

Supplemental information

Antibodies and complement are key drivers of thrombosis

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Supplementary Figure 1

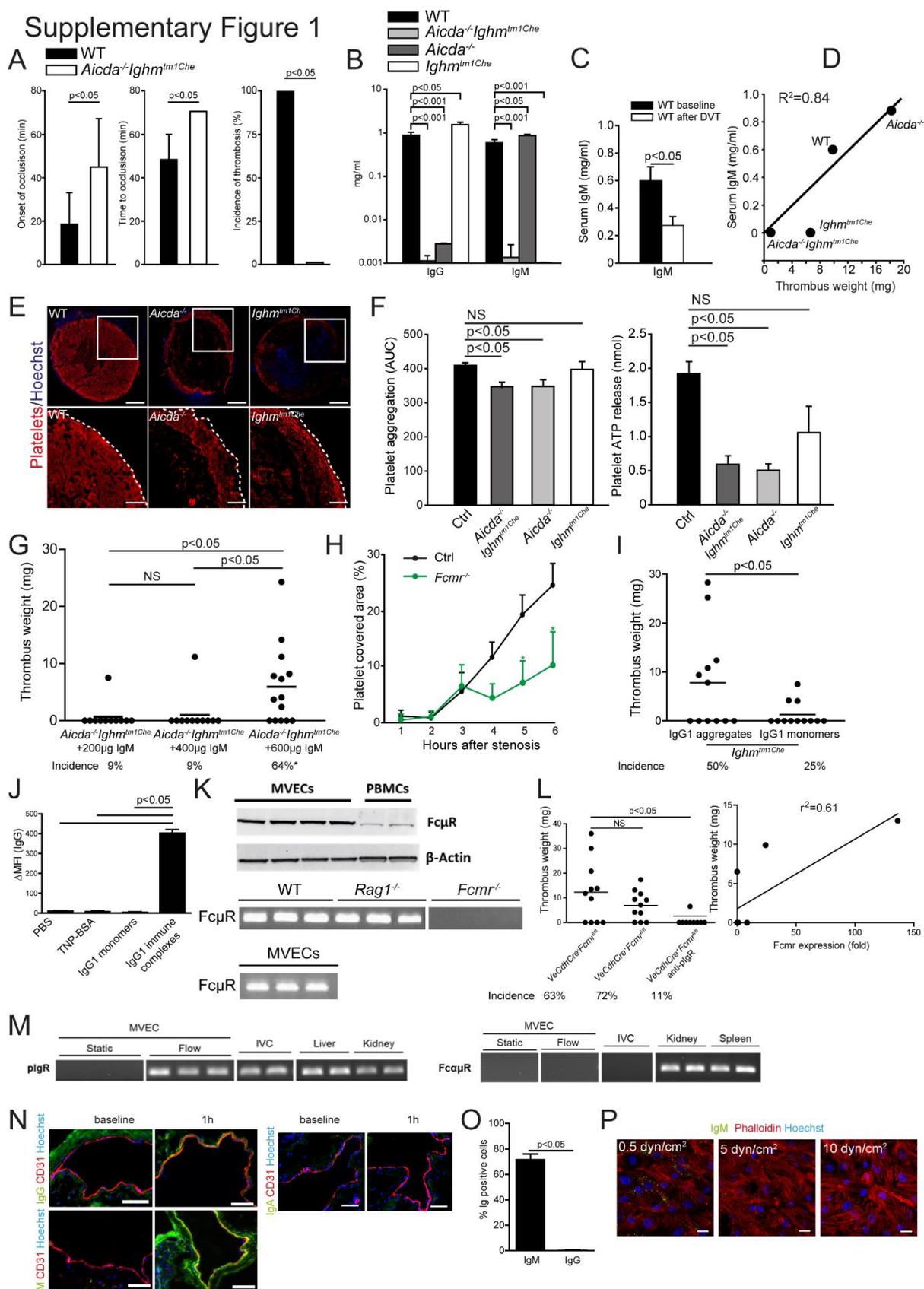


Figure S1.

