefly, glenzocimab was immobilized covalently on a CM5S sensor chip and GPVI-His at different concentrations was injected followed by dissociation and regeneration steps. For GPVI-Fc kinetic measurements dimeric GPVI-Fc was captured and glenzocimab at different concentrations was injected.

GPVI dimerization and oligomerization was analyzed by fluorescence correlation spectroscopy (FCS) as previously described.[32] Briefly, the molecular brightness of HEK293T cells transiently expressing monomeric CD86-eGFP, dimeric CD28-eGFP or GPVI-eGFP was compared and the effect of glenzocimab on the molecular brightness of GPVI-eGFP determined.

Fibrin-induced platelet aggregation: solubilized fibrin was obtained as described. [33] Washed human platelets were pre-incubated for 20 min at room temperature with vehicle, eptifibatide 9 µM or glenzocimab 50$\mu g.mL^{-1}$ before aggregation was initiated by the addition of the fibrin monomers solution (200 $\mu g.mL^{-1}$).

Solid phase binding assays were performed according to previously described protocols.[29 23]

Data were analysed using data Prism 9 software.

Crystallization and structure determination

To form the GPVIex and glenzocimab complex, a 1.3 fold molar excess of glenzocimab was mixed with GPVIex and incubated at 4°C for 1 hour. The complex was purified by size exclusion chromatography using a HiLoad 26/60 Superdex column (Cytiva) equilibrated with

20 mM Tris, 150 mM NaCl, pH 7.4. Two peaks were observed, one corresponding to the excess of Fab and the other to the binary complex GPVIex-Fab (Supplemental Figure 1). The complex was isolated and concentrated to 4.5 $mg.mL^{-1}$ for crystallography. Crystals were generated in 20% PEG 3350, 0.1 M Bis-Tris propane pH 8.5, 0.2 M KSCN, and diffraction data were collected at the station X06DA of the Swiss Light Source, Paul Scherrer Institute, Villigen, Switzerland, (λ = 1.0000 Å) equipped with a Pilatus 2M-F detector. The data were processed using XDS and Aimless.[34,35]

The structure was determined using the Phaser molecular replacement software.[36] The search models used for the heavy chain, light chain and GPVI were PDB entries 5AZE, 3GKW and 5OU7, respectively. One complex was found in the asymmetric unit, with a solvent content of 53 %. The structure was initially refined using the Refmac5 software [37] and model building was carried out in Coot.[38] Thereafter the model was subject to refinement using Buster while model building was pursued in Coot.[39] The Qt-PISA and NCONT programs were used to investigate the antigen-antibody interactions.[40] The atomic coordinates and structure factors (PDB ID codes 7R58) have been deposited in the Protein Data Bank (www.wwpdb.org). Structural models were generated from PDB coordinates using UCSF Chimera v1.15.[41]

Results

Inhibitory properties of glenzocimab

Glenzocimab has been reported to inhibit GPVI binding to collagen and collagen-induced platelet aggregation. [23] Here we confirm that glenzocimab inhibits collagen-induced platelet activation as indicated by the inhibition of P-selectin exposure at the platelet surface (Figure 1A). The monoclonal Fab 3J24 which targets the D2 domain of GPVI [29] had no inhibitory effect indicating that the mechanism of inhibition by glenzocimab is specific.

GPVI is predominantly monomeric on resting platelets with a minor fraction present as a dimer. [30-32] Dimers of recombinant soluble GPVI have been reported to have a greater affinity for collagen than monomers. [30,42] We have thus investigated whether glenzocimab could impact GPVI dimerization. We have analyzed the affinity of glenzocimab for soluble recombinant GPVI monomers and dimers by SPR (Supplemental Figure 2). Glenzocimab bound to GPVI to both monomers and dimers with a K_D of 12.60 ±0.04 nM and 1.310 ±0.005 nM (mean±SD), respectively. We also examined whether glenzocimab could impact GPVI

dimerization and clustering by FCS (Figure 1B). HEK293T cells expressing similar levels GPVI-eGFP, or monomeric CD86-eGFP, or dimeric CD28-eGFP (Supplemental Figure 3) were compared for molecular brightness with the level of brightness governed by the degree of receptor dimerization/clustering. The molecular brightness of cells expressing GPVI-eGFP was intermediate between the brightness of cells expressing monomeric CD86-eGFP and cells expressing dimeric CD28-eGFP confirming that GPVI is present at the cell surface as a mixture of monomers and dimers. [32] In the presence of glenzocimab the molecular brightness of GPVI-eGFP was lowered to the level of CD86-eGFP, indicating that glenzocimab inhibits GPVI dimerization and clustering.

GPVI interaction with fibrin has also been shown to promote platelet procoagulant activity and platelet spreading. [13,14] Here, light transmission aggregometry showed that fibrin induces aggregation of washed platelets (Figure 1C). In these experiments, fibrin solubilized in acidic conditions repolymerizes when added to the platelet suspension. When the experiment was repeated in the presence of eptifibatide, a peptidic antagonist of αIIbβ3, no change in light transmission was observed confirming that fibrin triggered αIIbβ3-dependent platelet aggregation. Glenzocimab completely inhibited fibrin-induced platelet aggregation.

Glenzocimab binds to the D2 domain of GPVI away from the collagen-binding site

Studies were performed to solve the crystal structure of glenzocimab-bound GPVI. One complex of GPVIex-glenzocimab was found in the asymmetric unit (PDB ID: 7R58). The structure was determined to a resolution of 1.9 Å with the entire interface region between glenzocimab and GPVI well resolved. The data collected and refinement statistics are summarized in Table 1. No dimeric GPVI structures are present in the crystal (Figure 2A). The interaction area between GPVI and glenzocimab is 940 $Å^2$ (503.6 $Å^2$ for the heavy chain and 436.4 $Å^2$ for the light chain). Glenzocimab bound largely to the D2 domain C-C' loop region, with several residues in the C-C' loop of GPVI situated within a distance of 4 Å from atoms of the Fab CDRs H1 (P131, A132, Y134) and H3 (Y134, K135, N136); the flanking amino acid P137 is in vicinity of both CDRH3 and CDR L2. The multiple hydrophobic interactions between these residues and with both the light and heavy chain of glenzocimab are shown in Figure 2B. Epitope residues having atoms within a distance of 4 Å from atoms in glenzocimab are shown in Table 2. Ten potential hydrogen bonds were identified between

GPVI and the VL domain of glenzocimab as defined by QtPISA[35] (Figure 2C and Table 3). The discontinuous epitope of glenzocimab on GPVI is depicted in Figure 2D.

Superimposing the GPVIex-glenzocimab complex (PDB ID: 7R58) on the original crystal structure of the GPVI dimer (PDB ID: 2GI7) revealed a root mean square deviation (RMSD) of 1.4 $Å^2$ for 161 aligned Cα atoms (Figure 3A). The 99-109 region which forms a loop between the βA and βB strands of D2 in 2GI7 contains several residues involved in the interaction with glenzocimab CDR-L1 (N31, N33, N35 and Y37 are within hydrogen bonding distance) which results in a conformational change within this loop and the formation of a short β strand ($A^{103}VS^{105}$) named βA' (Figure 3B) that interacts with the βG strand (residues 178-183) . In 2GI7, the βG strands of the D2 domain from both subunits formed a continuous β sheet at the dimer interface (Figure 3C). In the GPVIex-glenzocimab complex, this β-sheet was hampered by the newly-formed βA' strand which makes polar contacts with the βG strand and prevents the interaction of βG with a second βG from an adjacent subunit (Figure 3D). As a consequence, no dimer formation was observed, and this is consistent with the FCS data. As the C-C' loop is part of the glenzocimab epitope and was shown to be involved in the formation of dimers of GPVI complexed to the Nb2 nanobody, the structures of 7R58 and 7NMU were compared (supplemental Figure 4A). [31] This shows that the swapped domain structure cannot be formed in the presence of glenzocimab. The orientation of the C-C' loop in the glenzocimab bound structure also differs to that in the unbound non-domain swapped dimer (2GI7) (supplemental Figure 4B) highlighting that engaging glenzocimab causes a visible shift in this flexible loop resulting in minor changes within the D2 domain secondary structure.

Glenzocimab creates steric hindrance preventing a CRP chain from binding to adjacent GPVIs

The GPVI-CRP crystal structures (PDB ID: 5OU8 and 5OU9) reveal that the CRP-binding motif of GPVI is present within a groove on D1 with an orientation that allows the binding of multiple GPVIs along a single triple helix.[12] In the glenzocimab-GPVI co-crystal the variable domain of the Fab light chain bound to D2 lies directly in the path of the D1-bound CRP chain, thus preventing the binding of longer CRP or collagen chains (Figure 4 and supplemental video).

