Supplementary Material

Supplementary Table S1 Animals (*in vivo* studies)

Species	Source	Background strain	Sex
Mouse	Jackson Laboratory (#000664)	C57BL/6	M
Mouse	In-house generation	clec1b$^{fl/fl}$GPIbα-Cre (C57BL/6 background)	M/F
Mouse	In-house generation	GP6$^{-/-}$clec1b$^{fl/fl}$GPIbα-Cre (C57BL/6 background)	M/F

Supplementary Table S2 Genetically modified animals—CLEC-2$^{fl/fl}$GPIbα-Cre mouse

	Species	Source	Background strain
Parent—male	Mouse	In-house generation	GPIbα-Cre
Parent—female	Mouse	In-house generation	clec1b$^{fl/fl}$

Supplementary Table S3 Genetically modified animals—GP6$^{-/-}$:CLEC-2$^{fl/fl}$GPIbα-Cre mouse

	Species	Source	Background Strain
Parent—male	Mouse	In-house generation	clec1b$^{fl/fl}$GPIbα-Cre
Parent—female	Mouse	In-house generation	GP6$^{-/-}$

Supplementary Table S4 Cultured cells

Name	Sex	Vender	Reference
HUVEC	F	Promocell	C-12208

Supplementary Table S5 Antibodies

Target antigen	Source	Catalog #	Working concentration
Murine GPIbβ (*in vivo*-AF488)	Emfret	#X488	0.1 μg/kg
Murine GPIbβ (AF647)	Emfret	#X647	1:100
Murine GPIbα (platelet-depleting)	Emfret	#R300	0.1 mg/kg
Murine CD41-APC	Biolegend	#133913	1:100
Murine GPVI-AF647	R&D	#FAB6758R	1:100
Murine CLEC-2-PE	BioLegend	#146104	1:100
Murine GPIb-PE	BioLegend	#148503	1:100
αIIbβ3-FITC	Dako	#F011102-2	1:100
CD62P-AF647	BioLegend	#304918	1:100
Phosphatidylserine-AF568	Thermofisher	#A23204	1:100
F(ab) AYP1	In-house generation	–	10 μg/mL
F(ab)$_2$ AYP1	In-house generation	–	10 μg/mL
ICAM-1 (HM1)	Invitrogen	#69-74-9	1:100
VCAM-1-APC	Invitrogen	#A15721	1:50
VE-Cadherin	R&D	#MAB9381	1:100
Hoescht 33342	Thermofisher	#62249	1:10,000
CD41	BioLegend	#303709	1:100
CD62P	BioLegend	#304905	1:100

Recombinant Protein

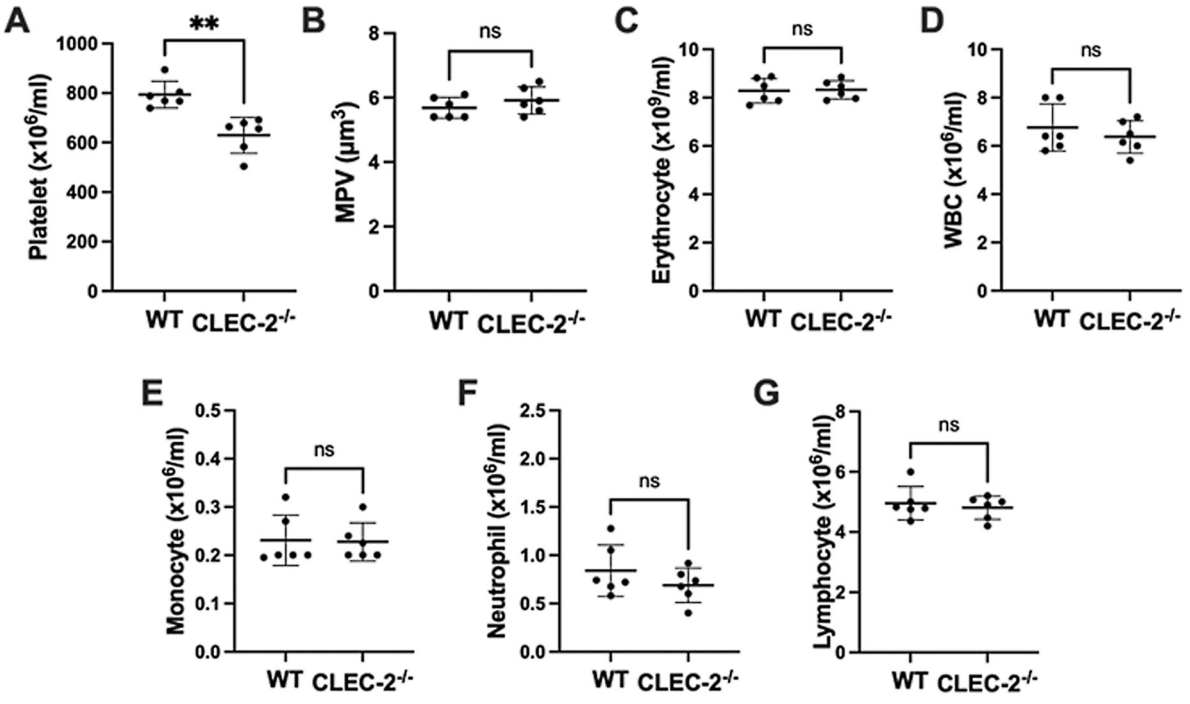
Human recombinant CLEC-2-Fc was generated in-house with a sequence:

