HHS Public Access

Author manuscript

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2023 February 01.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2022 February ; 42(2): 193–204. doi:10.1161/ATVBAHA.121.316668.

Atomic level dissection of the PECAM-1 homophilic binding interface: Implications for endothelial cell barrier function

Danying Liao[1,2,*], **Jesse Sundlov**[1,*], **Jieqing Zhu**[1,3], **Heng Mei**[2], **Yu Hu**[2], **Debra K. Newman**[1,4,5,7], **Peter J. Newman**[1,2,4,6,7]

[1]Blood Research Institute, Versiti Blood Center of Wisconsin, Milwaukee, WI

[2]Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

[3]Department of Biochemistry, Medical College of Wisconsin, Milwaukee

[4]Department of Pharmacology, Medical College of Wisconsin, Milwaukee

[5]Department of Microbiology Medical College of Wisconsin, Milwaukee

[6]Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee

[7]Department of The Cardiovascular Center, Medical College of Wisconsin, Milwaukee

Abstract

Objective: PECAM-1 is a 130-kDa member of the immunoglobulin (Ig) gene superfamily that is expressed on the surfaces of platelets and leukocytes and concentrated at the intercellular junctions of confluent endothelial cell monolayers. PECAM-1 Ig domains 1 and 2 (IgD1 and IgD2) engage in homophilic interactions that support a host of vascular functions, including support of leukocyte transendothelial migration and the maintenance of endothelial junctional integrity. The recently solved crystal structure of PECAM-1 IgD1 and IgD2 revealed a number of intermolecular interfaces predicted to play important roles in stabilizing PECAM-1/PECAM-1 homophilic interactions and in formation and maintenance of endothelial cell-cell contacts. We sought to determine whether the protein interfaces implicated in the crystal structure reflect physiologically-important interactions.

Approach and Results: We assessed the impact of single amino acid substitutions at the interfaces between opposing PECAM-1 molecules on homophilic binding and endothelial cell function. Substitution of key residues within the IgD1-IgD1 and IgD1-IgD2 interfaces, but not those within the smaller IgD2-IgD2 interface, markedly disrupted PECAM-1 homophilic binding

Address correspondence to: Peter J. Newman, Ph.D., Blood Research Institute, Versiti Blood Center of Wisconsin, 8727 Watertown Plank Rd, Milwaukee, Wisconsin 53266-3548, Phone: (414) 937-6237, Fax: (414) 937-6284, pjnewman@Versiti.org.

*Co-first authors contributed equally to this manuscript

Disclosures

The authors declare no competing financial interests.

Supplemental Materials

Online Table I (Primers)

Online Figure I – III

Major Resources Table

and its downstream effector functions, including the ability of PECAM-1 to localize at endothelial cell-cell borders, mediate the formation of endothelial tubes, and restore endothelial barrier integrity.

Conclusion: Taken together, these results validate the recently described PECAM-1 IgD1/IgD2 crystal structure by demonstrating that specific residues visualized within the IgD1-IgD1 and IgD1-IgD2 interfaces of opposing molecules in the crystal are required for functionally important homophilic interactions. This information can now be exploited to modulate functions of PECAM-1 *in vivo*.

Introduction

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kDa glycoprotein member of the immunoglobulin (Ig) superfamily that is comprised of six extracellular Ig-like homolog domains (IgD), a 19-residue transmembrane domain, and a 118 amino acid cytoplasmic tail containing motifs that mediate interactions with cytosolic signaling molecules.[1, 2] PECAM-1 is widely expressed on most hematopoietic cells, including neutrophils, monocytes, subsets of lymphocytes, and platelets.[3] In addition to its expression in circulating blood cells, PECAM-1 is the most abundant component of the endothelial cell-cell junction, where it plays a major role in angiogenesis,[4, 5] maintenance of vascular integrity under inflammatory and thrombotic stress,[6–11], mechanosensation[12–16], and regulating leukocyte transendothelial migration.[17–20]

While it has been known for some time that amino-terminal Ig domains 1 and 2 are required for PECAM-1/PECAM-1 homophilic interactions[21–23] and the functions that they support[10, 17, 18, 24–30], the structural basis for PECAM-1 homophilic binding was not clear until the crystal structure of PECAM-1 Ig domains 1 and 2 was determined.[31] This structure revealed how both IgD1 and IgD2 are aligned to form a set of *trans* homophilic-binding interfaces with a total buried interface area of >2300 $Å^2$ that is comprised of a 1608 $Å^2$ interface between adjacent IgD1 molecules, a rigid intrachain interface between IgD1 and IgD2 that governs the orientation of IgD1, a 550 $Å^2$ buried surface between IgD1 and IgD2, and a small homophilic interface of uncertain significance between adjacent IgD2 domains. Functional validation that the interfaces observed in the crystal structure are of physiological importance, especially in endothelial cell biology, where PECAM-1 is known to play an important role in junctional integrity, however, remain to be determined.

The purpose of the present investigation, therefore, was to validate the functional importance of the interactions revealed by the Paddock *et al.* crystal structure[31]. To do so, we generated a carefully selected series of mutations to form amino acid substitutions at key sites within each of the proposed interfaces, and subjected the resulting mutant PECAM-1 species to a series of well-characterized functional assays known to be dependent upon PECAM-1 homophilic binding, and that play a role in regulating endothelial cell barrier function. Our results substantiate the proposed model and suggest a mechanism at the atomic-level of how PECAM-1 homophilic interactions and adhesive properties mediate endothelial biology and vascular homeostasis.

Material and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Antibodies and fusion proteins

The mouse monoclonal antibody (mAb) specific for human PECAM-1 Ig domain 5 (PECAM-1.1) has been previously described.[27] Normal mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Normal human IgG was purchased from Sigma-Aldrich (St. Louis, MO). A soluble PECAM-1/IgG fusion protein was expressed in Chinese hamster ovary (CHO) cells and used for flow cytometric analysis of PECAM-1 homophilic binding.[28] Secondary antibodies used for flow cytometry and confocal microscopy included Alexa Fluor 647-conjugated goat anti-mouse IgG (AF647-GAM-IgG; Thermo Fisher Scientific, Waltham, MA) and mouse anti-human IgG (AF647-MAH-IgG; Jackson Immuno Research Laboratories, West Grove, PA).

Cell lines, transfection, and transduction

Cell culture reagents were obtained from Mediatech (Manassas, VA) unless otherwise specified. Immortalized wild-type human umbilical vein endothelial cells (WT-iHUVECs) were generated by transducing HUVECs with recombinant retrovirus LXSN16 E6/E7 as previously described.[32] PECAM-1-deficient iHUVECs (KO-iHUVECs) were generated from WT-iHUVECs using CRISPR/Cas9 technology as previously described.[33] To generate iHUVECs expressing variant forms of PECAM-1, KO-iHUVECs were seeded in 6-well plates and grown to 70–80% confluence. On the day of transduction, fresh medium that contained 8 μg/ml Polybrene was added to each well and lentiviral particles encoding single amino acid-substituted forms of PECAM-1 were added (MOI = 5). After 18–20 hours at 37°C, the medium was replaced, and cells were expanded and maintained as previously described.[10]

To generate human embryonic kidney 293 (HEK293) cells expressing WT and variant forms of PECAM-1, cultured HEK293FT cells were lifted with 0.05% trypsin-EDTA and 10^6 cells/well were seeded in 6-well plates. Plasmids encoding WT or single amino acid-substituted forms of PECAM-1 were added at 3 μg/well along with 6 μl of Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). After 24–30 hours at 37°C, cells were subjected to flow cytometric analysis for GFP expression. HEK293 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA).

Plasmids and constructs

For transfection of HEK293 cells, a cDNA encoding full-length human PECAM-1 was inserted between *Sal*I and *Spe*I of the pIRES2-EGFP-ET1 vector, which enables expression of PECAM-1 and EGFP from one transcript. Point mutations encoding single amino acid substitutions were introduced using QuickChange II site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) and primers listed in the Supplementary Table. For transduction of KO-iHUVECs, the full-length human *PECAM1*

cDNA was cloned into the lentiviral plasmid pLenti CMV/TO Neo (Addgene # 17485) and variant forms of PECAM-1 were made using the In-Fusion® HD Cloning kit (Takara, Mountain View, CA). The primers for In-Fusion cloning are: Fwd, 5'-ACTCGAGAT-ATCTAGAGCCACCATGCAGCCGAGGTGG-3'; Rev, 5'-GAAAGCTGGGTCTAGACTAAG-TTCCATCAAGGGAG-3'. Since KO-iHUVECs were generated with CRISPR/Cas9 technology, the guide RNA (gRNA) that targets PECAM-1 for excision was incorporated into the genome of this cell line. To avoid excision of the transduced sequences needed to rescue PECAM-1 expression in KO-iHUVECs, two silent mutations were introduced into the region targeted by the PECAM-1-targeting gRNA using QuickChange II site-directed mutagenesis and the following primers: Fwd, 5'-GGTGGGCCCAAGGGGCGACCATGTGGCTT-GGAGTC-3'; Rev, 5'-GACT-CCAAGCCACATGGTCGCCCCTTGGGCCCACC-3' (silent mutation sites are underlined). Point mutations encoding single amino acid substitutions were introduced as described above for HEK293 cells. Lentiviruses were generated by the Viral Vector Core facility of the Blood Research Institute.