In addition, on binding to glenzocimab, the GPVIex D1 domain undergoes small structural changes. In the GPVI-CRP co-crystals, the CRP binding groove within D1 is largely made up of βC and βF strands (Figure 5A). In the GPVI-glenzocimab complex, R38, a key CRP binding residue, shifts 3.5 Å towards the CRP groove where it interacts with E40 and stacks against Y47 (Figure 5 B,C). Combining the major steric hindrance caused by glenzocimab, the small conformational shifts and inhibition of dimerisation explains the strong inhibition caused by glenzocimab.

Truncating the C-C' loop of the GPVI D2 domain alters the binding of glenzocimab

In order to validate the binding site for the antibody, we investigated whether truncating D2 C-C' loop residues 129-136 affected the interaction of GPVI with glenzocimab. No binding of glenzocimab to a GPVI-Fc Δ129-136 coated surface was detected, even at concentrations up to 1 $\mu g.mL^{-1}$ (20.5 nM) which exceed the 88 $ng.mL^{-1}$ (1.8 nM) EC50 observed for its binding to GPVI-Fc by an order of magnitude (Figure 6A). Similarly, the binding of GPVI-Fc Δ129-136 to immobilized glenzocimab was profoundly impaired, with very low levels of binding for concentrations higher than 1 $\mu g.mL^{-1}$ (10.7 nM) while the EC50 observed with GPVI-Fc was 7.5 $ng.mL^{-1}$ (0.05 nM) (Figure 6B). Taken together these findings are consistent with the crystal structure of the complex and confirm the critical role of the D2 C-C' loop in the glenzocimab epitope. The ability of glenzocimab to inhibit the binding of GPVI-Fc and GPVI-Fc Δ129-136 to collagen was then analyzed (Figure 6C). As previously reported, GPVI-Fc Δ129-136 bound to collagen but to a lesser degree than GPVI-Fc [31] necessitating a higher concentration (20 $\mu g.mL^{-1}$). No inhibition of GPVI-Fc Δ129-136 binding was observed in the presence of glenzocimab at levels up to 1 $\mu g.mL^{-1}$ (20.5 nM). By contrast, glenzocimab efficiently inhibited the binding to GPVI-Fc at 2 and 20 $\mu g.mL^{-1}$, with IC50s values of 10.52 and 121 $ng.mL^{-1}$ (0.22 nM , 2.52 nM) respectively.

Discussion

We have demonstrated that glenzocimab binds to an epitope located in the D2 domain of GPVI with a high affinity, and we propose a novel mechanism regarding the inhibition of GPVI interactions with its major ligands (Visual Abstract).

Consistent with previous data obtained with the Fab 9O12, we show that its humanized version (ACT017) of clinical grade (glenzocimab) inhibits platelet activation by collagen and

by fibrin. Importantly, we show that glenzocimab inhibits GPVI dimerization and oligomerization.

Amino acid residues involved in the glenzocimab epitope were identified in the D2 domain of GPVI. Out of the four residues (Q99, D109, T111, S144) involved in ten hydrogen bonds at the GPVI-glenzocimab interface, all are well-preserved in non-human primate GPVI while two are substituted for non-conservative residues in rodent GPVI (Q99>H; S144>N) leading to potential alteration of these hydrogen bonds. In addition, several residues involved in hydrophobic interactions are not conserved in rodent GPVI. Overall, these data provide direct evidence as to why glenzocimab does not cross-react with rodent GPVI.

X-ray analysis provided a detailed view of the GPVI extracellular domain in complex with glenzocimab. The epitope is discontinuous. All the GPVI residues involved in interactions with glenzocimab belong to the D2 domain of GPVI, as previously foreseen for the parental mouse IgG 9O12.[29] Because the CRP/collagen binding site on GPVI has been consistently identified within the D1 domain as deduced from mutagenesis, docking and the analysis of crystallographic structures, one can conclude that the collagen binding site and the epitope targeted by glenzocimab do not overlap at all.[11,43,44] The mechanism by which glenzocimab inhibits GPVI activation results from two major effects: (i) glenzocimab prevents GPVI dimer formation through D2 and potentially promotes their dissociation, (ii) the light chain of glenzocimab provokes steric hindrance that prohibits the binding to adjacent GPVI-binding sites on CRP and collagen fibers. The loss of dimerization would also limit activation as it lowers the numbers of GPVI receptors in a cluster.[45]

Glenzocimab and the GPVI D2 domain

Several studies have established that the avidity of GPVI for collagen is increased by its dimerization. All these studies have been performed using the recombinant extracellular domain of GPVI fused to the Fc domain of human IgG.[30,42] The ability of GPVI to form dimers was confirmed by different studies during which the GPVI ectodomain dimerized upon crystallization (PDB ID: 2GI7, 5NMU, 5OU7). In the first wild type GPVI crystal described (PDB ID: 2GI7), two GPVI subunits formed homotypic interactions and were associated back-to-back via a β-sheet interaction between the parallel G strands (residues 178-183) of both D2 domains. The interaction between the two D2 domains no longer seems possible when glenzocimab binds to GPVI, and consistent with this, no GPVI dimers were observed in

crystals from the GPVI-glenzocimab complex or when analyzed by FCS microscopy. This is in line with the observation that the deletion of D2 prevents the formation of dimers at the cell surface.[32] Glenzocimab could even favor the dissociation of dimers by breaking the parallel β-sheet made of two G strands.

As regards to GPVI-Fc, it has never been crystalized and the degree to which this construct mimics the structures of dimers in crystals remains unclear. In GPVI-Fc, both D2 domains are artificially maintained in close proximity via the Fc moiety and with some flexibility so that contacts between the G strands of the D2 domains are most likely not maintained. In this way, the binding of one Fab to GPVI-Fc should be sufficient to decrease the avidity for collagen. Furthermore, two Fabs could bind both D2 domains of GPVI-Fc.

The structure of the GPVIex-glenzocimab crystal indicates that the epitope includes five residues of the C-C' loop (P131, A132, Y134, K135, N136) and the flanking P137. The C-C' loop (G^{129}DPAPYKN^{136}) has recently been shown to contribute to the formation of a domain swap between the D2 hinge regions of two GPVI subunits in the crystal of GPVI in complex with the nanobody Nb2 (PDB ID: 7NMU).[31] Glenzocimab would prevent any access to, and structural rearrangement of this loop. Slater and colleagues truncated this region to test its functional significance. GPVI-Fc Δ129-136 bound with weaker affinity to collagen in DT-40 cells expressing full-length GPVI Δ129-136. Signaling in response to CRP or collagen was abolished. [31] As expected, we observed that the deletion of residues 129-136 blocked the binding of glenzocimab, confirming the important contribution of the C-C' loop to the epitope. Altogether, these observations argue for a significant contribution of the C-C' loop in the GPVI function.

Glenzocimab and the collagen-binding domain

The GPVI-CRP complex has recently been solved leading to a better understanding of the mechanism of GPVI activation by collagen. [12] During these experiments a truncated GPVI, in which the 102-105 and 131-136 loops (ΔPAVS ΔPAPYKN) were deleted, was complexed to CRPs of different lengths (GPO_5 or GPO_3). Analysis of the crystals (PDB ID: 5OU8, 5OU9) indicated how the repeated GPVI binding motifs on parallel triple helix can interact with adjacent GPVI. The most striking observation on the GPVI-glenzocimab complex is the position of the variable domain of the light chain that obstructs the passage of the triple

helix. This steric hindrance prevents collagen binding to adjacent GPVI impeding the clustering of GPVI that is critical for downstream signaling. Minor conformational changes also affected the collagen binding groove, with the protrusion of the R38 side chain susceptible to clash with a hydroxyproline residue of CRP. This may contribute to alter the interactions between GPVI and fibrillar collagen in addition to steric clashes caused by glenzocimab VL domain.

It should also be noted that the glenzocimab epitope does not contain residues known to be involved in natural variants of human GPVI which makes it possible to predict that the efficacy of glenzocimab will not be affected by GPVI polymorphisms.[46] The GP6 sequence presents five non-synonymous single nucleotide polymorphisms (*rs1613662*, *rs1654416*, *rs2304167*, *rs1654413*, and *rs1671152*) which are far distant from the residues involved in the glenzocimab epitope (Supplemental Figure 5). This enables the prediction that glenzocimab binding to the five major GPVI haplotypes derived from the 1000 Genomes Project will be conserved.[47] The clinical effect of glenzocimab should not therefore be dependent on the currently known GPVI polymorphisms.