SVMQRNYLQGENENRTGTLQQLAKRFCQYVVKQSELKGT
FKGHKCSPCDTNWRYY
GDSCYGFFRHNLTWEESKQYCTDMNATLLKIDNRNIVEYI-
KARTHLIRWVGLSRQKSN

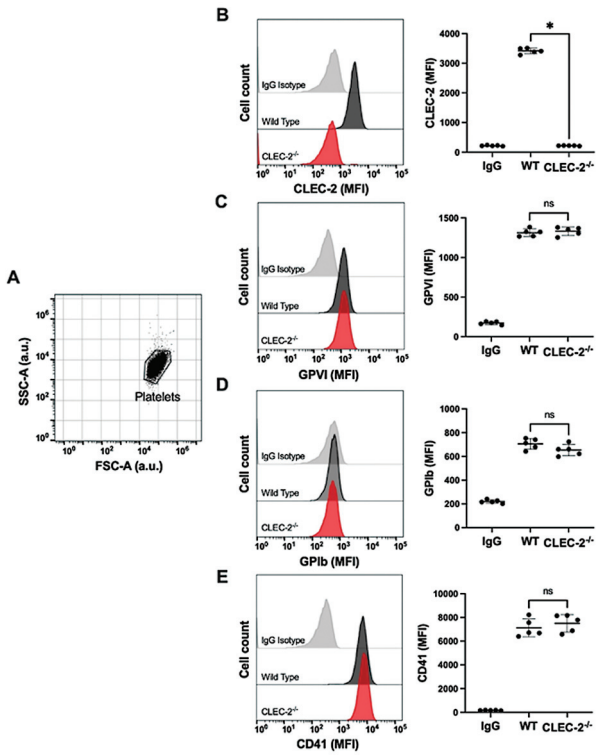
EVWKWEDGSVISENMFEFLEDGKGNMNCAYFHNGKMHPT
FCENKHYLMCERKAGM
TKVDQLP—fused to the Fc domain of IgG. Amino acids 55-229.

All methodology for Supplementary Data is included in the main manuscript.