Characterization of PECAM-1 expression and homophilic binding by flow cytometry

Transfected HEK293 cells were lifted with 0.05% trypsin-EDTA and resuspended in 100 μl of phosphate-buffered saline containing 2% FBS (PBS-FBS) at a density of 1×10^6 cells/ml. PECAM-1 expression was detected by staining with PECAM-1.1 mAb at a final concentration of 35 μg/ml, using normal mouse IgG as a negative control. PECAM-1 homophilic binding was detected by staining with PECAM-1/IgG at a final concentration of 100 μg/ml, using normal human IgG as a negative control. After incubating for 1 hour at 4°C, stained cells were suspended in 1 ml of 2% FBS-PBS, centrifuged, and resuspended in 100 μl of 2% FBS-PBS that contained AF647-GAM-IgG for assessment of PECAM-1 expression or AF647-MAH-IgG for assessment of PECAM-1 homophilic binding. Following 30 minutes at 4°C, cells were washed, resuspended and subjected to flow cytometric analysis using a Becton-Dickenson LSRII (BD Biosciences, San Jose, CA).

Sorting of iHUVECs expressing variant forms of PECAM-1

After lentivirus transduction and expansion, iHUVECs were lifted with 0.05% trypsin-EDTA, resuspended in 2% FBS-PBS that contained PECAM-1.1 mAb (35 μg/ml) and kept on ice for 30 min. Cells were washed twice, resuspended in 2% FBS-PBS that contained AF647-GAM-IgG, and kept on ice for 30 min. Cells were washed and resuspended in 5% FBS-PBS with 25mM HEPES. iHUVECs expressing comparable levels of variant forms of PECAM-1 (Online Figure II) were obtained by fluorescence-activated cell sorting using an Aria IIIu (Becton Dickinson, San Jose, CA).

Assessment of PECAM-1 localization at cell-cell borders

Slide chambers (BD Biosciences, San Jose, CA) were treated with 0.2% gelatin (Invitrogen, Waltham, MA) for 1 hour at 37°C. iHUVECs were plated on gelatin-coated slides at a density of 2×10^5/well and grown to confluence. Confluent cells were rinsed with Dulbecco's phosphate-buffered saline (D-PBS), and fixed with 2% paraformaldehyde for 20 minutes. After permeabilization with ice-cold 0.5% Triton X-100 for 2 minutes, cells were blocked with PBS that contained 3% bovine serum albumin. Monolayers were then

incubated with PECAM-1.1 mAb (10 μg/ml) for 1 hour at room temperature followed by AF647-GAM-IgG (2 μg/ml) for 30 minutes at room temperature. In some cases, monolayers were also stained with FITC-Phalloidin and DAPI to visualize the cytoskeleton and nuclei, respectively (Online Figure III). Images were obtained with a FluoView FV1000 multi-photon emission microscope (Olympus, Center Valley, PA). The reconstruction of cross sections was accomplished using MetaMorph (Molecular Devices, Inc., Nashville, TN) and the entire Z series was combined into a stacked projection.

Tube formation (*in vitro* angiogenesis)

Matrigel Matrix (Corning, Life Sciences, MA) was thawed overnight on ice at 4°C and added to pre-cooled 24 well culture plates (0.3 ml/well). Matrigel-coated plates were incubated at 37°C for 30–60 minutes before use. iHUVECs were grown to at least 80% confluence, trypsinized and resuspended in culture medium at a concentration of 4×10^5 cells/ml. Cell suspensions (300 μl/well) were added to Matrigel-coated wells and incubated at 37°C. After 18 hours, cells were stained with Corning Calcein AM dye following the manufacturer's protocol. Four random images from each well were taken with an EVOS FL microscope (Thermo Fisher Scientific, Waltham, MA) and each experiment was repeated three times. Images were analyzed by ImageJ (https://imagej.nih.gov/ij/index.html).

Measurements of endothelial barrier function

To measure endothelial barrier function, iHUVECs were grown to confluence in 400 μl of human endothelial serum free medium on 8W10E+ electrode arrays (Applied Biosystems, Troy, NY) that had been coated with 0.1% gelatin. Confluent monolayers were stimulated with 1 unit of human thrombin (Sigma-Aldrich, St. Louis, MO) to disrupt endothelial barriers, and restoration of barrier function was measured in real time by Electric Cell-substrate Impedance Sensing (ECIS) using an ECIS Z-Theta instrument (Applied Biophysics, Troy, NY).

Molecular modeling

Analyses of protein structures, domain interactions, and residue selection for mutational analysis, were performed using *Coot* 0.9.5.[34] Protein structure figures were made using PyMOL 2.4.[35, 36]

Statistical analysis

An ordinary one-way ANOVA was performed using the Kruskal-Wallis test with the Dunn's test for multiple comparisons to determine the statistical significance of differences between groups. A non-parametric statistical test was used either because sample sizes (n = 3) were too small for analysis by parametric statistical tests (Figure 2) or because the data associated with at least one condition failed tests for normality. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Actual p values are reported unless $p < 0.001$.

Results

The two most membrane-distal Ig domains of PECAM-1 engage in homophilic interactions that support endothelial vascular barrier integrity and mediate leukocyte transendothelial migration; however, the precise residues that mediate homophilic binding have not yet been structurally and functionally validated. The recently solved crystal structure of PECAM-1 Ig domains 1 and 2 revealed several interfaces that, while not functionally verified in the context of endothelial cell biology in the original paper, appeared to offer a model of *trans* homophilic binding (Figure 1A & B) occurring between PECAM-1 ectodomains emanating from opposing cell surfaces. These include a large 1608 $Å^2$ interface formed by the first Ig domains of two opposing PECAM-1 molecules (IgD1-IgD1 interaction), a smaller but substantial 550 $Å^2$ buried surface formed by domain 1 of one molecule and domain 2 of the opposing molecule (IgD1-IgD2 interaction), and a much smaller homophilic interface formed by IgD2-IgD2 interactions between adjacent molecules. As depicted in Figure 1C, the side chains of residue D_{33} within IgD1 molecule 1 forms inter-chain hydrogen bonds with S_{66} and Q_{29} of the adjacent IgD1 molecule 2, while K_{62} bonds with A_{32} and S_{63}. Within the IgD1-IgD2 interface, the side and main chains of D_{52} and the side chain of R_{122} form inter-chain hydrogen bonds with each other, while E_{165} bonds with R_{122} and R_{152} within the same domain, creating a network of interactions between these residues (Figure 1D). Within the IgD2-IgD2 interface, the side chains of D_{158} and K_{131} form an inter-chain hydrogen bond with each other, while P_{133} has the potential to ensure formation of a kink in the loop that properly positions the K_{131}-D_{158} interaction (Figure 1E).

To validate the functional importance of these residues in supporting PECAM-1/PECAM-1 *trans* homophilic interactions, specific directed amino acid substitutions were made by replacing existing wild-type residues with those having an opposite charge, specifically: D_{33}→R and K_{62}→D at the IgD1-D1 interface; D_{52}→R, R_{122}→D, and R_{152}→D within the IgD1-IgD2 interface; and D_{158}→K, K_{131}→D, and P_{133}→G within the IgD2-IgD2 interface, the latter of which should enable but does not ensure formation of a kink. The effect of these amino acid substitutions on homophilic binding was then assessed in HEK293 cells that had been transiently transfected with plasmids encoding these WT and mutant forms of full-length PECAM-1.

Cell surface expression of each mutant form of PECAM-1 was quantified (Figure 2A), and the variant cell lines compared for their ability to support the homophilic binding of PECAM-1/IgG – a well-characterized reagent used for semiquantitative assessment of homophilic binding[28]. As shown in Figure 2B, D33R substitution within the IgD1-IgD1 interface, as well as all the substitutions within the IgD1-IgD2 interface (D52R, R122D, and R152D) dramatically reduced or abolished PECAM-1 homophilic binding. In contrast, the K62D substitution within the IgD1-IgD1 interface and all of the substitutions within the IgD2-IgD2 interface (K131D, P133G, and D158K) had little or no effect on PECAM-1 homophilic binding (Figure 2B). These results demonstrate that residues located within the IgD1-IgD1 (D_{33}) and IgD1-IgD2 (D_{52}, R_{122}, and R_{152}) interfaces between opposing molecules described in the recently described PECAM-1 IgD1/IgD2 crystal structure are in fact required for PECAM-1 homophilic interactions.

Previous studies have shown that PECAM-1-mediated homophilic binding is critical for its concentration at endothelial cell intercellular junctions[29, 37]. To functionally validate that the homophilic binding residues identified on the basis of the PECAM-1 IgD1/IgD2 crystal structure are important for the ability of PECAM-1 to concentrate at endothelial cell-cell borders, mutations encoding single amino acid substitutions at a predicted representative position within each interface (D33R for IgD1-IgD1, R152D for IgD1-IgD2, and K131D for IgD2-IgD2) were introduced into a lentiviral construct that was used to reconstitute PECAM-1 expression in PECAM-1-deficient immortalized human umbilical endothelial cells (iHUVECs – described in reference 33). Following lentiviral transduction, iHUVECs reconstituted with wild-type or mutant forms of PECAM-1 were grown to confluence, and the ability of PECAM-1 to accumulate at cell-cell borders was visualized by confocal microscopy. As has been shown previously in NIH 3T3[38] and REN mesothelioma[29] cells expressing recombinant WT PECAM-1, PECAM-1 accumulated normally at the borders between iHUVECs expressing WT PECAM-1 (Figure 3A). Similarly, substitution of a residue within the IgD2-IgD2 interface that did not affect PECAM-1 homophilic binding (K131D) had no effect on PECAM-1 border localization (Figure 3D). In contrast, substitution of D33R (Figure 3B) within the IgD1-IgD1 and K152D (Figure 3C) at the IgD1-IgD2 interface both abolished PECAM-1 homophilic binding, as evidenced by the diffuse distribution of PECAM-1 on the surface of iHUVECs, indicative of the failure of PECAM-1 to engage homophilically and concentrate at cell-cell borders. These results demonstrate that residues predicted by the crystal structure to be important for formation of the IgD1-IgD1 (D_{33}) and IgD1-IgD2 (R_{152}) interfaces are, in fact, functionally required for PECAM-1 homophilic interactions and endothelial cell-cell junctional localization.