IgM-endothelial interactions initiate DVT, related to Figure 1. (A) Microvascular thrombosis in venules of the cremaster muscle shown as time to onset of thrombosis (left), time until complete vessel occlusion (middle), and incidence of thrombosis (right) in ctrl and *Aicda*^{-/-} *Ighm*^{tm1Che} mice (n=5 each). Data compared by two-tailed unpaired two-sample t-test or Fisher exact test. (B) ELISA for IgG and IgM from serum of *Aicda*^{+/+} *Ighm*^{wt/wt}, *Aicda*^{-/-} *Ighm*^{tm1Che}, *Aicda*^{-/-}, and *Ighm*^{tm1Ch} mice (n=3 each) compared by one-way ANOVA followed by LSD-post hoc-test. (C) Serum IgM levels in C57Bl6 (WT) before and 48 hours after flow reduction (n=4 each) compared by paired t-test. (D) Correlation between mean serum IgM levels (n=3 each) from of C57Bl6 (WT), *Aicda*^{-/-} *Ighm*^{tm1Ch}, *Aicda*^{-/-}, and *Ighm*^{tm1Ch} and thrombus weight (n=8-16). (E) Immunofluorescence staining for platelets (CD41, red) and nuclei (Hoechst, blue) of cross sections of the inferior vena cava 48 hour after flow reduction in control (ctrl), *Aicda*^{-/-}, and *Ighm*^{tm1Ch} mice. Top: Overview, Bar 200µm; Bottom: Higher magnification of boxed areas, dotted line indicates endothelium. Bar 40 µm. Images representative of n=5 experiments each. (F) Aggregometry of platelet rich plasma from ctrl (n=4), *Aicda*^{-/-} (n=4), *Ighm*^{tm1Ch} (n=4), or *Aicda*^{-/-} *Ighm*^{tm1Ch} (n=7) mice showing platelet aggregation (left) and ATP release (right) compared by one-way ANOVA followed by Tukey's Multiple Comparison Test. (G) Thrombus weight and incidence in *Aicda*^{-/-} *Ighm*^{tm1Ch} mice supplemented different amounts of polyclonal IgM (200µg, n=11; 400µg, n=11; 600µg, n=14) compared by one-way ANOVA followed by LSD-post hoc-test. or Fisher exact test. Line indicates mean. *indicates p<0.05. (H) Quantification of platelet recruitment over the first 6 hours after flow reduction in *Fcμr*^{-/-} or ctrl mice (n=4 each) compared by two-tailed unpaired two-sample t-test; * indicates p<0.05. (I) Thrombus weight and incidence in *Ighm*^{tm1Ch} mice intravenously supplemented with mouse anti-HIV gp120 IgG1 monomers (n= 12) or aggregates (n= 12) (600µg each) compared by two-tailed unpaired two-sample t-test or Fisher exact test. Line indicates mean. (J) Binding of TNP-specific mouse IgG1 immune complexes and monomers compared to PBS and bovine serum albumin TNP to FcμR expressing HEK cells assessed by flow cytometry. Mean fluorescence intensities quantified and background binding to non-FcμR expressing cells was subtracted from IgG binding to obtain ΔMFI (n=4 each). Data compared by one-way ANOVA followed by LSD-post hoc-test followed by Tukey's Multiple Comparison Test. (K) Western blot of a FcμR staining from MVECs and PBMCs including beta-actin, representative of n=4 experiments. Right top: Expression of FcμR in the IVC from WT (C57Bl6), B cell deficient *Rag1*^{-/-}, or *Fcμr*^{-/-} mice (representative of n=5 each) as shown on gels from rtPCR. Right bottom: Expression of FcμR in murine venous endothelial cells (MVECs) as shown by a representative gel from rtPCR (n=5). (L) Left: Thrombus weight and incidence in *VeCdhCre* *Fcμr*^{fl/fl} mice (n=11) compared to *VeCdhCre*⁺ *Fcμr*^{fl/fl} mice (n=11) and *VeCdhCre*⁺ *Fcμr*^{fl/fl} mice with pIgR blocking antibody (n=11) by one-way ANOVA followed by LSD-post hoc-test or Fisher exact test. Line indicates mean. Right: Correlation between thrombus weight and FcμR expression in the IVC of *VeCdhCre*⁺ *Fcμr*^{fl/fl} mice given as fold increase compared to *VeCdhCre*⁻ *Fcμr*^{fl/fl} mice. (M) rtPCR for pIgR (left) and FcαμR (right) in MVECs under flow and static conditions *in vitro* as well as *in vivo* in the IVC and liver, kidney, or spleen, respectively (representative of n=4-5 mice/experiments). (N) Representative immunofluorescence stainings of the IVC under baseline conditions (top) and one hour after flow reduction (bottom) for IgM, IgG, IgA (green), CD31 (red), and Hoechst (blue); Bar 30 µm, representative of n=5 mice. (O) Quantification of isotype IgG1 or IgM binding to murine endothelial cells *in vitro* by FACS (n=4 each) compared by two-tailed unpaired two-sample t-test. (P) Representative images from confocal microscopy of FITC-labeled