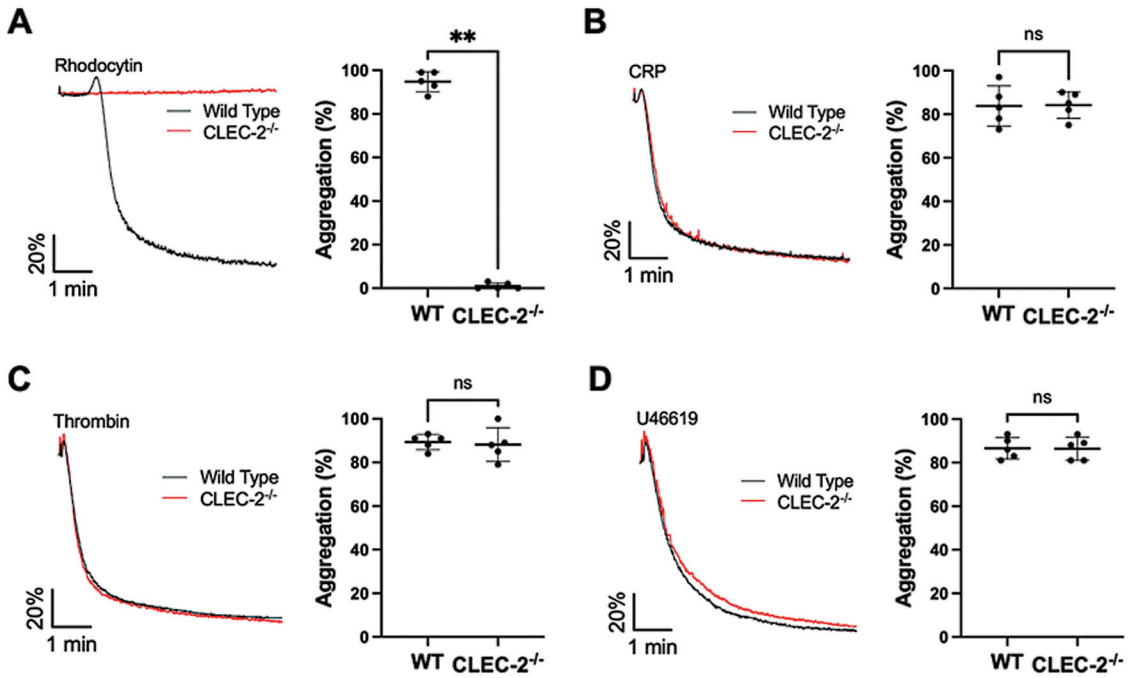
Supplementary Data



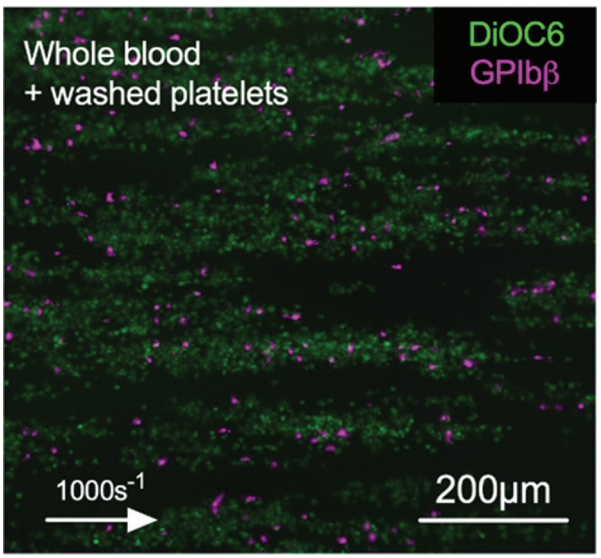
Supplementary Fig. S1 Platelet-CLEC-2-deficiency leads to a reduction in platelet count. Whole blood from wild type (WT; clec1b$^{fl/fl}$GPIbα-Cre^{-}) or platelet-CLEC-2-deficient (CLEC-2$^{-/-}$; clec1b$^{fl/fl}$GPIbα-Cre^{+}) mice was analyzed for **(A)** platelet count, **(B)** mean platelet volume (MPV), **(C)** erythrocyte count, **(D)** white blood cell (WBC) count, **(E)** monocyte count, **(F)** neutrophil count, and **(G)** lymphocyte count ($n = 6$). The statistical significance between two groups was analyzed using a *t*-test. $^{**}p < 0.01$.