Endothelial cells are able to form organized tube structures resembling small capillaries when cultured in a Matrigel matrix – an assay often used as a surrogate *in vitro* measure of angiogenesis.[39] Previous studies using blocking antibodies specific for PECAM-1 Ig domains 1 and 2 have demonstrated that PECAM-1 homophilic interactions contribute importantly to *in vitro* endothelial tube formation.[40, 41] To determine whether the homophilic binding residues identified on the basis of the PECAM-1 IgD1/IgD2 crystal structure were also important for endothelial cell angiogenesis, iHUVECs expressing wild-type or mutant forms of PECAM-1 were grown in Matrigel and their ability to form tube structures was assessed. As shown in Figure 4, iHUVECs that do not express PECAM-1 (KO-iHUVECs) formed poorly organized multicellular aggregates (Figure 4A) and fragmented tubes (Figure 4B) with few nodes (Figure 4C) and meshes (Figure 4D). Reconstitution of KO-iHUVECs with either a WT or K131D form of PECAM-1, both of which are capable of homophilic binding (**see** Figure 2), resulted in formation of well-organized structures (Figure 4A) with significantly more tubes, nodes and meshes than were observed in KO-iHUVECs (Figure 4B–D). In contrast, reconstitution of KO-iHUVECs with either D33R or R152D forms of PECAM-1, neither of which support homophilic binding, resulted in formation of fragmented structures (Figure 4A) with numbers of tubes, nodes and meshes that were significantly fewer than were observed in iHUVECs expressing WT PECAM-1 and that did not differ significantly from those observed in KO-iHUVECs (Figure 4B–D). Based on these findings, we conclude that key residues found at the IgD1-IgD1 (D_{33}) and IgD1-IgD2 (R_{152}) interfaces in the PECAM-1 IgD1/IgD2 crystal

structure that were predicted from the Paddock et al. crystal structure, and then proven to be important for PECAM-1/PECAM-1 trans homophilic interactions, are also required for *in vitro* angiogenesis by endothelial cells.

Homophilic interactions mediated by the extracellular portion of PECAM-1 have been shown to be important for maintenance of the vascular permeability barrier.[10] To further validate that key homophilic binding residues at the IgD1-IgD1 and IgD1-IgD2 interface of the PECAM-1 IgD1/IgD2 crystal structure actually play an important functional role, endothelial barrier function of iHUVECs expressing wild-type or mutant forms of PECAM-1 was measured both before and after barrier disruption with thrombin (Figure 5A) using Electric Cell-substrate Impedance Sensing technology (ECIS). As shown in the real-time barrier resistance tracings (Figure 5A), the steady state impedance supported by iHUVECs expressing either WT or K131D mutant forms of PECAM-1 was similarly robust, and both formed significantly stronger barriers than those formed by iHUVECs that either did not express PECAM-1 (KO) or that expressed the D33R or R152D mutant forms of PECAM-1 (quantified in Figure 5B). Figure 5C quantifies the relative rate of recovery of barrier formation following thrombin challenge, and shows that iHUVECs expressing WT PECAM-1 re-established its permeability barrier at a significantly faster rate than did cells expressing either no PECAM-1 (KO) or the D33R and R152D mutant forms of PECAM-1. The rate of barrier reformation of cells expressing K131D PECAM-1, while lower than that of cells expressing WT PECAM-1, did not reach statistical significance. As was observed with baseline barrier function, barrier restoration following thrombin challenge was particularly slow for iHUVECs expressing the D33R mutant form of PECAM-1. These results demonstrate that PECAM-1 homophilic interactions, particularly those involving residue D_{33} within the IgD1-IgD1 interface, are critical for maintenance and restoration of endothelial junctional integrity.

Discussion

As a major component of endothelial cells, and strategically concentrated at intercellular junctions, PECAM-1 functions importantly in a number of biological functions, including angiogenesis,[4, 5] maintenance of vascular integrity under inflammatory and thrombotic stress,[6–10] and regulation of leukocyte transendothelial migration.[17–20] PECAM-1/PECAM-1 homophilic interactions, mediated by IgD1 and IgD2, are necessary for these activities.[10, 17, 18, 24–30] However, the structural basis for how PECAM-1 interacts homophilically was not clear until the crystal structure of PECAM-1 Ig domains 1 and 2 was determined.[31] This structure revealed that IgD1-IgD1, IgD1-IgD2, and IgD2-IgD2 form buried surfaces with presumed functional significance for mediating PECAM-1 trans-homophilic interactions. Functional validation that the three interfaces observed in the crystal structure are of physiological importance, especially in endothelial cell biology, where PECAM-1 is known to play an important role in junctional integrity, however, remained to be determined.

The extracellular elements of PECAM-1 that are required for homophilic binding were established decades ago, beginning with antibody blocking studies[27, 28, 30] and continuing with domain-deletion and domain-swap analysis.[25, 28] all of which implicated IgD1 as

necessary for PECAM-1 self-association. Likewise, the importance of PECAM-1 IgD2 was demonstrated when antibodies against it were shown to be equally efficient as anti-IgD1 antibodies in inhibiting homophilic binding using either PECAM-1-expressing cells[28] or purified PECAM-1.[17] These experiments suggested that IgD2 either acted as a structural support for positioning IgD1 to mediate homophilic binding, or was directly involved in binding. A robust mutational analysis of IgD1 and IgD2 identified a series of residues in IgD1 that were necessary for homophilic adhesion to occur.[26] These residues were predicted to be located on opposite faces of IgD1, suggesting the existence of multiple binding interfaces. The first published structure of PECAM-1 IgD1/IgD2 confirmed this hypothesis – demonstrating a large 1600 $Å^2$ IgD1-IgD1 *trans* interface, two IgD1-IgD2 *trans* interfaces with a total buried area of >2300 $Å^2$, as well as a smaller 550 $Å^2$ *trans* IgD2-IgD2 interface. Additionally, multiple intrachain interactions between IgD1 and IgD2 demonstrated the latter's role in providing rigidity as a structural scaffold to IgD1. Importantly, mutations at residues D_{33} and D_{51}, which crippled homophilic interactions in the prior study,[26] were shown to be centrally located within the IgD1-IgD1 and IgD1-IgD2 interfaces, respectively. In order to build upon these established biochemical data, the present study was designed to elucidate the biological relevance of the interchain domain interfaces observed in the PECAM-1 IgD1/IgD2 structure previously published by our group.[31]

Based on an analysis of putative key residues found at these structural interfaces (Figure 1), we strategically selected an amino acid residue in each for mutagenesis, expressed these PECAM-1 mutants in HEK293 cells, and subjected them to a series of PECAM-1-dependent homophilic binding assays. Cells expressing PECAM-1 variants with mutations within the IgD1-IgD1 and IgD1-IgD2 interfaces showed remarkably decreased levels of homophilic binding, while the IgD2-IgD2 mutations had surprisingly little effect (Figure 2). Based on the result of this homophilic binding assay, single-residue PECAM-1 mutants with the most pronounced effects were introduced into iHUVEC cells for further functional validation using a series of cell biological assays previously shown to be reliant on PECAM-1/PECAM-1 homophilic binding interactions. In the cell-cell border localization assay, the PECAM-1 mutants predicted to disrupt IgD1-IgD1 and IgD1-IgD2 interactions resulted in diffuse PECAM-1 staining over the surface of the cell, whereas the IgD2-IgD2 mutant still localized to the cell junctions (Figure 3), consistent with our previously proposed model of homophilic binding in which multiple protein interfaces are employed in the process of PECAM-1 diffusion trapping at cell-cell junctions.[31] Further support for the functional importance of residues identified in the crystal structure was provided using an *in vitro* angiogenesis assay employing mutants targeting all three potential PECAM-1 binding interfaces (Figure 4). In these studies, the D33R mutation at the IgD1-IgD1 interface, and the R152D mutation at the IgD1-IgD2 interface had significant effects on the ability of endothelial cells to form tubes, nodes, and meshes, while mutation of K_{131} in the IgD2-IgD2 interface had little effect. These studies are consistent with the findings of DeLisser and colleagues showing that antibodies against N-terminal domains 1 and 2 inhibit endothelial tube formation in vitro, and tumor angiogenesis in vivo.[4] Finally, ECIS assays revealed that both steady-state endothelial cell junctional integrity and barrier reformation following thrombin challenge were affected by mutations within the IgD1-IgD1 and IgD1-IgD2 interfaces to a much greater extent than was a mutation of K_{131}, located within the

IgD2-IgD2 interface. Taken together, these data strongly suggest that the IgD1-IgD1 and IgD1-IgD2 interfaces revealed in the crystal structure of Paddock et al.[31] are functionally important for forming and supporting PECAM-1/PECAM-1 *trans* homophilic interactions, while the smaller IgD2-IgD2 interface is expendable.