isotype IgM (green) deposition on MVECs exposed to different shear stress stained for nuclei (Hoechst, blue) and phalloidin (red). Bar 5 μ m. (**A-C, F, H, J, O**) Data given as mean \pm s.e.m.

Supplementary Figure 2

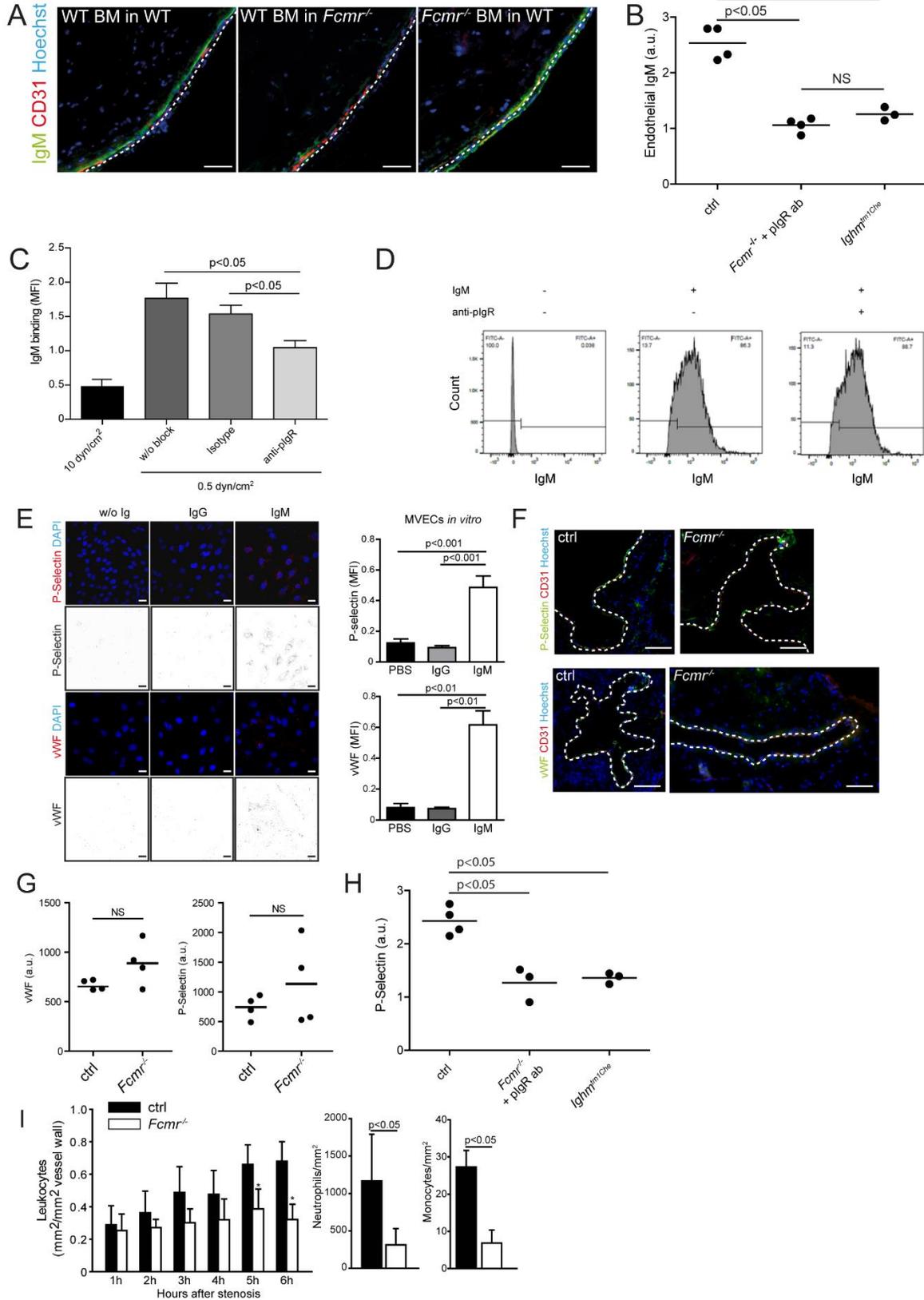


Figure S2.