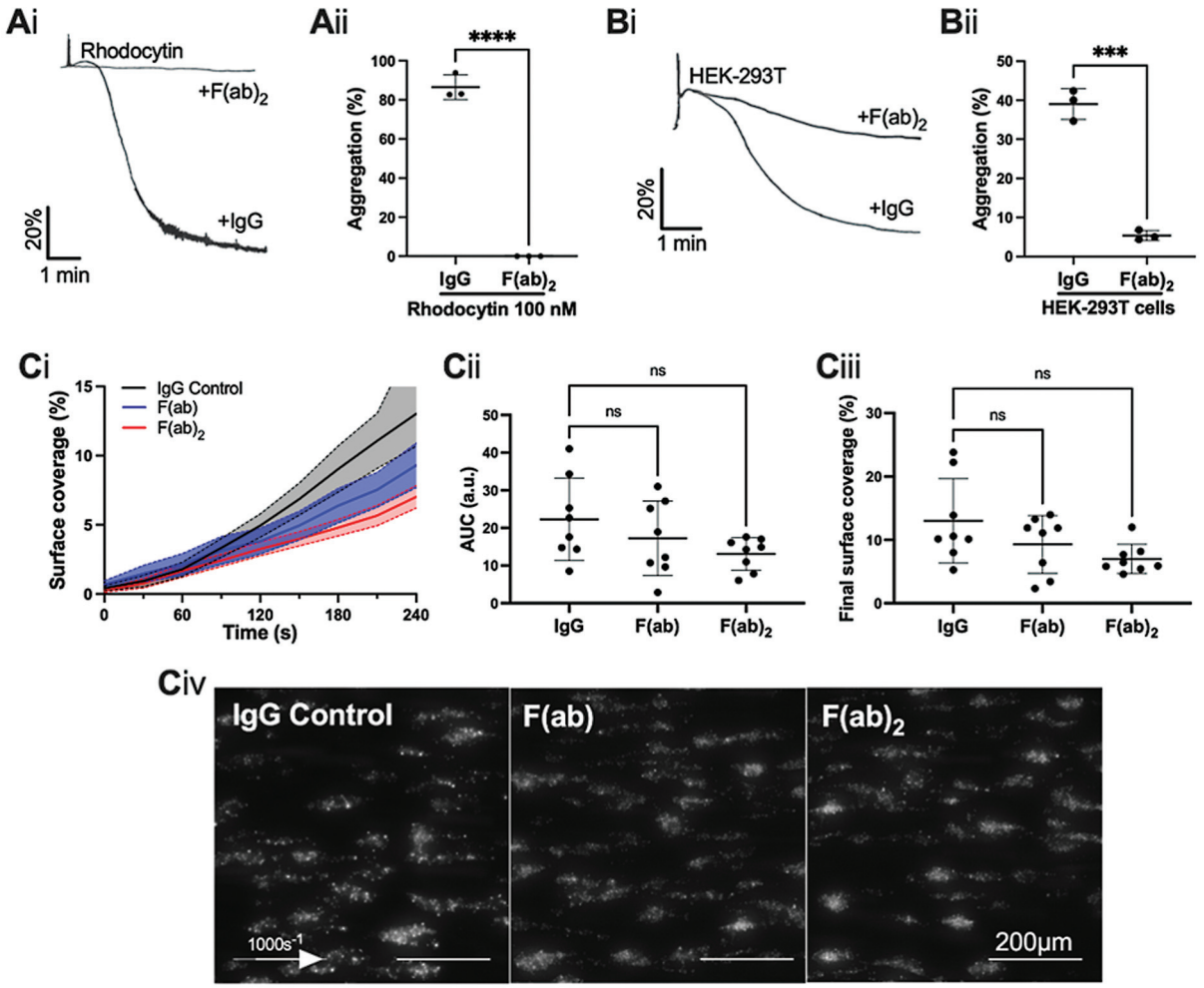
Supplementary Fig. S2 Platelet-CLEC-2-deficiency in mice does not impact the expression of classical platelet receptors. (A–E) Platelet receptor expression from wild type (WT; clec1b$^{fl/fl}$GPIbα-Cre^{-}) or platelet-CLEC-2-deficient (CLEC-2$^{-/-}$; clec1b$^{fl/fl}$GPIbα-Cre^{+}) mice was quantified using washed platelets by flow cytometry and compared to a conjugated IgG isotype control antibody. (A) Platelets were identified by size using forward- (FSC) and side-scatter (SSC; a.u. = arbitrary units). The mean fluorescence intensity (MFI) of (B) CLEC-2, (C) GPVI, (D) GPIb, and (E) CD41 was analyzed using an Accuri C6 flow cytometer, and representative histograms were made using FlowJo (n = 5 mice). The statistical significance between groups was analyzed a one-way ANOVA. $^{*}p < 0.05$.