Fibrin or fibrinogen have been identified as ligands for GPVI with important functional effects on thrombus growth and stability. Platelet GPVI is activated by fibrin, leading to the exposure of platelet procoagulant activity and the accretion of new platelets.[13,14] GPVI has also been shown to allow the spreading of human platelets on immobilized fibrinogen and to trigger outside-in signaling following fibrinogen binding to integrin αIIbβ3.[15] Recent studies are in favor of a greater avidity of dimeric GPVI for fibrin(ogen) compared to monomeric GPVI, and a mechanism involving GPVI clustering by fibrin fibers for platelet activation.[48,49] The present observation that glenzocimab inhibits platelet aggregation by fibrin is in line with previous data.[14,25] Moreover, we observed that an αIIbβ3 integrin-specific inhibitor and glenzocimab block the aggregation to a similar level in agreement with the results of Perrella and colleagues.[50] This confirms that the integrin αIIbβ3 and GPVI play complementary roles in fibrin-induced platelet activation/aggregation. Both receptors are required and Syk is activated downstream of both. At the low concentration of fibrin used in our experiments to prevent clotting, its binding to integrin αIIbβ3 can trigger Syk activation but at a level insufficient to induce platelet aggregation. The interaction of fibrin with GPVI in turn triggers

a sufficient level of Syk activation for the full signaling cascade to occur but only when the integrin αIIbβ3 is functional. Together, functional and structural data indicate that glenzocimab alters the interaction between GPVI and fibrin and that the hinge region of the D2 domain may also regulate this interaction as well.

Conclusion

Resolving the crystal structure of the glenzocimab-GPVI complex has allowed us to identify a new mechanism by which the binding of glenzocimab to the hinge region of the D2 domain prevents interactions between GPVI and its main ligands, collagen and fibrin(ogen), and enables the inhibition of cell signaling downstream of GPVI and potentially the destabilization of platelet-platelet interactions. This original mechanism may be the basis of the powerful antithrombotic effect of glenzocimab *in vitro* and *in vivo* in animal models of thrombosis and supports its clinical development in thrombotic diseases such as acute ischemic stroke.

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Authorship contributions

PB and MJP designed the study, interpreted the data and wrote the manuscript. AS and SW provided GPVI-FcΔ129-136, interpreted the data and edited the manuscript. MW, JCC, MP, designed experiments, interpreted the data, and edited the manuscript; IGJ contributed to

structural analysis; NR, SL and DF performed experiments. ET edited the manuscript. KL contributed to design the heterologous expression and purification of GPVIex.

Conflict of interest disclosure

PB and MJP are founders and scientific advisers for Acticor-Biotech

DF, KL and ET are employees at Acticor-Biotech

MW and NR are employees at SARomics Biostructures

Other authors report no other conflicts

ORCID profiles

PB., 0000-0001-8148-5297. AS., 0000-0001-7166-438X. JCC., 0000-0002-1912-7816. SPW., 0000-0002-7846-7423.

MJP., 0000-0002-8450-9247.

References

1. Lebas H, Yahiaoui K, Martos R, Boulaftali Y. Platelets Are at the Nexus of Vascular Diseases. *Front Cardiovasc Med*. 2019;6:132.
2. McFadyen JD, Schaff M, Peter K. Current and future antiplatelet therapies: emphasis on preserving haemostasis. *Nat Rev Cardiol*. 2018;15(3):181-191.
3. Borst O, Gawaz M. Glycoprotein VI - novel target in antiplatelet medication. *Pharmacol Ther*. 2021;217:107630.
4. Jandrot-Perrus M, Busfield S, Lagrue AH, et al. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood*. 2000;96(5):1798-1807.
5. Nieswandt B, Watson SP. Platelet-collagen interaction: is GPVI the central receptor? *Blood*. 2003;102(2):449-461.
6. Bergmeier W, Stefanini L. Platelet ITAM signaling. *Curr Opin Hematol*. 2013;20(5):445-450.
7. Cosemans JM, Kuijpers MJ, Lecut C, et al. Contribution of platelet glycoprotein VI to the thrombogenic effect of collagens in fibrous atherosclerotic lesions. *Atherosclerosis*. 2005;181(1):19-27.
8. Penz S, Reininger AJ, Brandl R, et al. Human atheromatous plaques stimulate thrombus formation by activating platelet glycoprotein VI. *FASEB J*. 2005;19(8):898-909.
9. Kuijpers MJ, Gilio K, Reitsma S, et al. Complementary roles of platelets and coagulation in thrombus formation on plaques acutely ruptured by targeted ultrasound treatment: a novel intravital model. *J Thromb Haemost*. 2009;7(1):152-161.
10. Hechler B, Gachet C. Comparison of two murine models of thrombosis induced by atherosclerotic plaque injury. *Thromb Haemost*. 2011;105 Suppl 1:S3-12.

11. Horii K, Kahn ML, Herr AB. Structural basis for platelet collagen responses by the immune-type receptor glycoprotein VI. *Blood*. 2006;108(3):936-942.
12. Feitsma LJ, Brondijk HC, Jarvis GE, et al. Structural insights into collagen binding by platelet receptor glycoprotein VI. *Blood*. 2022;139(20):3087-3098.
13. Alshehri OM, Hughes CE, Montague S, et al. Fibrin activates GPVI in human and mouse platelets. *Blood*. 2015;126(13):1601-1608.
14. Mammadova-Bach E, Ollivier V, Loyau S, et al. Platelet glycoprotein VI binds to polymerized fibrin and promotes thrombin generation. *Blood*. 2015;126(5):683-691.
15. Mangin PH, Onselaer MB, Receveur N, et al. Immobilized fibrinogen activates human platelets through glycoprotein VI. *Haematologica*. 2018;103(5):898-907.
16. Ahmed MU, Kaneva V, Loyau S, et al. Pharmacological Blockade of Glycoprotein VI Promotes Thrombus Disaggregation in the Absence of Thrombin. *Arterioscler Thromb Vasc Biol*. 2020;40(9):2127-2142.
17. Nieswandt B, Schulte V, Bergmeier W, et al. Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. *J Exp Med*. 2001;193(4):459-469.
18. Massberg S, Gawaz M, Gruner S, et al. A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J Exp Med*. 2003;197(1):41-49.
19. Jandrot-Perrus M, Hermans C, Mezzano D. Platelet glycoprotein VI genetic quantitative and qualitative defects. *Platelets*. 2019;30(6):708-713.
20. Nurden AT. Clinical significance of altered collagen-receptor functioning in platelets with emphasis on glycoprotein VI. *Blood Rev*. 2019;38:100592.
21. Zahid M, Mangin P, Loyau S, et al. The future of glycoprotein VI as an antithrombotic target. *J Thromb Haemost*. 2012;10(12):2418-2427.
22. Mangin PH, Tang C, Bourdon C, et al. A humanized glycoprotein VI (GPVI) mouse model to assess the antithrombotic efficacies of anti-GPVI agents. *J Pharmacol Exp Ther*. 2012;341(1):156-163.
23. Lebozec K, Jandrot-Perrus M, Avenard G, Favre-Bulle O, Billiald P. Design, development and characterization of ACT017, a humanized Fab that blocks platelet's glycoprotein VI function without causing bleeding risks. *MAbs*. 2017;9(6):945-958.
24. Lebozec K, Jandrot-Perrus M, Avenard G, Favre-Bulle O, Billiald P. Quality and cost assessment of a recombinant antibody fragment produced from mammalian, yeast and prokaryotic host cells: A case study prior to pharmaceutical development. *N Biotechnol*. 2018;44:31-40.
25. Lehmann M, Schoeman RM, Krohl PJ, et al. Platelets Drive Thrombus Propagation in a Hematocrit and Glycoprotein VI-Dependent Manner in an In Vitro Venous Thrombosis Model. *Arterioscler Thromb Vasc Biol*. 2018;38(5):1052-1062.
26. Voors-Pette C, Lebozec K, Dogterom P, et al. Safety and Tolerability, Pharmacokinetics, and Pharmacodynamics of ACT017, an Antiplatelet GPVI (Glycoprotein VI) Fab. *Arterioscler Thromb Vasc Biol*. 2019;39(5):956-964.
27. Renaud L, Lebozec K, Voors-Pette C, et al. Population Pharmacokinetic/Pharmacodynamic Modeling of Glenzocimab (ACT017) a Glycoprotein VI Inhibitor of Collagen-Induced Platelet Aggregation. *J Clin Pharmacol*. 2020;60(9):1198-1208.
28. Mazighi M, Richard S, Molina C, et al. Glenzocimab, an novel antithrombotic, is associated with reduced intracrnial hemorrhage and mortality rates when combined with Standard-of-Care reperfusion therapies: The ACTIMIS study. Eur Stroke Jounal; 2022:574.
29. Dumont B, Minullina I, Loyau S, et al. Chimeric Fc Receptors Identify Ligand Binding Regions in Human Glycoprotein VI. *J Mol Biol*. 2006;361(5):877-887.