The role of IgM-receptors in venous thrombosis, related to Figure 1. (A) Representative images of immunofluorescence staining of cross sections of the IVC 48 hours after flow reduction for CD31 (red), IgM (green), and Hoechst (blue) in bone marrow chimeras: wt bone marrow into wt mice, wt bone marrow into *FcμR*^{-/-} mice, *FcμR*^{-/-} bone marrow into wt mice (n=6 each). Dotted line indicates endothelium. Bar 50 μm. (B) Quantification of IgM on endothelial cells in the inferior vena cava 48 hours after flow reduction in wildtype mice treated with isotype antibody (n=4), *FcμR*^{-/-} mice treated with the pIgR blocking antibody (n=4) or IgM deficient *Ighm*^{tm1Ch} mice (n=3). Line indicates mean. Data compared by one-way ANOVA followed by Tukey's Multiple Comparison Test (C, D) Binding of FITC labeled IgM to endothelial cells under static (± pIgR blocking antibody, representative of n=5 independent experiments) and flow conditions *in vitro* (± pIgR blocking antibody/isotype control); n=5 each. Data compared by one-way ANOVA followed by Tukey's Multiple Comparison Test. (E) Left: Immunofluorescence staining of P-selectin and vWF exposure (red; inverted images at the bottom) on murine endothelial cells *in vitro* after incubation with PBS, isotype IgG1, or isotype IgM. Nuclei stained with DAPI (blue). Bar 20μm. Images representative of n=4 experiments. Right: Quantification of P-selectin (top) and vWF (bottom) exposure on MVECs *in vitro* exposed to PBS, isotype IgG1, or isotype IgM (n=3-5 each) compared by one-way ANOVA followed by LSD-post hoc-test. (F) Immunofluorescence of the vessel wall of the IVC 3 hours after flow reduction stained for CD31 (red), DAPI (blue), and P-selectin (top, green) or vWF (bottom, green) in *FcμR*^{-/-} or control mice. Bar 50 μm. Representative of n=5 experiments each. (G) Quantification of P-selectin and vWF exposure on the IVC endothelium under baseline conditions in *FcμR*^{-/-} mice compared to control mice (n=4 each) compared by two-tailed unpaired two-sample t-test. (H) Quantification of P-Selectin on endothelial cells in the inferior vena cava 48 hours after flow reduction in WT +isotype treated (n=4), *FcμR*^{-/-} + pIgR blocking antibody treated (n=3) or IgM deficient *Ighm*^{tm1Ch} mice (n=3). Data compared by one-way ANOVA followed by Tukey's Multiple Comparison Test. (I) Left: Quantification of leukocyte recruitment over the first 6 hours after flow reduction in the IVC in *FcμR*^{-/-} or ctrl mice (n=6 each) compared by two-tailed unpaired two-sample t-test. Right: Quantification of neutrophils (left) and monocytes (right) by immunofluorescence staining of cross sections of the IVC 48 hours after flow reduction in *FcμR*^{-/-} or ctrl mice (n=5 each). (E, I) Data given as mean ± s.e.m.