Several months after the publication of the Paddock *et al.* structure of PECAM-1 IgD1/IgD2 and their associated homophilic molecular interactions[31], a second structure of PECAM-1 IgD1/IgD2 was deposited in the PDB (PDB ID:5gni) by Hu *et al.*[42] While the individual IgD1/IgD2 monomers are nearly identical to those in the Paddock *et al.* structure (In chain A, RMSD of 0.93 Å for Cα 5–183. In chain B, RMSD of 0.64 Å for Cα 4–184), the interactions *between* monomers are substantially different (Figure 6). In the Hu *et al.* structure (Figure 6B), the most extensive interface is between IgD1 and IgD2, and it lacks the major IgD1-IgD1 trans interface as well as the smaller IgD2-IgD2 interface that were observed in Paddock *et al.* (Figure 6A). A likely explanation for this difference is the substitution by Hu *et al.*, using site-directed mutagenesis, of three Asn residues with Gln residues, resulting in the elimination of well-known N-linked glycosylation sites. Two of these, N57Q and N124Q, are buried in the IgD1-IgD2 homophilic binding surface in their structure, which, if left as wild type Asn residues with full glycosylation, would have caused steric clashes with surrounding residues, and therefore could not likely occur in nature. The third N-linked glycosylation site, N_{25}, is present at the cap of the IgD1-IgD1 interface in the Paddock *et al.* structure, and shown to be functionally important in the ability of transfected REN cells to reestablish cell-cell contacts following thrombin challenge.[43] In contrast, N_{25} is not located at a homophilic binding interface in the Hu et al. structure, and is instead protruding into the surrounding solvent with no apparent functional or structural interaction. Moreover, to validate their structure, Hu *et al.* expressed a single mutant form of PECAM-1 containing four mutations (L_{47}→E, I_{85}→E, F_{161}→E, I_{163}→E) located within the homophilic binding interface of their crystal structure. Indeed, these mutations together had a statistically significant impact on the ability of PECAM-1 to homophilically interact. While we did not target the same group of residues for mutagenesis, two of those four residues (F_{161} and I_{163}) are also present within the trans-homophilic interface in the Paddock *et al.* structure (see Online Figure I). That being the case, the functional studies performed by Hu *et al.*, are unfortunately unable to functionally discriminate between the two published structures.

In contrast, the functional analyses performed on point mutation variants of IgD1 and IgD2 of PECAM-1 reported herein identify the minimal domain interactions necessary for forming the PECAM-1/PECAM-1 homophilic binding interface of Paddock *et al.*[31] These interacting residues by themselves, however, cannot totally account for the cell adhesive properties of this receptor, as both antibodies[28, 44] and the neutrophil-derived NB1/PR3 complex[45], both of which target membrane proximal IgD6 of PECAM-1[46], have been shown to markedly increase homophilic binding mediated by PECAM-1 IgD1. This phenomenon cannot be explained through the current model where PECAM-1 binding is entirely dependent upon IgD1-IgD2 interactions. Interestingly, PECAM-1 IgD6 has recently been implicated as the major receptor for *Clostridium perfringens* B-toxin, the virulence factor responsible for fatal, necro-hemorrhagic enteritis in both animals and humans,[47] and the pneumococcal adhesin RrgA also binds PECAM-1, mediating bacterial brain invasion.[48]

It is tempting to speculate that the extracellular domains of PECAM-1 utilize a form of affinity modulation, conceptually similar to what is observed in integrins, to regulate cell-cell adhesion. Thus, while the current work validates the PECAM-1 homophilic interactions observed in the published structure of Paddock et al., future studies that address higher-order complex molecular interactions governing PECAM-1 homophilic binding need to focus on the entire six-domain structure using both through x-ray crystallography and cryo-EM imaging techniques. Incorporation of domain-specific anti-PECAM-1 antibodies into such structural studies may allow for the elucidation of distinct conformational rearrangements that are likely to be involved in regulating PECAM-1-mediated homophilic interactions in the blood and vascular cells in which it is expressed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

DL designed and performed research, analyzed data and wrote the manuscript; PJN and JZ designed research and analyzed data. JS and DKN analyzed data and wrote the manuscript. HM and YH funded the research and provided valuable review of the manuscript. We thank Cathy Paddock (Blood Research Institute (BRI), Versiti Blood Center of Wisconsin) for consultation and advice during the early stages of this investigation, and the BRI Lentiviral Core for production of lentiviral vectors.

Sources of Funding

This work was supported by grants HL40926 and R35 HL139937 (to PJN) and grant HL131836 (to JZ) from the National Heart, Lung, and Blood Institute of the National Institutes of Health, and by Grant 201406160062 (to DL) from the China Scholarship Council.

Non-standard Abbreviations and Acronyms

Cas9	CRISPR-associated protein 9
CHO	Chinese hamster ovary cells
CRISPR	clustered regularly interspaced short palindromic repeats
ECIS	electric cell-substrate impedance sensing
FBS	fetal bovine serum
GFP	green fluorescent protein
HEK293	human embryonic kidney 293 cells
HUVEC	human umbilical vein endothelial cells
Ig	immunoglobulin
IgD	immunoglobulin-like homolog domain
iHUVEC	immortalized human umbilical vein endothelial cells
PECAM-1	Platelet endothelial cell adhesion molecule-1

References

1. Newman PJ, Berndt MC, Gorski J, White GC, Lyman S, Paddock C, Muller WA. PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. Science. 1990;247:1219–1222 [PubMed: 1690453]
2. Newman PJ, Newman DK. Signal transduction pathways mediated by PECAM-1. New roles for an old molecule in platelet and vascular cell biology. ArteriosclerThrombVascBiol. 2003;23:953–964
3. Newman PJ. The biology of PECAM-1. JClinInvest. 1997;99:3–8
4. Zhou Z, Christofidou-Solomidou M, Garlanda C, DeLisser HM. Antibody against murine PECAM-1 inhibits tumor angiogenesis in mice. Angiogenesis. 1999;3:181–188 [PubMed: 14517436]
5. Solowiej A, Biswas P, Graesser D, Madri JA. Lack of Platelet Endothelial Cell Adhesion Molecule-1 Attenuates Foreign Body Inflammation because of Decreased Angiogenesis. American Journal of Pathology. 2003;162:953–962
6. Mahooti S, Graesser D, Patil S, Newman P, Duncan G, Mak T, Madri JA. PECAM-1 (CD31) expression modulates bleeding time in vivo. American Journal of Pathology. 2000;157:75–81
7. Graesser D, Solowiej A, Bruckner M, Osterweil E, Juedes A, Davis S, Ruddle NH, Engelhardt B, Madri JA. Altered vascular permeability and early onset of experimental autoimmune encephalomyelitis in PECAM-1-deficient mice. Journal of Clinical Investigation. 2002;109:383–392
8. Maas M, Stapleton M, Bergom C, Mattson DL, Newman DK, Newman PJ. Endothelial cell PECAM-1 confers protection against endotoxic shock. American Journal of Physiology - Heart and Circulatory Physiology. 2005;288:H159–H164 [PubMed: 15319204]
9. Carrithers M, Tandon S, Canosa S, Michaud M, Graesser D, Madri JA. Enhanced susceptibility to endotoxic shock and impaired STAT3 signaling in CD31-deficient mice. American Journal of Pathology. 2005;166:185–196
10. Privratsky JR, Paddock CM, Florey O, Newman DK, Muller WA, Newman PJ. Relative contribution of PECAM-1 adhesion and signaling to the maintenance of vascular integrity. Journal of Cell Science. 2011;124:1477–1485 [PubMed: 21486942]
11. Ferrero E, Ferrero ME, Pardi R, Zocchi MR. The platelet endothelial cell adhesion molecule-1 (PECAM1) contributes to endothelial barrier function. Febs Lett. 1995;374:323–326 [PubMed: 7589563]
12. Kaufman DA, Albelda SM, Sun J, Davies PF. Role of lateral cell-cell border location and extracellular/transmembrane domains in PECAM/CD31 mechanosensation. Biochem Biophys Res Commun. 2004;320:1076–1081 [PubMed: 15249199]
13. Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhardt B, Cao G, DeLisser H, Schwartz MA. A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. Nature. 2005;437:426–431 [PubMed: 16163360]
14. Collins C, Guilluy C, Welch C, O'Brien ET, Hahn K, Superfine R, Burridge K, Tzima E. Localized tensional forces on PECAM-1 elicit a global mechanotransduction response via the integrin-RhoA pathway. Curr Biol. 2012;22:2087–2094 [PubMed: 23084990]
15. Conway DE, Breckenridge MT, Hinde E, Gratton E, Chen CS, Schwartz MA. Fluid shear stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1. Curr Biol. 2013;23:1024–1030 [PubMed: 23684974]
16. Conway DE, Schwartz MA. Mechanotransduction of shear stress occurs through changes in VE-cadherin and PECAM-1 tension: implications for cell migration. Cell Adh Migr. 2015;9:335–339 [PubMed: 25482618]
17. Liao F, Huynh HK, Eiroa A, Greene T, Polizzi E, Muller WA. Migration of monocytes across endothelium and passage through extracellular matrix involve separate molecular domains of PECAM-1. Journal of Experimental Medicine. 1995;182:1337–1343
18. Liao F, Ali J, Greene T, Muller WA. Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo. Journal of Experimental Medicine. 1997;185:1349–1357