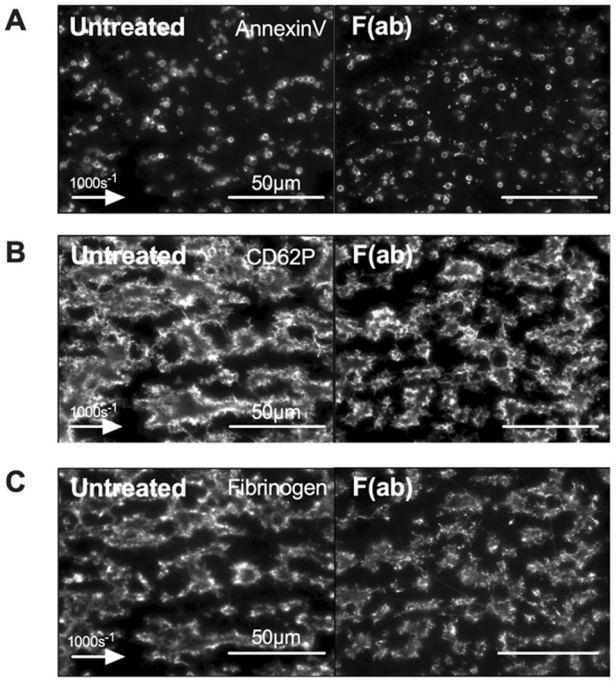
Supplementary Fig. S3 Platelet-CLEC-2-deficient mice respond normally to PAR, GPVI, and TxA_2 receptor agonists. (A–D) Washed platelet aggregation from wild type (WT; clec1b$^{fl/fl}$GPIbα−Cre^{-}) or platelet-CLEC-2-deficient (CLEC-2$^{-/-}$; clec1b$^{fl/fl}$GPIbα-Cre^{+}) mice was assessed by light transmission aggregometry. Representative platelet aggregation trace and maximum aggregation shown, induced by (A) rhodocytin (100 nM), (B) CRP (10 µg/mL), (C) thrombin (0.1 U/mL), or (D) U46619 (2.5 µM) for 6 minutes. The statistical significance between two groups was analyzed using a paired *t*-test. $^{**}p < 0.01$.



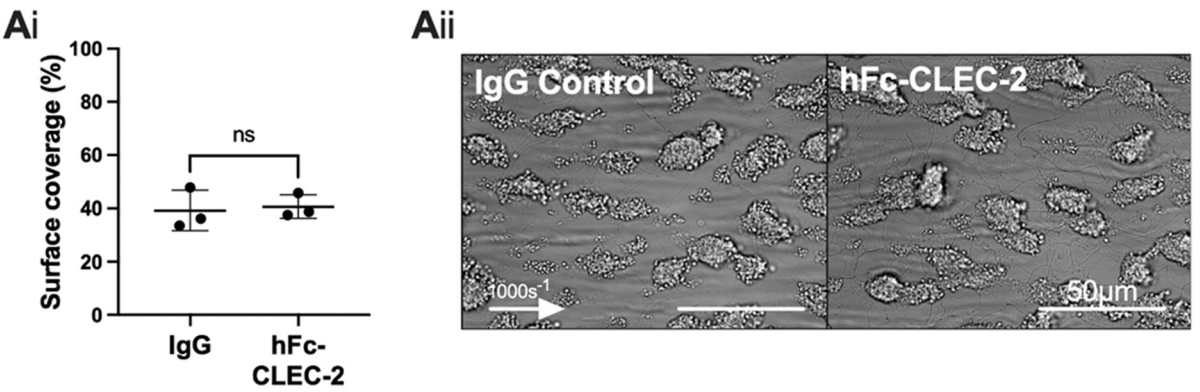
Supplementary Fig. S4 Transfused washed platelets contribute to thrombus formation in murine whole blood when perfused over collagen at arterial shear. Washed mouse platelets were donated to whole mouse blood, before perfusion through a Horm collagen-coated (100 µg/mL) µ-Slide VI 0.1 flow chamber at 1,000 s^{-1}. The washed platelets (*magenta*) were incubated with a GPIbβ-AF647 conjugated antibody, before addition to whole blood containing DiOC6 (*green*). *Arrow* indicates direction of flow. Image is representative of three mice.