30. Loyau S, Dumont B, Ollivier V, et al. Platelet glycoprotein VI dimerization, an active process inducing receptor competence, is an indicator of platelet reactivity. *Arterioscler Thromb Vasc Biol*. 2012;32(3):778-785.
31. Slater A, Di Y, Clark JC, et al. Structural characterization of a novel GPVI-nanobody complex reveals a biologically active domain-swapped GPVI dimer. *Blood*. 2021;137(24):3443-3453.
32. Clark JC, Neagoe RAI, Zuidscherwoude M, et al. Evidence that GPVI is Expressed as a Mixture of Monomers and Dimers, and that the D2 Domain is not Essential for GPVI Activation. *Thromb Haemost*. 2021;121(11):1435-1447.
33. Jandrot-Perrus M, Aurousseau MH, Rabiet MJ, Josso F. Fibrinogen Bondy: a new case of dysfibrinogenemia. Isolation of the abnormal fibrinogen molecules. *Thromb Res*. 1982;27(6):659-670.
34. Kabsch W. Xds. *Acta Crystallogr D Biol Crystallogr*. 2010;66(Pt 2):125-132.
35. Evans PR, Murshudov GN. How good are my data and what is the resolution? *Acta Crystallogr D Biol Crystallogr*. 2013;69(Pt 7):1204-1214.
36. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J Appl Crystallogr*. 2007;40(Pt 4):658-674.
37. Murshudov GN, Skubak P, Lebedev AA, et al. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr D Biol Crystallogr*. 2011;67(Pt 4):355-367.
38. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr*. 2010;66(Pt 4):486-501.
39. Bricogne G, Blanc E, Brandl M, et al. BUSTER version 2.11.7.: Global Phasing Ltd, Cambridge, United Kingdom.; 2011.
40. Winn MD, Ballard CC, Cowtan KD, et al. Overview of the CCP4 suite and current developments. *Acta Crystallogr D Biol Crystallogr*. 2011;67(Pt 4):235-242.
41. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem*. 2004;25(13):1605-1612.
42. Miura Y, Takahashi T, Jung SM, Moroi M. Analysis of the interaction of platelet collagen receptor glycoprotein VI (GPVI) with collagen. A dimeric form of GPVI, but not the monomeric form, shows affinity to fibrous collagen. *J Biol Chem*. 2002;277(48):46197-46204.
43. Smethurst PA, Joutsi-Korhonen L, O'Connor MN, et al. Identification of the primary collagen-binding surface on human glycoprotein VI by site-directed mutagenesis and by a blocking phage antibody. *Blood*. 2004;103(3):903-911.
44. O'Connor MN, Smethurst PA, Farndale RW, Ouwehand WH. Gain- and loss-of-function mutants confirm the importance of apical residues to the primary interaction of human glycoprotein VI with collagen. *J Thromb Haemost*. 2006;4(4):869-873.
45. Slater A, Jandrot-Perrus M. GPVI and collagen: the final word? *Blood*. 2022;139(20):3005-3007.
46. Watkins NA, O'Connor MN, Rankin A, et al. Definition of novel GP6 polymorphisms and major difference in haplotype frequencies between populations by a combination of in-depth exon resequencing and genotyping with tag single nucleotide polymorphisms. *J Thromb Haemost*. 2006;4(6):1197-1205.
47. Genomes Project C, Auton A, Brooks LD, et al. A global reference for human genetic variation. *Nature*. 2015;526(7571):68-74.

48. Moroi M, Induruwa I, Farndale RW, Jung SM. Dimers of the platelet collagen receptor glycoprotein VI bind specifically to fibrin fibers during clot formation, but not to intact fibrinogen. *J Thromb Haemost*. 2021.
49. Xu RG, Gauer JS, Baker SR, et al. GPVI (Glycoprotein VI) Interaction With Fibrinogen Is Mediated by Avidity and the Fibrinogen alphaC-Region. *Arterioscler Thromb Vasc Biol*. 2021;41(3):1092-1104.
50. Perrella G, Huang J, Provenzale I, et al. Nonredundant Roles of Platelet Glycoprotein VI and Integrin alphaIIbbeta3 in Fibrin-Mediated Microthrombus Formation. *Arterioscler Thromb Vasc Biol*. 2021;41(2):e97-e111.

Table 1: Crystallographic data collection and refinement statistics.

Data collection	**Value**
Resolution (Å)	49.4 - 1.90 (1.95 - 1.90)
Wavelength (Å)	1,0000
Space group	$P2_1\ 2_1\ 2_1$
Unit cell	
a, b, c (Å)	68.7, 93.2, 110.4
Completeness (%)	100 (99.5)
Redundancy	13.2 (12.5)
No of observations/unique reflections	743962 / 56379
<I/ σ(I)>	9.9 (1.6)
CC (1/2) (%)	99.5 (59.8)
R merge (I) (%)	25.5 (177.7)
Rcryst (F) (%)	17.9 (28.6)
Rfree (F) (%)	22.5 (21.4)
No. of non-hydrogen atoms	5539
No. of water molecules	779
R.m.s deviations	
Bond lengths (Å)	0.010
Bond angles (°)	1.09
Mean B-factor all atoms ($Å^2$)	24.2
Mean B-factor protein chain A, H, L ($Å^2$)	21.4, 21.8, 23.1
Mean B-factor solvent ($Å^2$)	36.2
Mean B-factor others ($Å^2$)	37.3
Ramachandran plot quality [#]	
Favored regions (%)	98.1
Allowed regions (%)	1.9
Outliers (%)	0

Values in parentheses are for highest-resolution shell.

[#]Calculated using a local Molprobity server

Table 2: GPVI-glenzocimab interactions. Epitope residues were defined as GPVI residues having atoms within a distance of 4 Å from Fab atoms.

CDR	glenzocimab	GPVI-CBD residues
CDR-H1	Thr 28	Pro 131, Ala 132
	Thr 30	Pro 131
	Ser 31	Tyr 134
CDR-H2	Asn 55	Ala 153
CDR-H3	Thr 100	Tyr 134
	Val 101	Tyr 134, Ile147, His154
	Val 102	Leu 125, Tyr 134, Phe 145, Pro 146
	Gly 103	Pro 146
	Asp 104	Pro 146, Ile 148
	Trp 105	Lys 135, Asn 136, Pro 137
	Tyr 106	Ile 148
CDR-L1	Asn 31	Asp 109
	Asn 33	Gln 99, Pro 100, Asp 109
	Asn 35	Gln 99, Asp 109, Thr 111
	Tyr 37	Asp 109, Thr 111, Pro 146, Ile 148
CDR-L2	Tyr 54	Pro 137
	Arg 55	Ser 144, Pro 146
	Phe 60	Pro 137
	Ser 61	Pro 137
CDR-L3	Leu 96	Ile 148

Table 3: Hydrogen bonds involved in the GPVI - glenzocimab interface. Distance were calculated using Qt-PISA. * indicates a very long distance to being defined as a hydrogen bond

GPVI	glenzocimab	distance (Å)
Asp 109 (OD2)	Asn L31 (ND2)	2.9
Asp 109 (OD1)	Asn L33 (ND2)	2.8
Asp 109 (O)	Asn L33 (ND2)	3.2
Gln 99 (NE2)	Asn L33 (O)	3.3
Gln 99 (OE1)	Asn L35 (ND2)	3.2
Asp 109 (OD2)	Asn L35 (ND2)	3.0
Thr 111 (OG1)	Asn L35 (ND2)	3.7*
Asp 109 (OD2)	Tyr L37 (OH)	2.6
Thr 111 (OG1)	Tyr L37 (OH)	3.9*
Ser 144 (O)	Arg L55 (NH2)	3.4*

Figure legends

Figure 1: Inhibitory properties of glenzocimab

(A) Glenzocimab inhibits collagen-induced platelet activation. Washed human platelets were preincubated with glenzocimab or the 3J24 Fab (50 µg.mL^{-1}) before activation was triggered by the addition of collagen (25 µg.mL^{-1}) during 15 min at room temperature. Exposure of P-selectin was assessed by flow cytometry using an FITC-coupled anti P-selectin. Mean fluorescence intensity are shown. Data are the mean±SD of three experiments made in triplicate. Statistical analysis was performed using one way Anova followed by a Tukey's multiple comparisons test *** $p<0.001$. NS= not significant.

(B) Glenzocimab inhibits GPVI dimerization and clustering. Box plot showing the effect of glenzocimab (50 µg.mL^{-1}) on the molecular brightness (cpm s^{-1}) of GPVI-eGFP alongside the molecular brightness (cpm s^{-1}) of monomeric CD86-eGFP and dimeric CD28-eGFP control receptors in transfected HEK293T cells. For all box plots, centre lines represent the median; box limits indicate the 25th and 75th percentiles and whiskers extend to minimum and maximum points. Significance was measured with Kruskal-Wallis with Dunn's *post-hoc* where $p \leq 0.05$. NS = not significant. FCS measurements were taken in 35-49 cells from 3 independent experiments.

(C) Glenzocimab inhibits fibrin-induced platelet aggregation. Washed human platelets were pre-incubated with vehicle (red curves), eptifibatide 9µM (green curve) or glenzocimab (50µg.mL^{-1}) before aggregation was initiated by the addition of a solubilized fibrin (200 µg.mL^{-1}).