Supplementary Figure 3

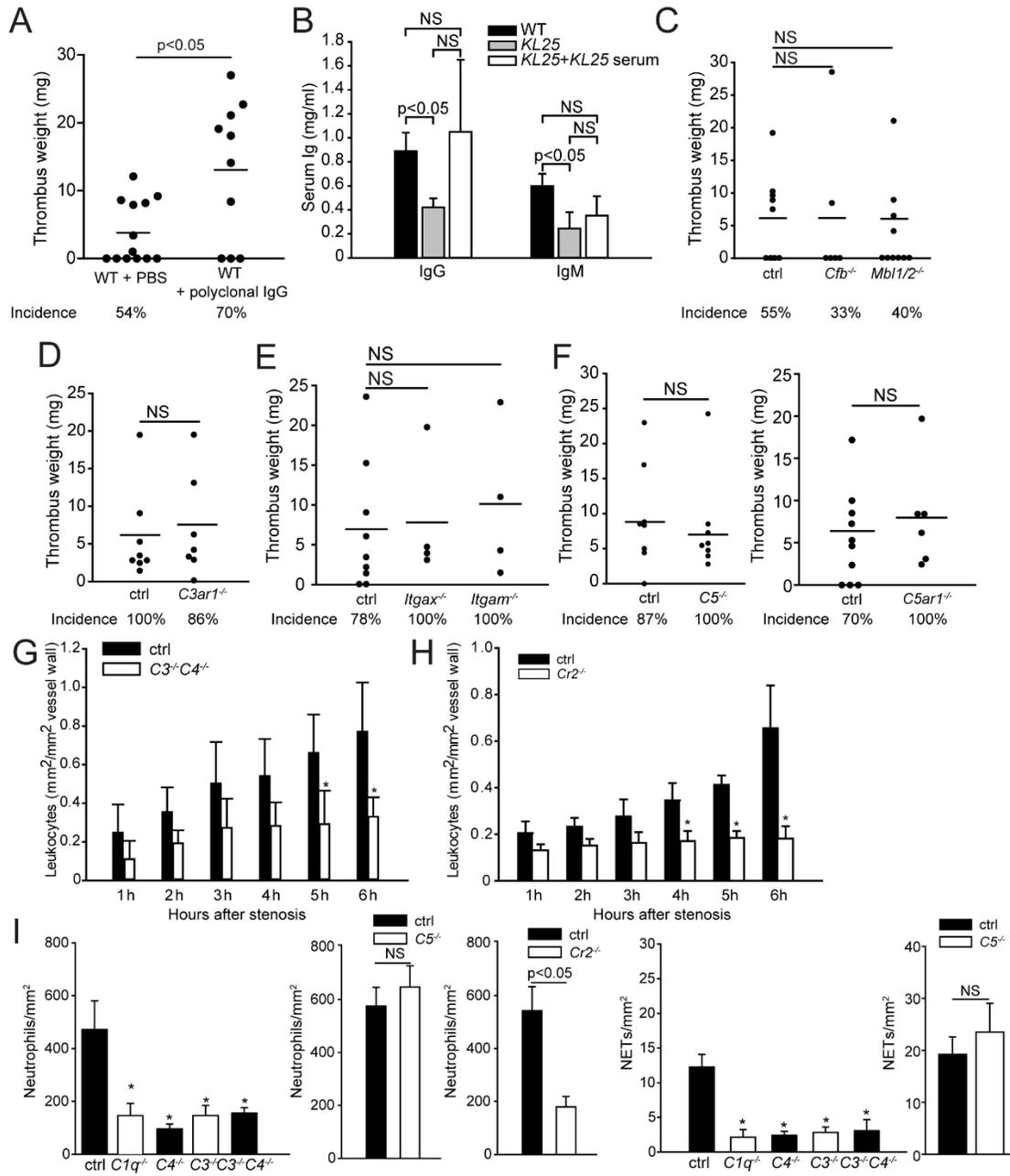


Figure S3.

Contribution of the complement system to venous thrombosis, related to Figure 2. (A) Thrombus weight and incidence in C57B16 mice supplemented with PBS (ctrl, n=13) or polyclonal IgG (n=10) compared by two-tailed unpaired two-sample t-test or Fisher exact test. Line indicates mean. (B) ELISA from plasma of wt, KL25, and KL25 mice supplemented with KL25 serum for IgG and IgM (n=4 each) compared by one-way ANOVA followed by LSD-post hoc-test. (C) Thrombus weight and incidence in C57B16 mice (ctrl, n=9) compared to *Cfb*^{-/-} mice (n=6) and *Mbl1/2*^{-/-} mice (n=10) by one-way ANOVA followed by LSD-post hoc-test or Fisher exact test. Line indicates mean. (D) Thrombus weight and incidence in C57B16 mice (ctrl, n=8) compared to *C3ar1*^{-/-} (n=7) mice by two-tailed unpaired two-sample t-test or Fisher exact test. Line indicates mean. (E) Thrombus weight and incidence in C57B16 mice (ctrl, n=9), *Itgax*^{-/-} (n=4), and *Itgam*^{-/-} (n=4) mice compared by one-way ANOVA followed by LSD-post hoc-test or Fisher exact test. Line indicates mean. (F) Thrombus weight and incidence in: Left: C57B110 mice (ctrl, n=8) and *C5*^{-/-} (n=8); Right: C57B16 mice (ctrl, n=10) and *C5ar1*^{-/-} (n=6) mice. Data compared by two-tailed unpaired two-sample t-test or Fisher exact test. Line indicates mean. (G) Quantification of leukocyte recruitment over the first 6 hours after flow reduction in *C3*^{-/-}*C4*^{-/-} (n=6) or ctrl mice (n=10) compared by two-tailed unpaired two-sample t-test. * P<0.05. (H) Quantification of leukocyte recruitment over the first 6 hours after flow reduction in the IVC in *Cr2*^{-/-} (n=5) or ctrl mice (n=7) compared by two-tailed unpaired two-sample t-test. * P<0.05. (I) Quantification of neutrophils (left) and NETs (right) by immunofluorescence staining of cross sections of the IVC 48 hours after flow reduction in *C1q*^{-/-}, *C4*^{-/-}, *C3*^{-/-}, *C3*^{-/-}*C4*^{-/-}, *Cr2*^{-/-} or ctrl mice (n=3-6), *C5*^{-/-} or C56B110 ctrl (n=5 each), * P<0.05. (B, G to I) Data given as mean ± s.e.m.