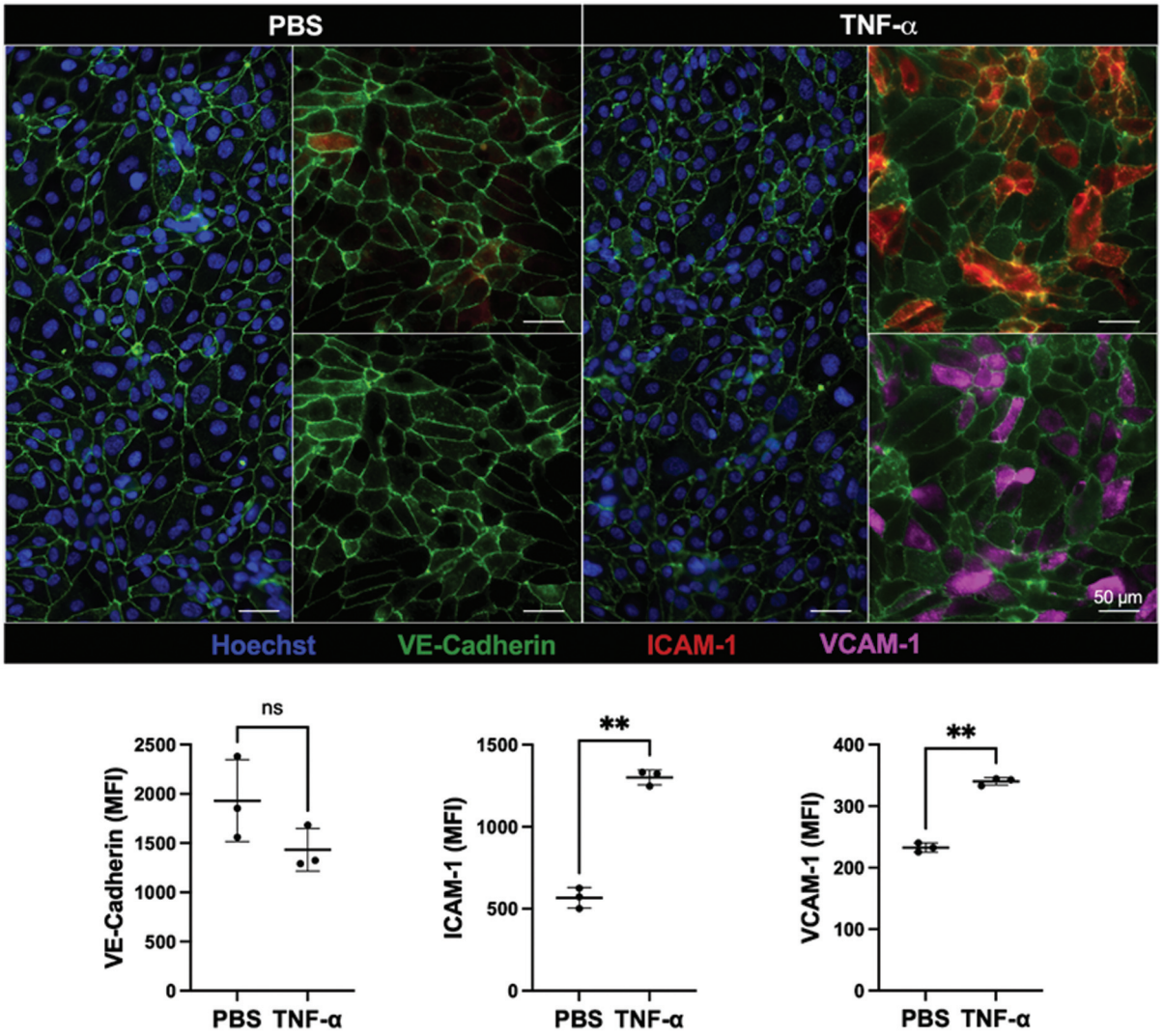
Supplementary Fig. S5 CLEC-2 inhibition by AYP1 fragments does not alter thrombus formation in human blood perfused over Horm collagen. (A, B) Washed platelet aggregation was assessed by light transmission aggregometry, in the presence or absence of AYP1 fragment, F $(ab)_2$ (10 µg/mL). (Ai) Representative platelet aggregation trace induced by rhodocytin (100 nM) and (Aii) maximum aggregation (n = 3 donors). (Bi) Representative platelet aggregation trace induced by HEK-293T cells and (Bii) final aggregation (n = 3 donors). (C) Whole blood from healthy volunteer donors was perfused over Horm collagen-coated (100 µg/mL) µ-Slide VI 0.1 flow chamber at 1,000 s^{-1}, in the presence or absence of F (ab) (10 µg/mL) and $F(ab)_2$ (10 µg/mL) for minutes prior to perfusion. (Ci) Thrombus surface coverage over 4 minutes, measured using DiOC6 fluorescence (2 µM), (Cii) subsequent area under the curve (AUC) was calculated (a.u. = arbitrary units), and (Ciii) thrombus formation after 4 minutes. (Civ) Representative images shown after 4 minutes (n = 8 donors; *arrow* indicates direction of flow). The statistical significance between two groups was analyzed using an unpaired t-test and the statistical significance between multiple groups was analyzed using one-way ANOVA with Tukey's multiple comparisons test. $^{****}p < 0.0001$.