Figure 2: Delineation of the glenzocimab epitope based on GPVIex/glenzocimab crystal analysis.

(A) The heavy chain (VH-CH1) of glenzocimab is depicted in red and the light chain (VL-CL) in blue. GPVI domain 2 (D2) bound to glenzocimab is represented in green as is GPVI domain 1 (D1). The D2 C-C' loop (residues 131-137) is shown in cyan. (B) Hydrophobic contacts made between the D2 C-C' loop (shown in green and the heavy and light variable chains of glenzocimab (red and light blue respectively). Hydrophobic contacts are shown as red dotted lines and represent residue at within 4 Å apart.

(C) Hydrogen bonds between glenzocimab light chain CDRs (grey) and GPVI (green) according to Qt-PISA.

(D) Representation of the glenzocimab epitope on the GPVIex sequence (top strand corresponds to D1 domain, bottom strand to D2 domain): the glenzocimab epitope is discontinuous. In red and in blue: residues having atoms within a distance of 4 Å from the VL and VH CDRs, respectively. In purple: residues with atoms within a distance of 4 Å from both VL and VH CDRs. Underlined are GPVI residues involved in hydrogen bonds with glenzocimab. The solid red box indicates the GPVI 129-136 truncation that results in the loss of glenzocimab binding and decreased binding to collagen. Red dashed boxes indicate GPVI residues truncated in the 5OU7, 5OU8 and 5OU9 crystals. * indicates N72.

Figure 3: Structural comparison between GPVIex/glenzocimab and 2GI7 crystals.

(A) Overview of the superimposed dimeric GPVIex (PDB ID: 2GI7) on the GPVIex/glenzocimab complex (PDB ID: 7R58). The heavy chain (VH-CH1) of glenzocimab is depicted in red and the light chain (VL-CL) in blue. GPVI domain 2 (D2) bound to glenzocimab is represented in green and GPVI without any ligand (PDB ID: 2GI7) in purple.

(B) Close up view of the overlayed D2 domains of dimeric GPVI (PDB:2GI7) (pale cyan) and glenzocimab bound GPVI (green) highlighting the rearrangements within D2 due to clashes between the βA-βB loop of GPVI and the light chain of glenzocimab (light blue). Contacts with the light chain of glenzocimab causes a conformational change βA-βB loop resulting in the formation of a new β-strand, βA', (shown in yellow).

(C) Zoomed in view of the dimeric interface formed between two βG strands from two GPVI subunits in the dimeric crystal structure (PDB:2GI7). Subunits are coloured pale cyan and light pink, respectively. Polar contacts between each strand are shown as red dashed lines with binding resides shown as sticks.

(D) Zoomed in view of the βG strand of glenzocimab bound GPVI (green) superimposed with the alternate GPVI subunit found in the dimeric structure (light pink). The conformational changes in the glenzocimab bound D2 domain results in the formation of a new βA' strand which blocks the interaction between the two βG strands. The formation of βA' also results in a shift in the βG strand resulting in an increased gap between the two βG strands of 0.6 Å. These factors combined explain how GPVI dimerisation is blocked by glenzocimab.

Figure 4: Glenzocimab inhibits CRP/collagen binding through steric hindrance.

(A) D1 domain of GPVI (grey) in complex with CRP (magenta) (PDB ID: 5OU8).

(B) D1 domain of GPVI (green) in complex with glenzocimab (PDB ID: 7R58). The VL domain of glenzocimab (light blue) at the front creates a barrier in the way of the CRP indicated by the arrow (magenta).

(C) Surface representation of the glenzocimab-bound structure of GPVI (PDB ID: 7R58) with CRP binding superimposed from PDB ID: 5OU8. GPVI, CRP and glenzocimab heavy and light chains are colored in green, magenta, orange and blue, respectively. The binding of glenzocimab to the D2 domains means the light variable region of the Fab lies directly in the way of the bound CRP chain. Larger CRP and collagen chains would be prevented from binding due to the obstruction by the light chain.

(D) Stereo view of (C)

Figure 5: The CRP binding groove in the GPVIex/glenzocimab complex

(A) Zoomed-in view of the CRP binding groove of the CRP-bound GPVI (PDB ID: 5OU8) (A) and glenzocimab-bound GPVI (PDB ID: 7R58) (B). Structures shown in grey and green, respectively, with CRP shown in magenta. In (C) both structures are superimposed: the CRP binding groove is largely made from the βC and βF strands within D1. In the glenzocimab-bound structure the R38 shifts 3.5 Å towards CRP and clashes with a CRP hydroxyproline residue. The side chain of R38 in both structures is shown as a stick and the shift is shown using a red dotted line.

Figure 6: Truncation of GPVI Δ129-136 disrupts the epitope of glenzocimab

GPVI-Fc Δ129-136 was compared to GPVI-Fc regarding interactions with glenzocimab in solid-phase assays.

(A) Dose dependent-binding of glenzocimab to immobilized GPVI-Fc (black circles) and GPVI-Fc Δ129-136 (black squares). The EC50 value of GPVI-Fc was of 85 $ng.mL^{-1}$ (1.88nM), in line with the K_D value. No binding between glenzocimab and GPVI-Fc Δ129-136 could be observed even at 1 $\mu g.mL^{-1}$ which is more than ten-fold the EC50 of GPVI-Fc.

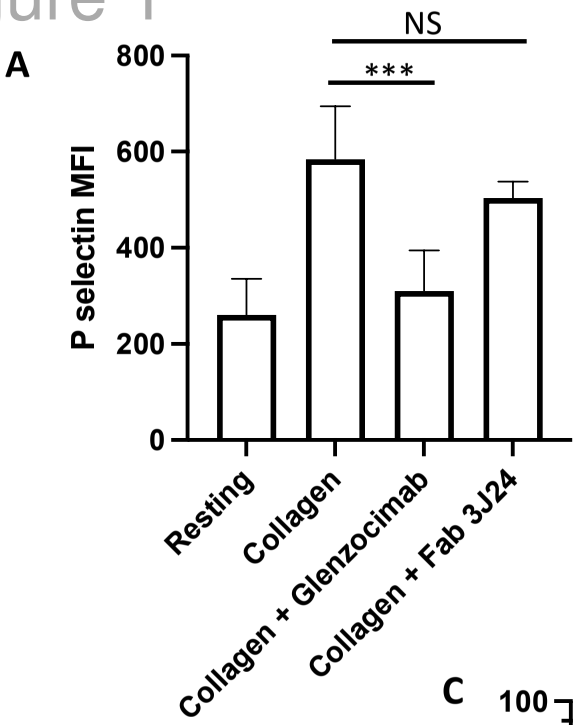
(B) Dose-dependent binding of GP-VI-Fc (black circles) and GPVI-Fc Δ129-136 (black squares) to immobilized glenzocimab confirms defective interactions between glenzocimab and GPVI-Fc Δ129-136. Results are mean±SEM of three experiments performed in triplicate.

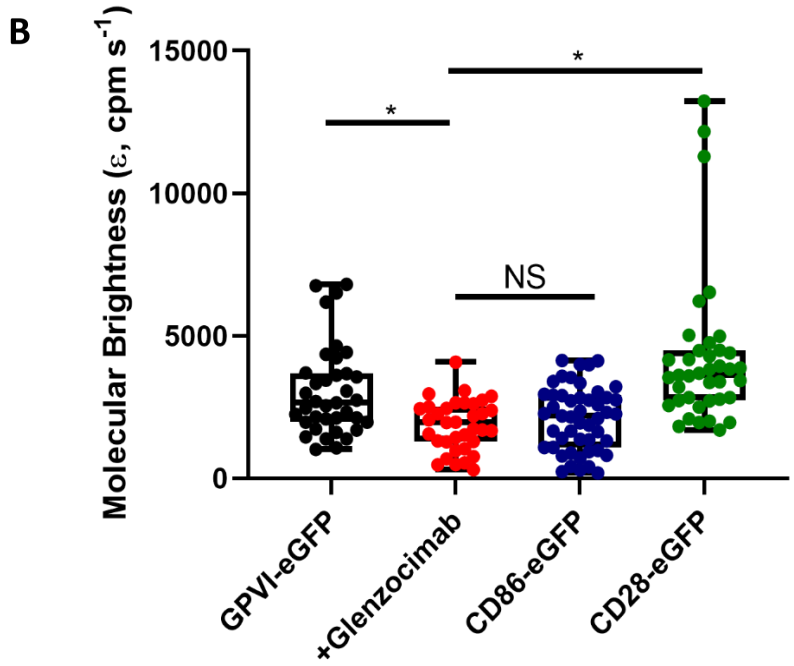
(C) Inhibition of GPVI-Fc binding to immobilized collagen by increasing concentrations of glenzocimab. Binding of GPVI-Fc at 2 $\mu g.mL^{-1}$ (black circles) or at 20 $\mu g.mL^{-1}$ (black squares) to collagen was dose-dependently inhibited by glenzocimab with IC50 values of 10.25 and 113.7 $\mu g.mL^{-1}$, respectively. In contrast, glenzocimab had no effect on the binding of GPVI-Fc Δ129-136 at 20 $\mu g.mL^{-1}$ (white triangles) to collagen.