Supplementary Figure 4

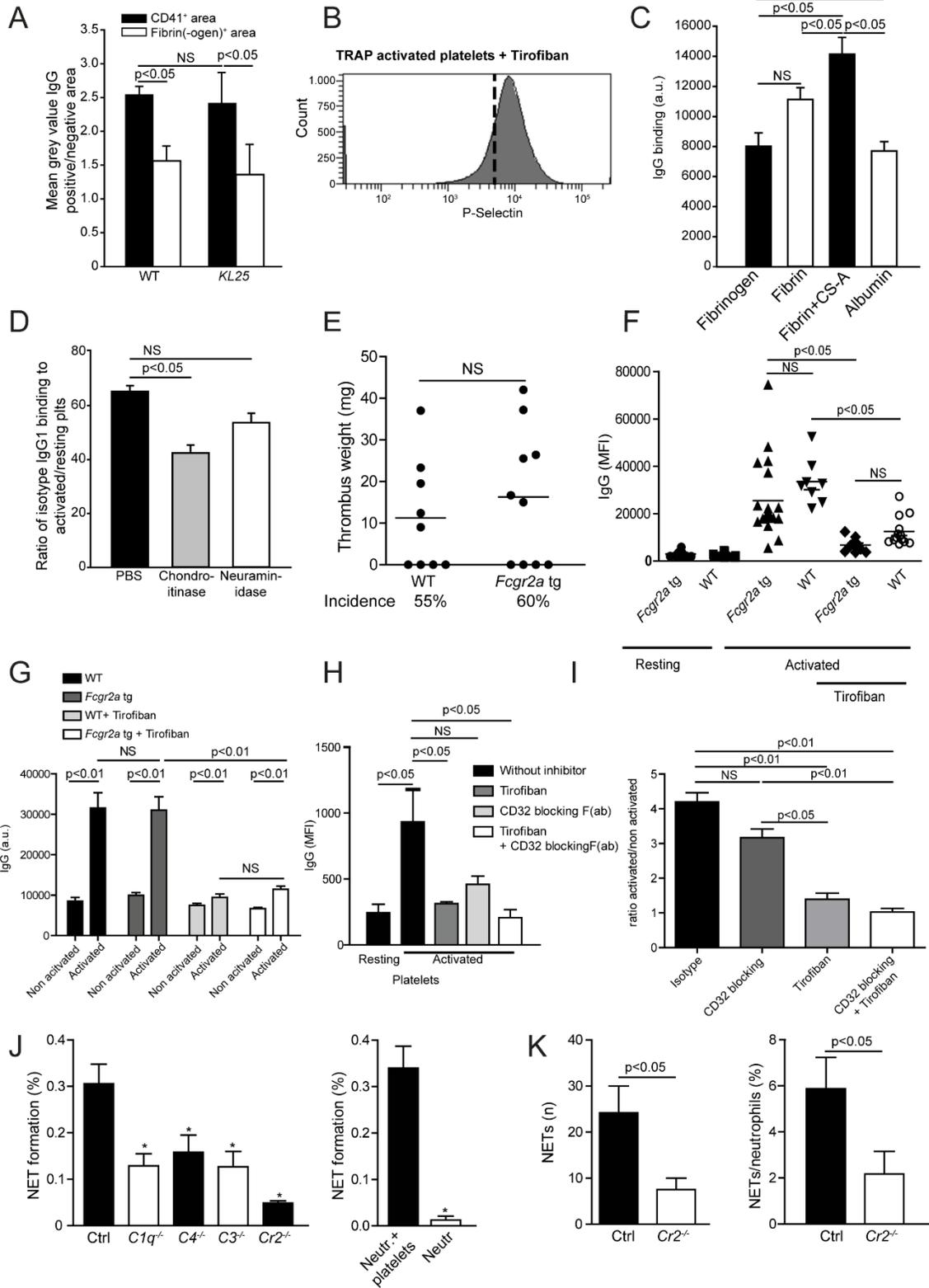


Figure S4.

Platelets provide a surface for antibody deposition, related to Figure 3 and 4. (A) Quantification of IgG mean gray value in CD41⁺ and fibrin(-ogen)⁺ areas from immunofluorescence staining of cross sections of the IVC 48 hours after flow reduction in ctrl mice (WT) and *KL25* mice (n=4 each) compared by two-tailed unpaired two-sample t-test. (B) FACS analysis of P-selectin exposure in PAR4 agonist (TRAP) activated washed platelets incubated with tirofiban. Representative of n=4 experiments. (C) Quantification of fluorescence intensity of isotype IgG1 deposition on surfaces covered with fibrin, fibrinogen, chondroitin sulfate-A, or albumin (n=3 each) compared by one-way ANOVA followed by LSD-post hoc-test. (D) Ratio of isotype IgG1 binding to PAR4 agonist activated compared to resting washed platelets from C57Bl6 mice assessed by FACS after incubation with PBS, chondroitinase, or neuraminidase (n= 5 each) analysed by one-way ANOVA followed by LSD-post hoc-test. (E) Thrombus weight and incidence in the inferior vena cava after stenosis in *hFcgr2a* mice expressing human *Fcgr2a* in platelets (n=10) compared to wildtype littermate controls (n=9) compared by two-tailed unpaired two-sample t-test or Fisher exact test. (F) Flow cytometry of FITC labeled IgG isotype antibodies on resting or PAR4 agonist stimulated platelets from *hFcgr2a* transgenic vs. wildtype (wt) mice with or without tirofiban (GPIIB/IIIa blocker). (n=8-10 independent experiments each). (G) ELISA of FITC labeled IgG