19. Dangerfield J, Larbi KY, Huang MT, Dewar A, Nourshargh S. PECAM-1 (CD31) Homophilic interaction up-regulates a6b1 on transmigrated neutrophils in vivo and plays a functional role in the ability of a6 Integrins to mediate leukocyte migration through the perivascular basement membrane. Journal of Experimental Medicine. 2002;196:1201–1212
20. Vaporciyan AA, DeLisser HM, Yan H-C, Mendiguren II, Thom SR, Jones ML, Ward PA, Albelda SM. Involvement of Platelet Endothelial Cell Adhesion Molecule-1 in neutrophil recruitment in vivo. Science. 1993;262:1580–1582 [PubMed: 8248808]
21. Sun Q-H, DeLisser HM, Zukowski MM, Paddock C, Albelda SM, Newman PJ. Individually distinct Ig homology domains in PECAM-1 regulate homophilic binding and modulate receptor affinity. JBiolChem. 1996;271:11090–11098
22. Sun J, Williams J, Yan H-C, Amin KM, Albelda SM, DeLisser HM. Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) homophilic adhesion is mediated by immunoglobulin-like domains 1 and 2 and depends on the cytoplasmic domain and the level of surface expression. JBiolChem. 1996;271:18561–18570
23. Newton JP, Buckley CD, Jones EY, Simmons DL. Residues on both faces of the first immunoglobulin fold contribute to homophilic binding sites of PECAM-1/CD31. J Biol Chem. 1997;272:20555–20563 [PubMed: 9252369]
24. Nakada MT, Amin K, Christofidou-Solomidou M, O'Brien CD, Sun J, Gurubhagavatula I, Heavner GA, Taylor AH, Paddock C, Sun QH, et al. Antibodies against the first Ig-like domain of human platelet endothelial cell adhesion molecule-1 (PECAM-1) that inhibit PECAM-1- dependent homophilic adhesion block In vivo neutrophil recruitment. Journal of Immunology. 2000;164:452–462
25. Sun J, Williams J, Yan H-C, Amin KM, Albelda SM, DeLisser HM. Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) homophilic adhesion is mediated by immunoglobulin-like domains 1 and 2 and depends on the cytoplasmic domain and the level of surface expression. Journal of Biological Chemistry. 1996;271:18561–18570
26. Newton JP, Buckley CD, Jones EY, Simmons DL. Residues on both faces of the first immunoglobulin fold contribute to homophilic binding sites on PECAM-1/CD31. Journal of Biological Chemistry. 1997;272:20555–20563
27. Yan H-C, Pilewski JM, Zhang Q, DeLisser HM, Romer L, Albelda SM. Localization of multiple functional domains on human PECAM-1 (CD31) by monoclonal antibody epitope mapping. Cell Adhesion and Communication. 1995;3:45–66 [PubMed: 7538415]
28. Sun Q-H, DeLisser HM, Zukowski MM, Paddock C, Albelda SM, Newman PJ. Individually distinct Ig homology domains in PECAM-1 regulate homophilic binding and modulate receptor affinity. Journal of Biological Chemistry. 1996;271:11090–11098
29. Sun J, Paddock C, Shubert J, Zhang H, Amin K, Newman PJ, Albelda SM. Contributions of the extracellular and cytoplasmic domains of platelet- endothelial cell adhesion molecule-1 (PECAM-1/CD31) in regulating cell- cell localization. Journal of Cell Science. 2000;113:1459–1469 [PubMed: 10725228]
30. Sun Q-H, DeLisser HM, Paddock C, Liu C-Y, Albelda SM, Newman PJ. Epitope mapping of CD31 monoclonal antibodies and a study of their effects on PECAM-1 homophilic interaction. In: Kishimoto T, ed. Leucocyte Typing. Garland Publishing, Inc; 1997.
31. Paddock C, Zhou D, Lertkiatmongkol P, Newman PJ, Zhu J. Structural basis for PECAM-1 homophilic binding. Blood. 2016
32. Moses AV, Fish KN, Ruhl R, Smith PP, Strussenberg JG, Zhu L, Chandran B, Nelson JA. Long-term infection and transformation of dermal microvascular endothelial cells by human herpesvirus 8. Journal of Virology. 1999;73:6892–6902 [PubMed: 10400787]
33. Liao D, Mei H, Hu Y, Newman DK, Newman PJ. CRISPR-mediated deletion of the PECAM-1 cytoplasmic domain increases receptor lateral mobility and strengthens endothelial cell junctional integrity. Life Sciences. 2018;193:186–193 [PubMed: 29122551]
34. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. Acta Crystallogr D Biol Crystallogr. 2010;66:486–501 [PubMed: 20383002]

35. Janson G, Zhang C, Prado MG, Paiardini A. PyMod 2.0: improvements in protein sequence-structure analysis and homology modeling within PyMOL. Bioinformatics. 2017;33:444–446 [PubMed: 28158668]

36. Janson G, Paiardini A. PyMod 3: a complete suite for structural bioinformatics in PyMOL. Bioinformatics. 2020

37. Wong CW, Wiedle G, Ballestrem C, Wehrle-Haller B, Etteldorf S, Bruckner M, Engelhardt B, Gisler RH, Imhof BA. PECAM-1/CD31 trans-homophilic binding at the intercellular junctions is independent of its cytoplasmic domain; evidence for heterophilic interaction with integrin avb3 in cis. Molecular Biology of the Cell. 2000;11:3109–3121 [PubMed: 10982404]

38. Albelda SM, Muller WA, Buck CA, Newman PJ. Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. Journal of Cell Biology. 1991;114:1059–1068

39. Folkman J, Haudenschild C. Angiogenesis in vitro. Nature. 1980;288:551–556 [PubMed: 6160403]

40. Matsumura T, Wolff K, Petzelbauer P. Endothelial cell tube formation depends on cadherin 5 and CD31 interactions with filamentous actin. Journal of Immunology. 1997;158:3408–3416

41. Sheibani N, Newman PJ, Frazier WA. Thrombospondin-1, a natural inhibitor of angiogenesis, regulates Platelet-Endothelial Cell Adhesion Molecule-1 expression and endothelial cell morphogenesis. Molecular Biology of the Cell. 1997;8:1329–1341 [PubMed: 9243511]

42. Hu M, Zhang H, Liu Q, Hao Q. Structural Basis for Human PECAM-1-Mediated Trans-homophilic Cell Adhesion. Science Report. 2016;6:38655

43. Lertkiatmongkol P, Liao D, Mei H, Hu Y, Newman PJ. Endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD31). Curr Opin Hematol. 2016;23:253–259 [PubMed: 27055047]

44. Mei H, Campbell JM, Paddock CM, Lertkiatmongkol P, Mosesson MW, Albrecht R, Newman PJ. Regulation of endothelial cell barrier function by antibody-driven affinity modulation of platelet endothelial cell adhesion molecule-1 (PECAM-1). J Biol Chem. 2014;289:20836–20844 [PubMed: 24936065]

45. von Vietinghoff S, Tunnemann G, Eulenberg C, Wellner M, Cristina Cardoso M, Luft FC, Kettritz R. NB1 mediates surface expression of the ANCA antigen proteinase 3 on human neutrophils. Blood. 2007;109:4487–4493 [PubMed: 17244676]

46. Sachs UJ, Andrei-Selmer CL, Maniar A, Weiss T, Paddock C, Orlova VV, Choi EY, Newman PJ, Preissner KT, Chavakis T, Santoso S. The neutrophil-specific antigen CD177 is a counter-receptor for platelet endothelial cell adhesion molecule-1 (CD31). Journal of Biological Chemistry. 2007;282:23603–23612

47. Bruggisser J, Tarek B, Wyder M, Muller P, von Ballmoos C, Witz G, Enzmann G, Deutsch U, Engelhardt B, Posthaus H. CD31 (PECAM-1) Serves as the Endothelial Cell-Specific Receptor of Clostridium perfringens beta-Toxin. Cell Host Microbe. 2020;28:69–78 e66 [PubMed: 32497498]

48. Iovino F, Engelen-Lee JY, Brouwer M, van de Beek D, van der Ende A, Valls Seron M, Mellroth P, Muschiol S, Bergstrand J, Widengren J, Henriques-Normark B. pIgR and PECAM-1 bind to pneumococcal adhesins RrgA and PspC mediating bacterial brain invasion. Journal of Experimental Medicine. 2017;214:1619–1630

Highlights

- Based on the PECAM-1 IgD1/IgD2 crystal structure, single amino acid substitutions were inserted into PECAM-1 with the intent of disrupting PECAM-1 homophilic binding.
- Mutations targeting the observed IgD1-IgD1 and IgD1-IgD2 interfaces disrupted PECAM-1 homophilic binding.
- These mutations significantly decreased the ability of PECAM-1 to localize at endothelial cell-cell borders, mediate the formation of endothelial tubes, and restore endothelial barrier integrity.
- The results validate the physiological relevance of the PECAM-1 IgD1/IgD2 crystal structure determined by Paddock *et al.* by demonstrating that specific residues observed in the protein interfaces are required for functionally important homophilic interactions.