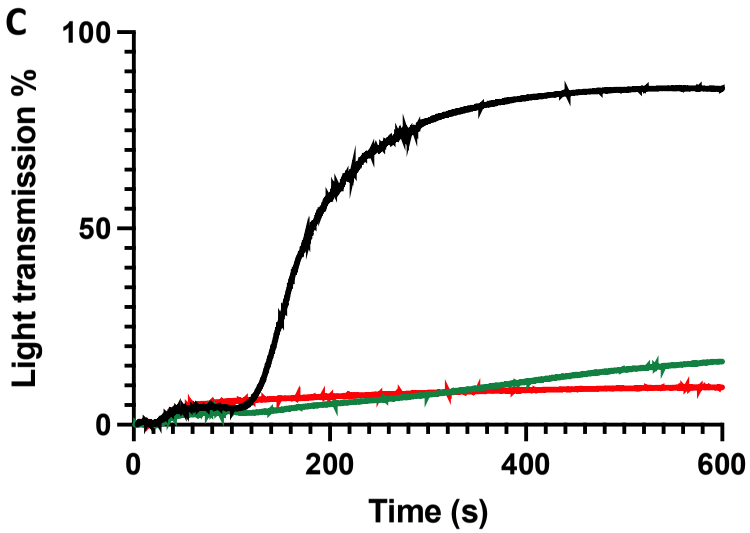
Visual Abstract: Dual mechanism of GPVI inhibition by glenzocimab

On the left is represented the clustering of GPVI by collagen. Collagen binds to the D1 domain of GPVI. On the right is represented a glenzocimab-bound GPVI. Glenzocimab binds to D2 and prevents the formation of GPVI dimers. The light chain of glenzocimab creates a steric hindrance blocking the binding of the triple helix of collagen.