Supplementary Fig. S6 The effect of inhibition of CLEC-2 inhibition by AYP1 fragments on platelet activation on collagen under flow conditions. Whole blood from healthy volunteer donors was perfused through a Maastricht flow chamber at 1,000 s^{-1} in the presence or absence of F(ab) (10 µg/mL) 10 minutes prior to perfusion. The flow chamber was coated with Horm collagen (100 µg/mL). Representative images of three donors shown after 3.5 minutes of (A) phosphatidylserine (PS) exposure, (B) P-selectin expression (CD62P), and (C) fibrinogen coverage. Quantification was assessed with a semi-automated ImageJ script from these images, shown in Fig. 3Div–vi.



Supplementary Fig. S7 Recombinant human CLEC-2-Fc does not alter thrombus formation in human blood perfused over Horm collagen. Whole blood from healthy volunteer donors was perfused through a Maastricht flow chamber at 1,000 s^{-1}, in the presence or absence of hFc-CLEC-2 (10 µg/mL) for 10 minutes prior to perfusion. **(Ai)** Thrombus surface coverage and **(Aii)** representative images shown after 4 minutes (n = 3 donors; *arrow* indicates direction of flow). The statistical significance between two groups was analyzed using an unpaired t-test.



Supplementary Fig. S8 TNF-α-stimulated HUVECs express adhesion receptors ICAM-1 and VCAM-1 in the Ibidi µ-Slide 0.4 model. A confluent monolayer of HUVECs was challenged with recombinant TNF-α (10 ng/mL) for 24 hours or PBS. The expression of VE-Cadherin (green), ICAM-1 (red), and VCAM-1 (magenta) was acquired using an epifluorescent microscope with a 20X and 40X lens, and representative images are shown. Protein expression was quantified using FIJI ($n = 3$). The statistical significance between two groups was analyzed using an unpaired *t*-test. $^{****}p < 0.01$.