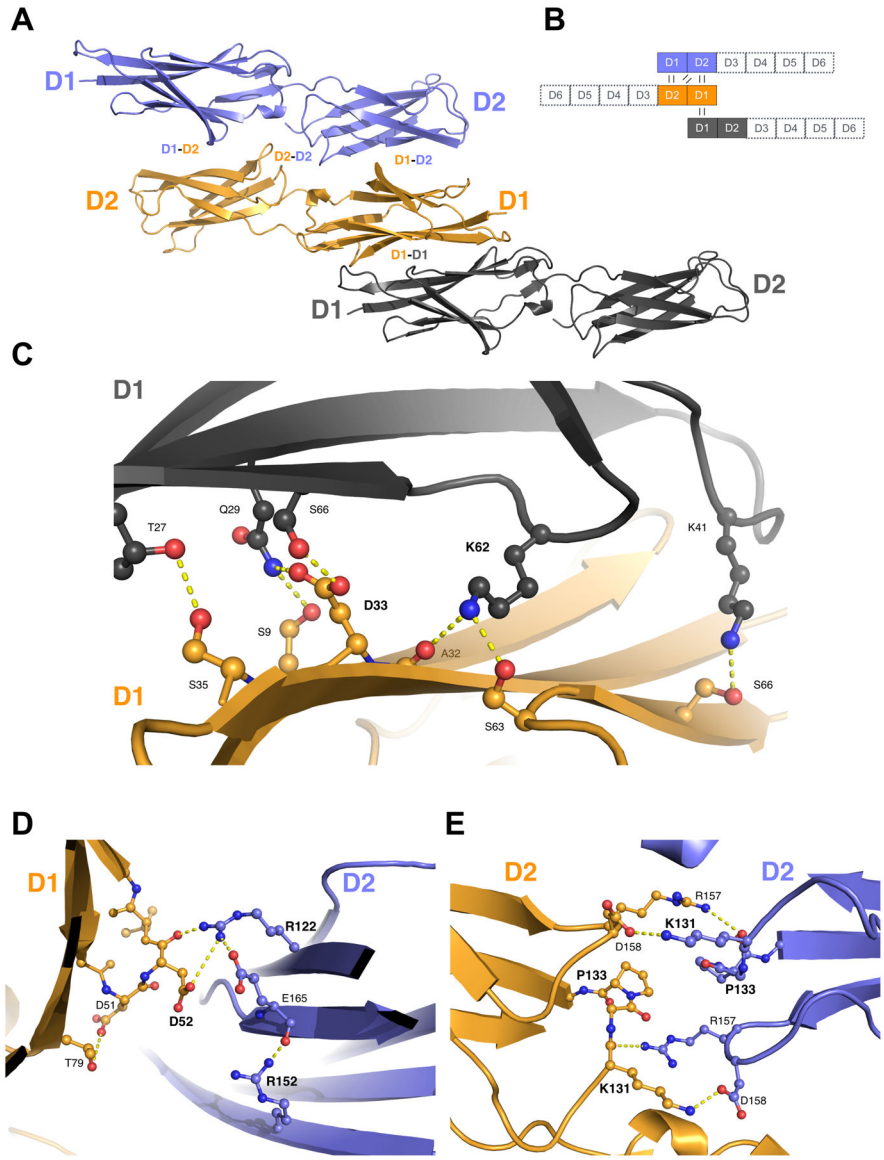

Figure 1. Homophilic binding interactions visualized in the crystal structure of PECAM-1.

(A) Ribbon model of the PECAM-1 Immunoglobulin Domain 1/Domain 2 (IgD1-IgD2) crystal structure (PDB ID: 5C14). Residues 4–185 are shown for each subunit. Each chain is uniquely colored blue, orange, and gray. IgD1-IgD1, IgD1-IgD2 and IgD2-IgD2 interfaces are labeled accordingly. **(B)** Schematic representation of the PECAM-1 IgD1-IgD1, IgD1-IgD2, and IgD2-IgD2 interfaces seen in the crystal structure as they would appear in the context of the full extracellular region (IgD1-IgD6) of PECAM-1. Colors of the IgD1/IgD2 subunits correspond with those shown in **(A)**. Dotted lines represent IgD3-IgD6, which are not present in the PECAM-1 IgD1/IgD2 crystal structure. **(C-E)** Atomic-level detail of PECAM-1 inter-chain homophilic interactions within the **(C)** IgD1-IgD1, **(D)** IgD1-IgD2, and **(E)** IgD2-IgD2 interfaces. Hydrogen bonds are shown as dashed lines. Residues that were targeted for mutation include D_{33} and K_{62} within the large IgD1-IgD1 interface **(C)**,

D_{52} in IgD1 and R_{122} and R_{152} in IgD2 at the IgD1-IgD2 interface **(D)**, and K_{131}, P_{133} and D_{158} at the IgD2-IgD2 interface **(E)**.

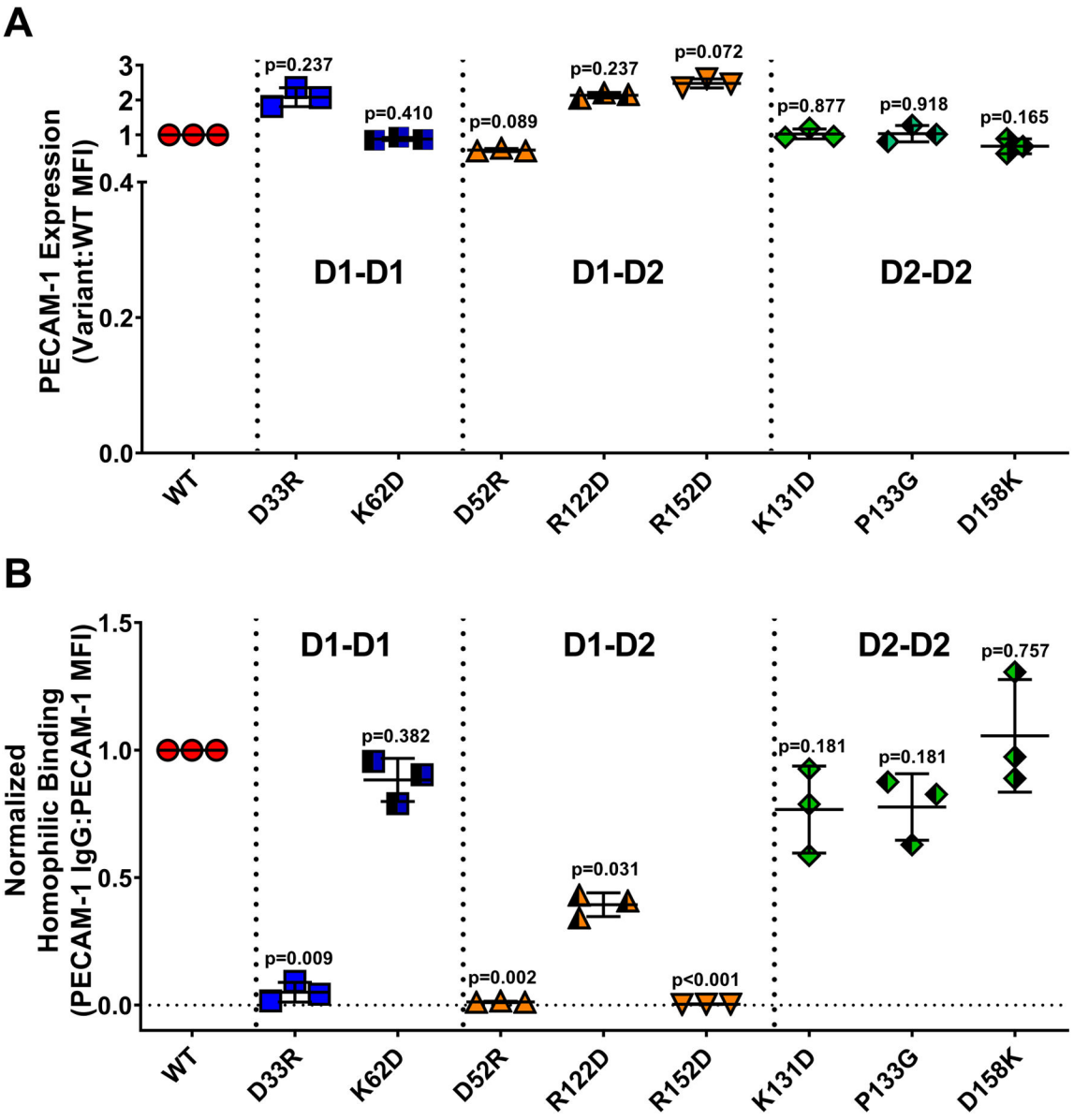

Figure 2. Residues within IgD1-IgD1 and IgD1-IgD2, but not IgD2-IgD2, interfaces in the PECAM-1 IgD1/IgD2 crystal structure are required for homophilic binding.

HEK293 cells were transfected with wild-type (WT) or variant forms of PECAM-1 bearing amino acid substitutions at positions located within the IgD1-IgD1 (D33R, K62D), IgD1-IgD2 (D52R, R122D, R152D), or IgD2-IgD2 (K131D, P133G, D158K) interface. **(A)** PECAM-1 expression was analyzed by flow cytometry following staining with a PECAM-1-specific primary antibody and a fluorescently tagged secondary antibody. Results are expressed as the ratio of PECAM-1 median fluorescence intensity (MFI) observed in transfectants expressing WT or variant forms of PECAM-1 relative to that observed in transfectants expressing WT PECAM-1. Note that none of the substitutions dramatically affected the level of PECAM-1 expression. **(B)** PECAM-1 homophilic binding was analyzed by flow cytometry following staining with PECAM-1-IgG and a fluorescently-tagged secondary antibody. PECAM-1-IgG MFI was normalized to PECAM-1 MFI for each

variant. Note that the D33R substitution within the IgD1-IgD1 interface and D52R and R152D substitutions within the IgD1-IgD2 interface, but not K131D, P133G or D158K substitutions within the IgD2-IgD2 interface, dramatically decreased PECAM-1 homophilic binding. Lines and error bars represent means ± standard deviations calculated from three independent experiments Statistically significant differences were determined by performing an ordinary one-way ANOVA ($p = 0.003$ for expression, $p = 0.002$ for normalized binding) using the Kruskal Wallis test with the Dunn's test multiple comparisons.