Figure 1

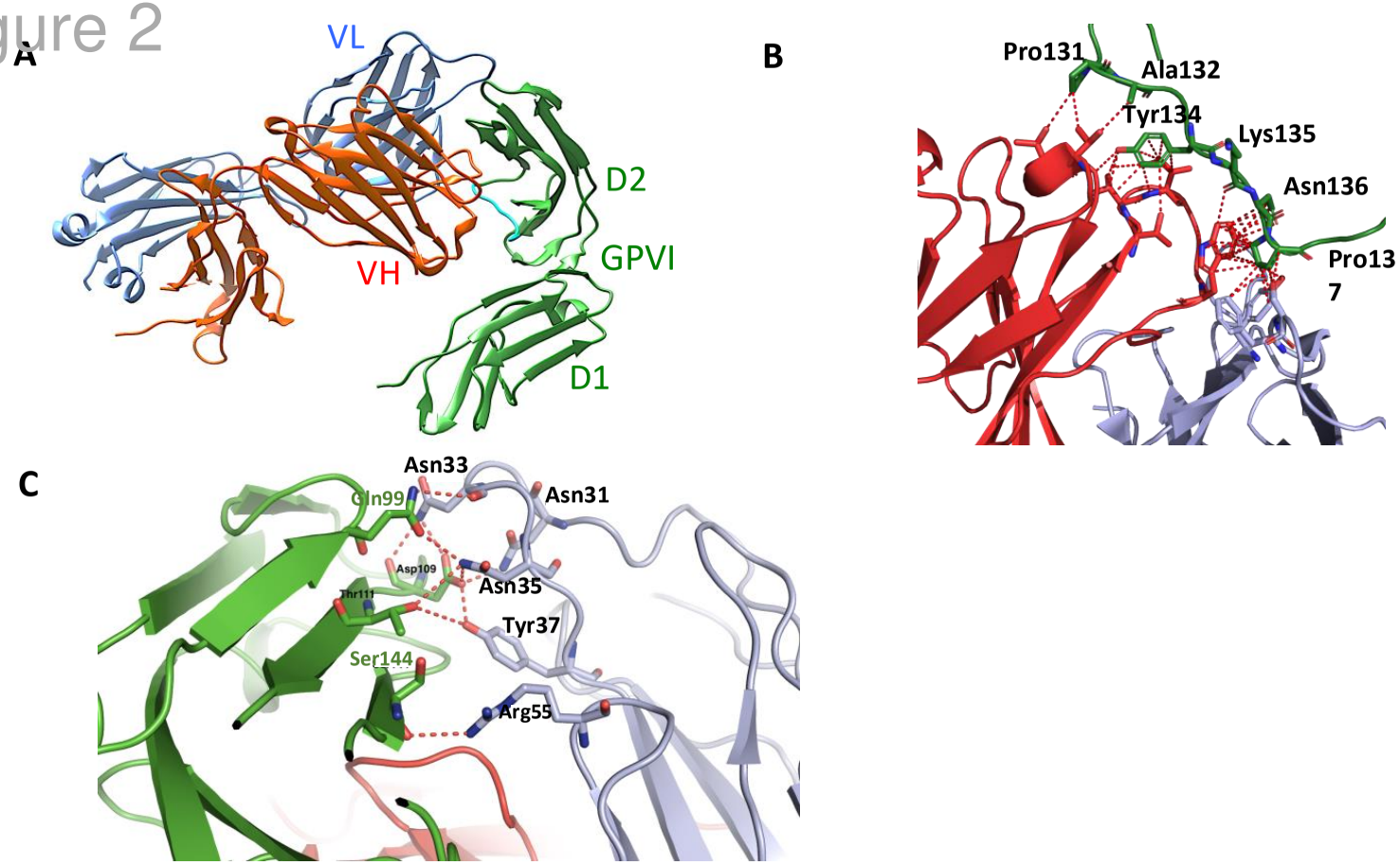






Billiald *et al.* Figure 1

Figure 2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VH
D1
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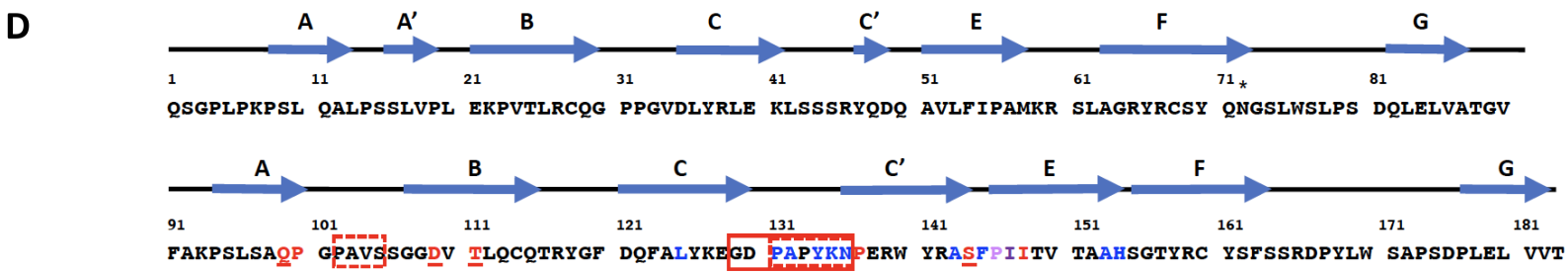
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A B C C' E F G
91 101 111 121 131 141 151 161 171 181
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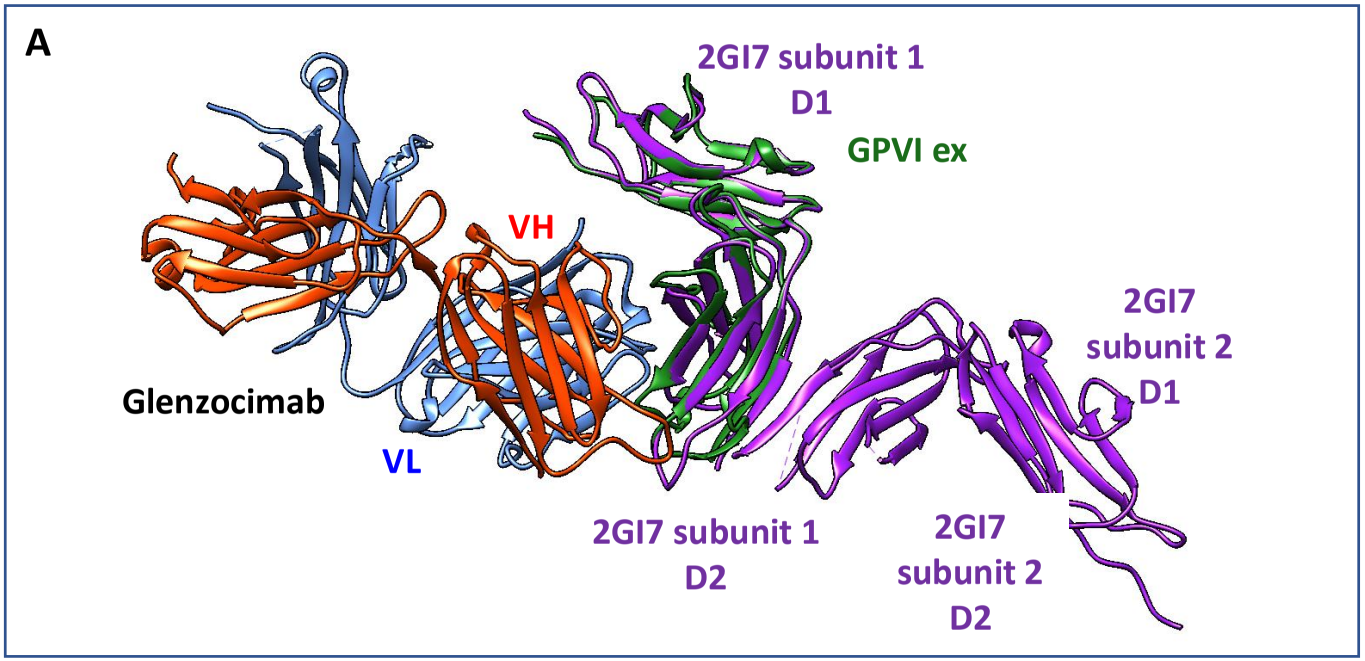
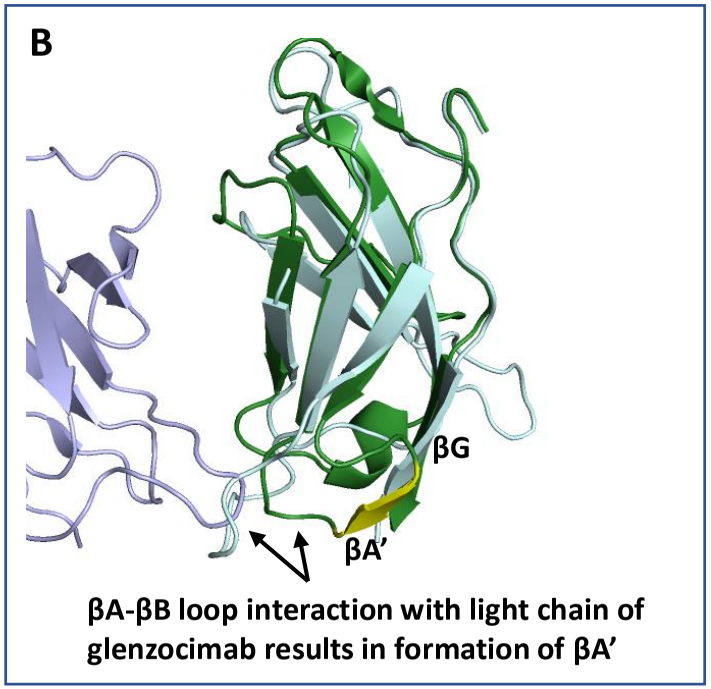
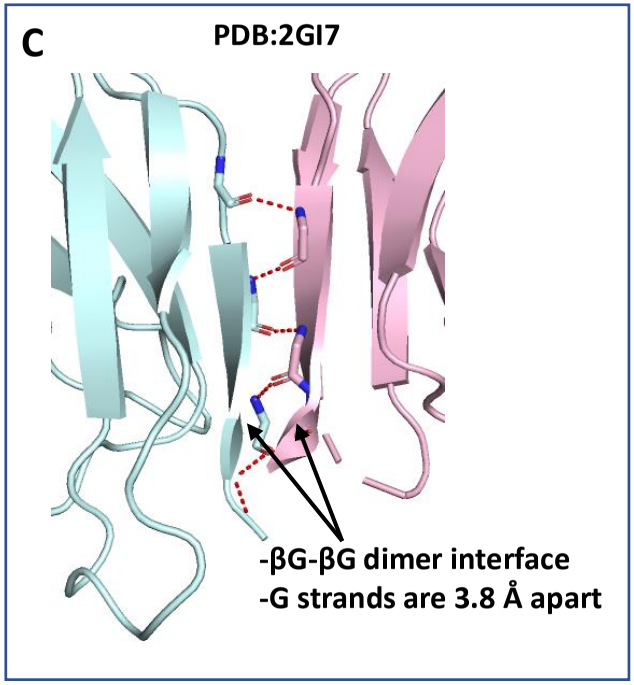
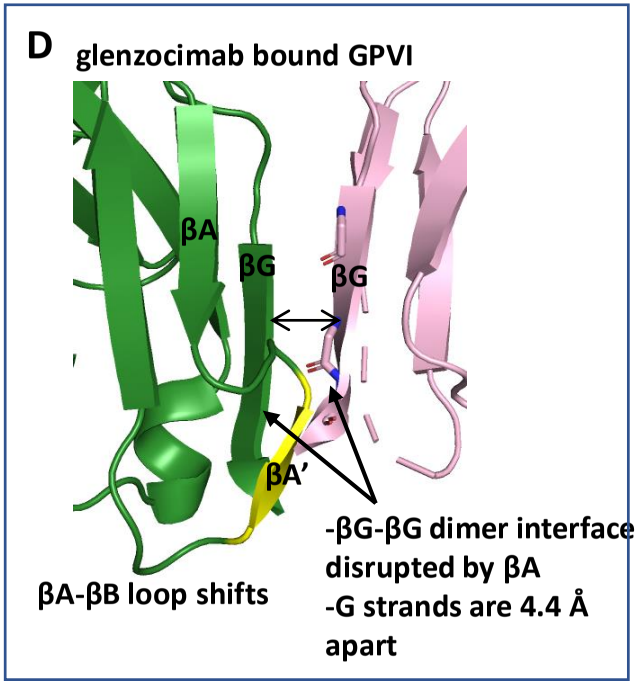
Figure 3