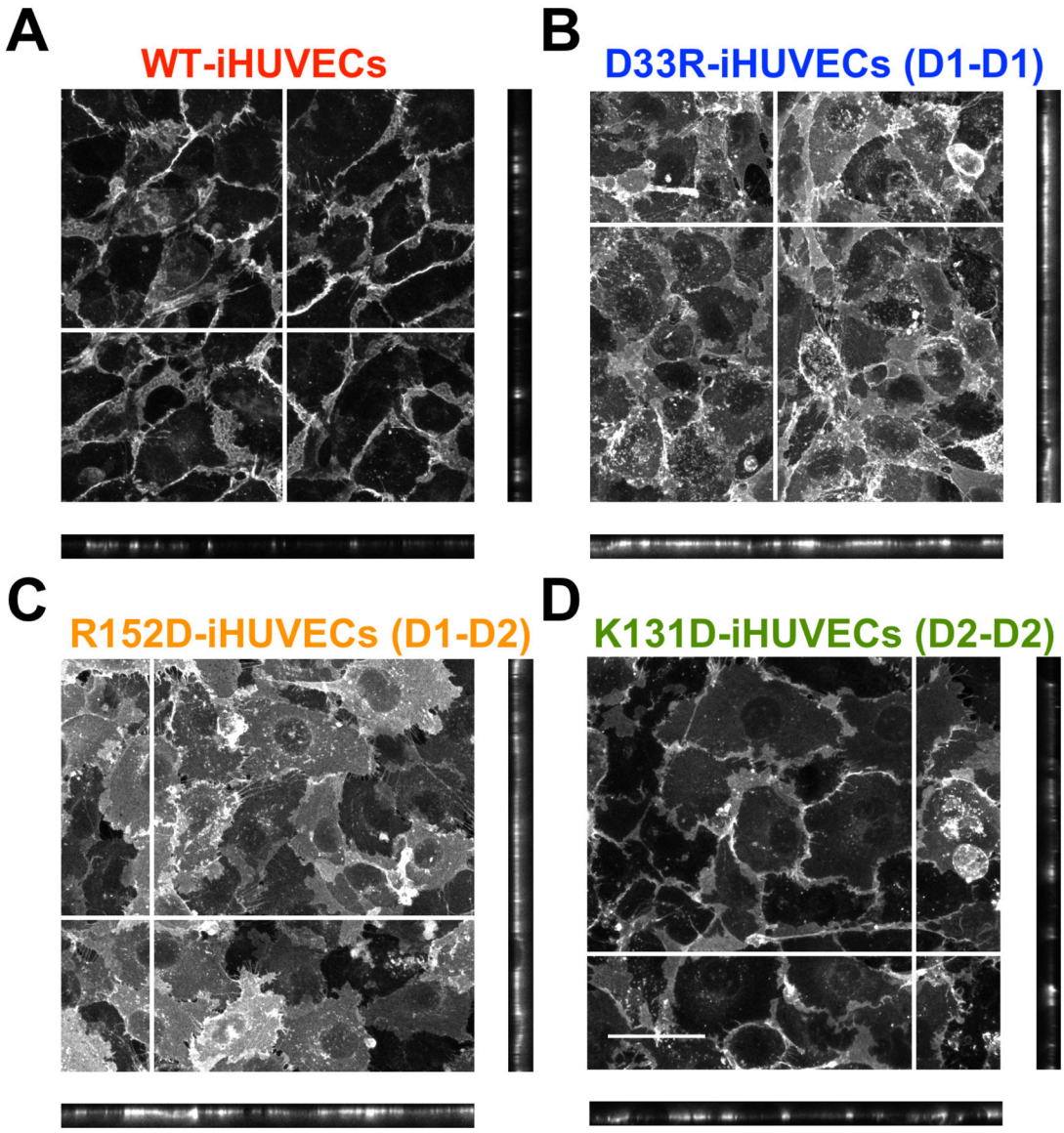

Figure 3. Homophilic binding residues within the PECAM-1 IgD1-IgD1 and IgD1-IgD2 interfaces are required for localization of PECAM-1 at endothelial cell-cell junctions.

Wild-type (WT) immortalized human umbilical vein endothelial cells (iHUVEC) or PECAM-1-deficient iHUVEC that were transduced with lentiviral vectors encoding variant forms of PECAM-1 bearing amino acid substitutions at positions located within the IgD1-IgD1 (D33R), IgD1-IgD2 (R152D), or IgD2-IgD2 (K131D) interface were grown to confluence, stained with an antibody specific for PECAM-1 and a fluorescently tagged secondary antibody, and imaged using confocal fluorescence microscopy. Representative *en face* projections and cross-sectional images, the latter reconstructed in the Z-axis to show border localization, are shown. Cross-section locations are indicated by vertical and horizontal white lines in the *en face* image. The scale bar represents 20 μm. Note that WT- and K131D-PECAM-1 concentrated at endothelial cell-cell junctions, whereas D33R- and R152D-PECAM-1 were diffusely distributed across the endothelial cell surface.

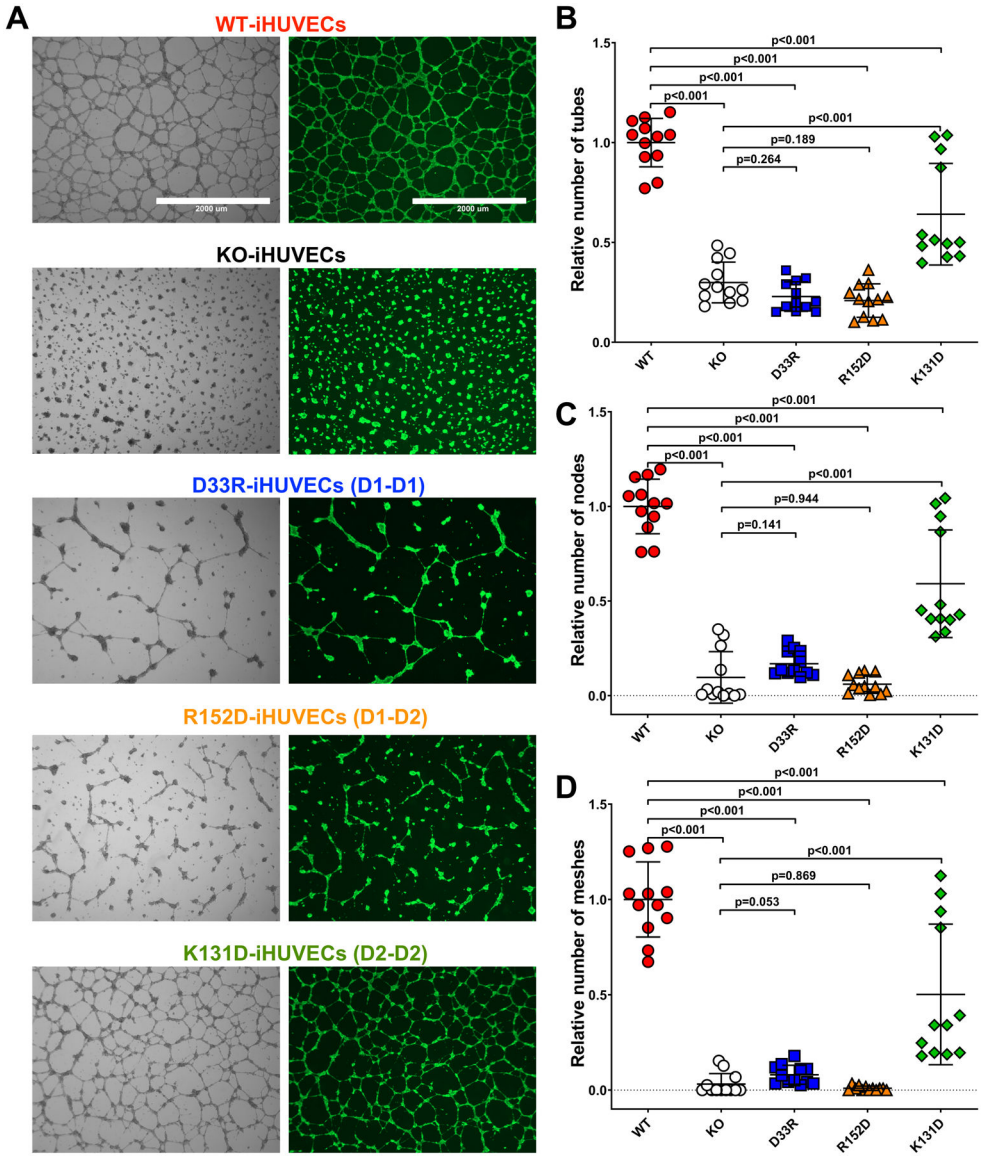

Figure 4. Homophilic binding residues within the PECAM-1 IgD1-IgD1 and IgD1-IgD2 interfaces are required for *in vitro* sprouting and tube formation by endothelial cells.

Wild-type (WT) immortalized human umbilical vein endothelial cells (iHUVEC), PECAM-1-deficient iHUVEC (KO), or KO iHUVEC that were transduced with lentiviral vectors encoding variant forms of PECAM-1 bearing amino acid substitutions at positions located within the IgD1-IgD1 (D33R), IgD1-IgD2 (R152D), or IgD2-IgD2 (K131D) interface were seeded in Matrigel-coated plates for 18h and imaged following staining with Calcein AM. **(A)** Representative bright-field (left) and fluorescent (right) images of networks and interconnecting tube structures. The scale bar represents 2 mm. Note that WT- and K131D-iHUVECs formed robust networks and interconnecting tube structures, whereas KO-, D33R-, and R152D-iHUVECs formed round aggregates with sprouts and discontinuous tubes. The numbers of tubes **(B)**, nodes **(C)**, and meshes **(D)** formed in four randomly chosen fields for each well were quantified using ImageJ software. Results are expressed as means ± standard deviations calculated from three independent experiments.

Statistically significant differences were determined by performing an ordinary one-way ANOVA ($p < 0.001$ for numbers of tubes, nodes and meshes) using the Kruskal Wallis test with the Dunn's test multiple comparisons. Note that the structures formed by KO-, D33R-, and R152D-iHUVECs had fewer tubes, nodes and meshes than those formed by WT and K131D-iHUVECs.

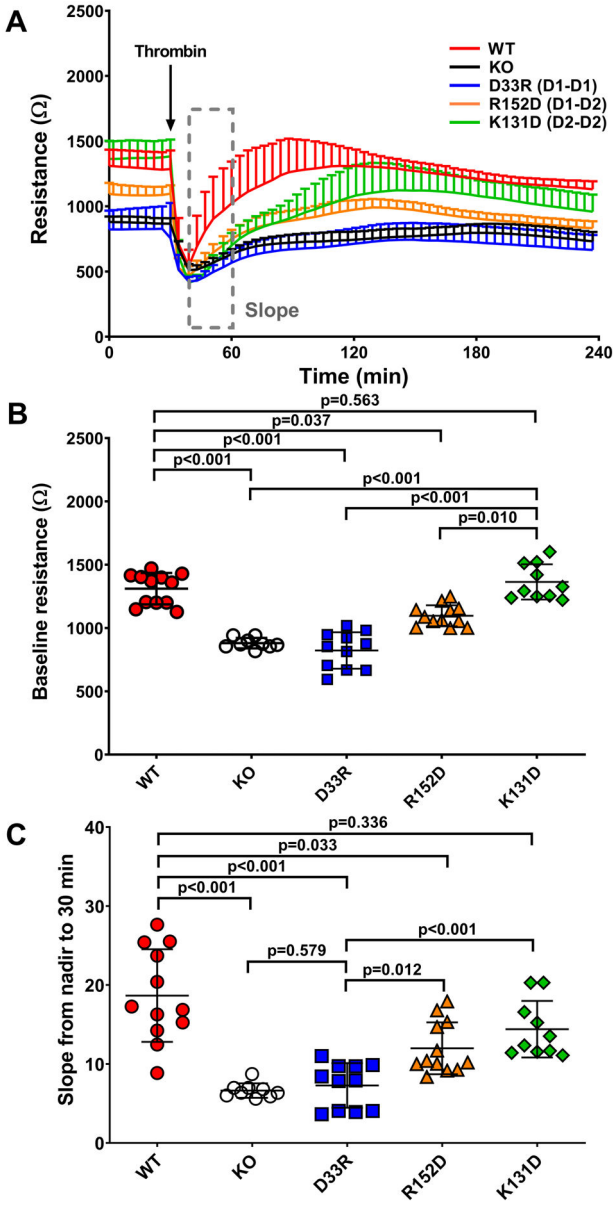

Figure 5. Homophilic binding residues within the PECAM-1 IgD1-IgD1 and IgD1-IgD2 interfaces are required for endothelial cell barrier function.

Confluent wild-type (WT) immortalized human umbilical vein endothelial cells (iHUVEC), PECAM-1-deficient iHUVEC (KO), or KO iHUVEC that were transduced with lentiviral vectors encoding variant forms of PECAM-1 bearing amino acid substitutions at positions located within the IgD1-IgD1 (D33R), IgD1-IgD2 (R152D), or IgD2-IgD2 (K131D) interface were tested for their ability to impede the flow of an electrical current (4000 Hz) by Electric Cell-substrate Impedance Sensing (ECIS). **(A)** Representative ECIS tracings before and after disruption of inter-endothelial cell interactions by treatment with thrombin (2.5 U/ml). Results are expressed as the mean ± standard deviation of resistance values in ohm/cm^2 (Ω) over time. The arrow indicates the time of exposure to thrombin and the dashed box represents the values used to calculate the initial rate of recovery of barrier

resistance following thrombin challenge. **(B)** Baseline resistance of iHUVEC expressing the indicated forms of PECAM-1. Results are expressed as means ± standard deviations calculated from 9–12 independent experiments. **(C)** Linear regression analysis of the rate of recovery of barrier function after thrombin challenge, measuring the slope from the point of lowest resistance following thrombin challenge (nadir) to 30 minutes after challenge. Results are expressed as means ± standard deviations calculated from 8–9 independent experiments. Statistically significant differences were determined by performing an ordinary one-way ANOVA ($p < 0.001$ for baseline and slope from nadir to 30 minutes) using the Kruskal Wallis test with the Dunn's test multiple comparisons. Note that KO-iHUVECs and iHUVECs expressing D33R and R152D variant forms of PECAM-1 had both lower baseline barrier resistance and slower rates of barrier recovery following thrombin challenge relative to WT-iHUVECs.

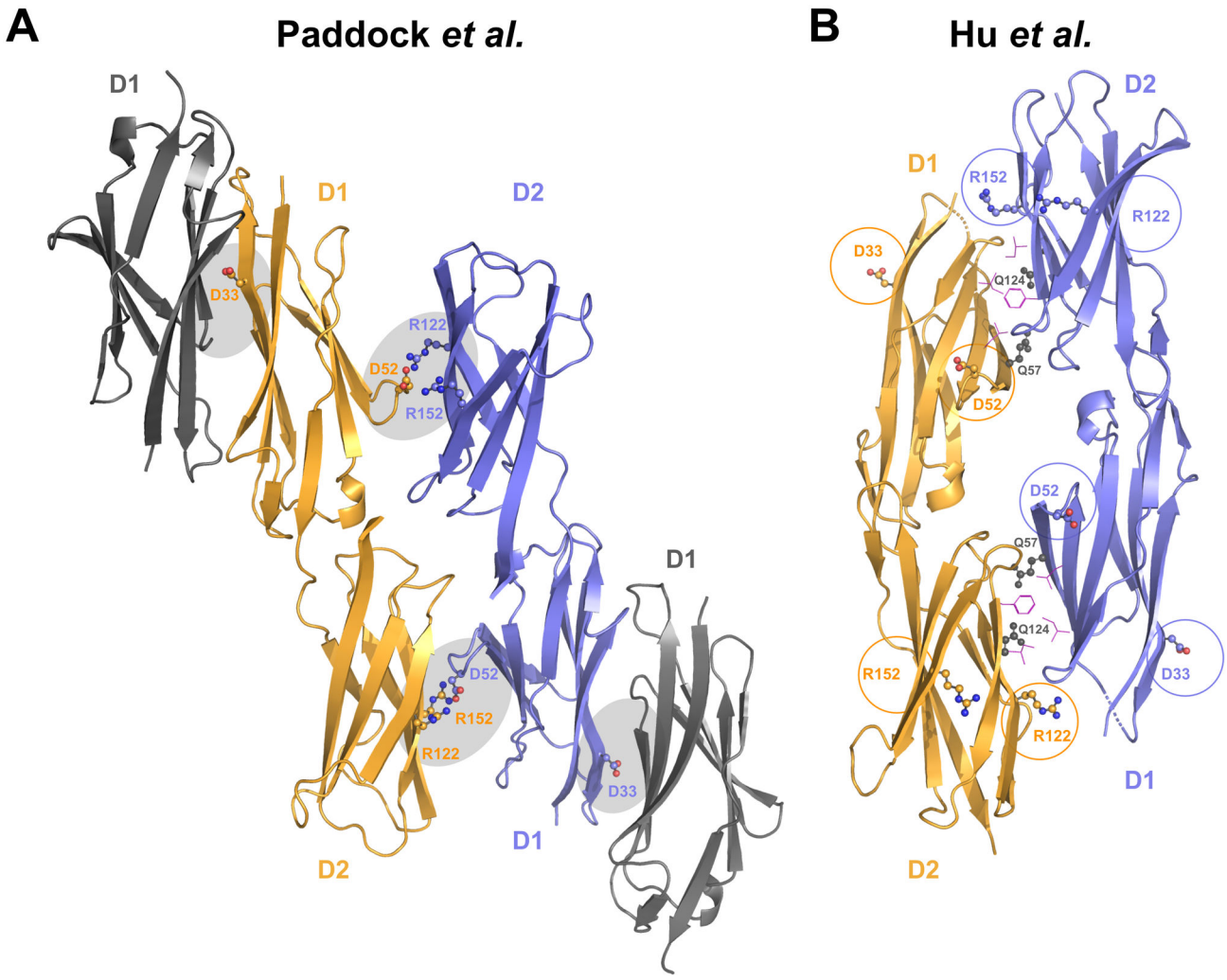

Figure 6. Location of key mutated residues in PECAM-1 IgD1/IgD2 structures from Paddock *et al.* and Hu *et al.*

In the Paddock *et al.* structure **(A)**, residues D52, R122, and R152 are clustered in the center of the two IgD1-IgD2 interfaces, whereas in the Hu *et al.* structure **(B)** the D52 and R152 sidechains project outward into the surrounding solvent, with R122 at the far boundary of the IgD1-IgD2 interface. In the Paddock *et al.* structure, the largest interface occurs between adjacent IgD1 domains in trans. D33 is prominently located in this interface, and mutation of this residue significantly decreases homophilic binding. The Hu *et al.* structure does not contain a IgD1-IgD1 interface, and the D33 sidechain is directed outward into the solvent. The residues targeted for mutation based on the Paddock *et al.* structure are displayed as ball and stick sidechains, and carbon atoms are shaded to match the domains in which they are located. In **(A)**, shaded areas encompass the portions of the domain interfaces targeted for mutational studies. Mutated residues are labeled. In **(B)**, labeled residues correspond to the mutations made in the current work. The hydrophobic residues targeted by Hu et al. in their lone mutant protein (L47E/I85E/F161E/I163E) are shown with sidechains drawn as purple

lines. Likewise, the N57Q and N124Q mutations are shown with gray sidechains. Protein images were created using PyMOL.