Billiald *et al*. Figure 3 A-D

Figure 4

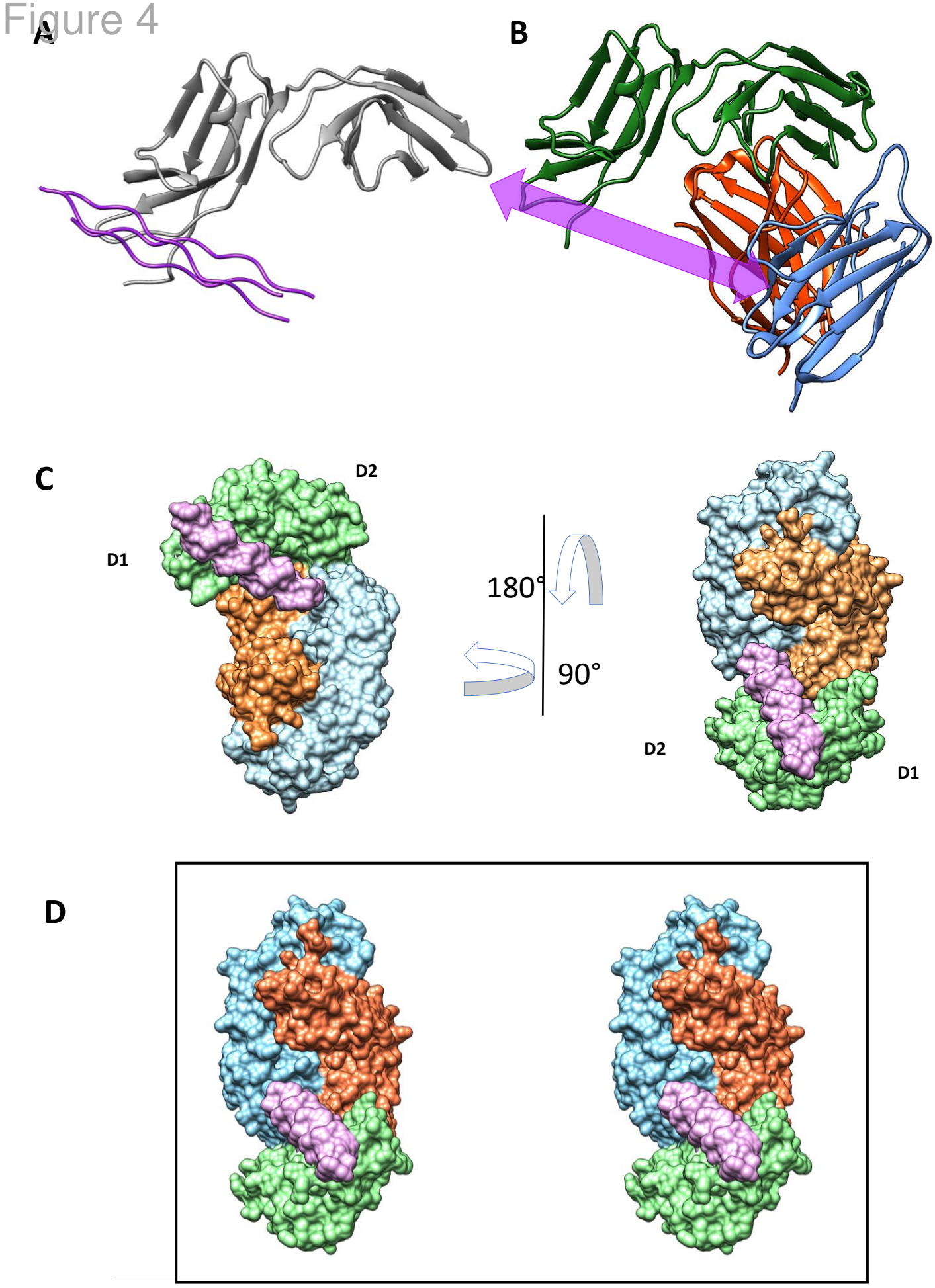
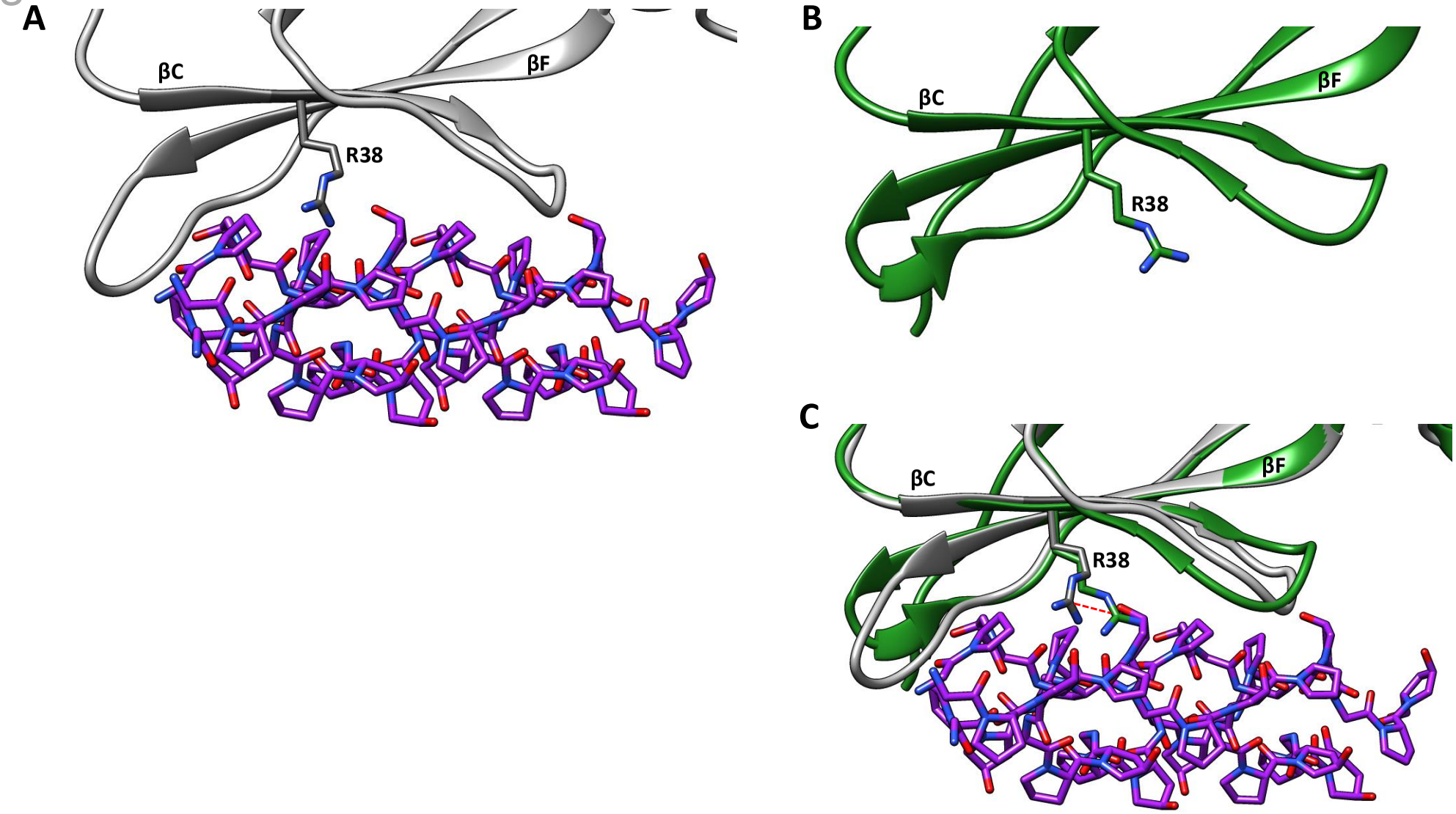
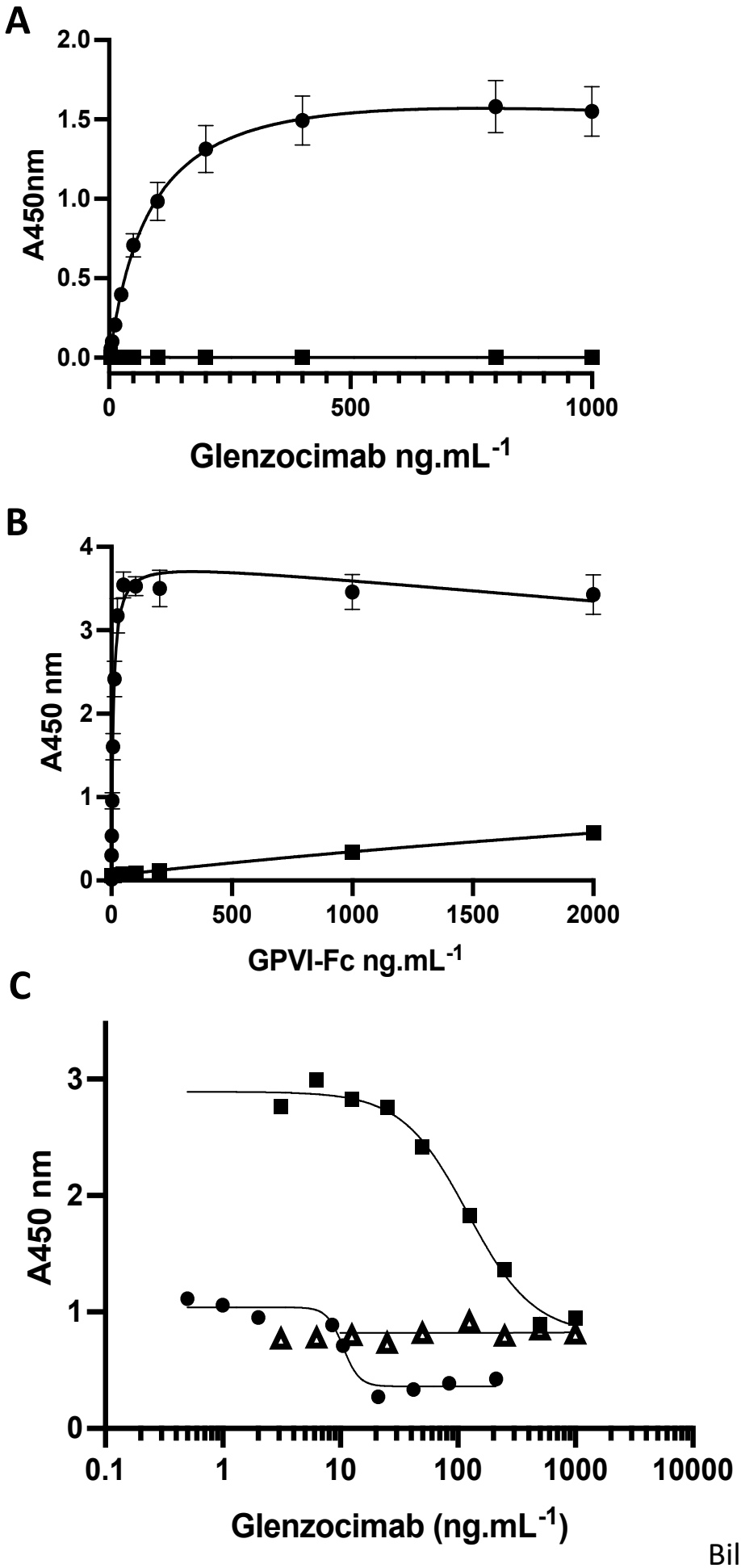


Figure 5



Billiald *et al.* Figure 5 A-C

Figure 6



Billiald *et al.* BA Figure 6