isotype antibodies with resting or PAR4 agonist stimulated platelets from *Fcgr2a* transgenic mice vs. FcγRII wildtype (wt) mice with or without tirofiban (GPIIB/IIIa blocker) (n=6-8 independent experiments each). (H) Flow cytometry of FITC labeled IgG isotype antibodies on resting or PAR4 stimulated human platelets treated with tirofiban (GPIIB/IIIa blocker) and/or FcγRII (CD32) blocking F(ab) fragment (n=12-16 independent experiments each). (I) ELISA of FITC labeled IgG isotype antibodies on resting or PAR4 stimulated human platelets treated with tirofiban (GPIIB/IIIa blocker) or FcγRII (CD32) blocking F(ab) fragment (n=8 independent experiments each). (E-I) Data compared by one-way ANOVA followed by Tukey's Multiple Comparison Test. (J) Left: *In vitro* NET formation assay with TRAP activated platelets from C57Bl6 (ctrl, n=8); C1q^{-/-} (n=5), C4^{-/-} (n=6), C3^{-/-} mice (n=11) with C57Bl6 neutrophils or *Cr2*^{-/-} neutrophils and TRAP activated platelets (n=4). Data compared by one-way ANOVA followed by Tukey's Multiple Comparison Test. Right: *In vitro* NET formation assay with TRAP activated WT platelets and neutrophils or WT neutrophils only (n=4) compared by two-tailed unpaired two-sample t-test. * P<0.05. (K) NET formation in immunofluorescence staining of cross sections of the IVC 48 hours after flow reduction of *Cr2*^{-/-} compared to ctrl mice (n=4 each) given as total number of NETs (left) and NETs/neutrophil (right) compared by two-tailed unpaired two-sample t-test. (A, C, D to G to K) Data given as mean ± s.e.m.

Supplementary Figure 5

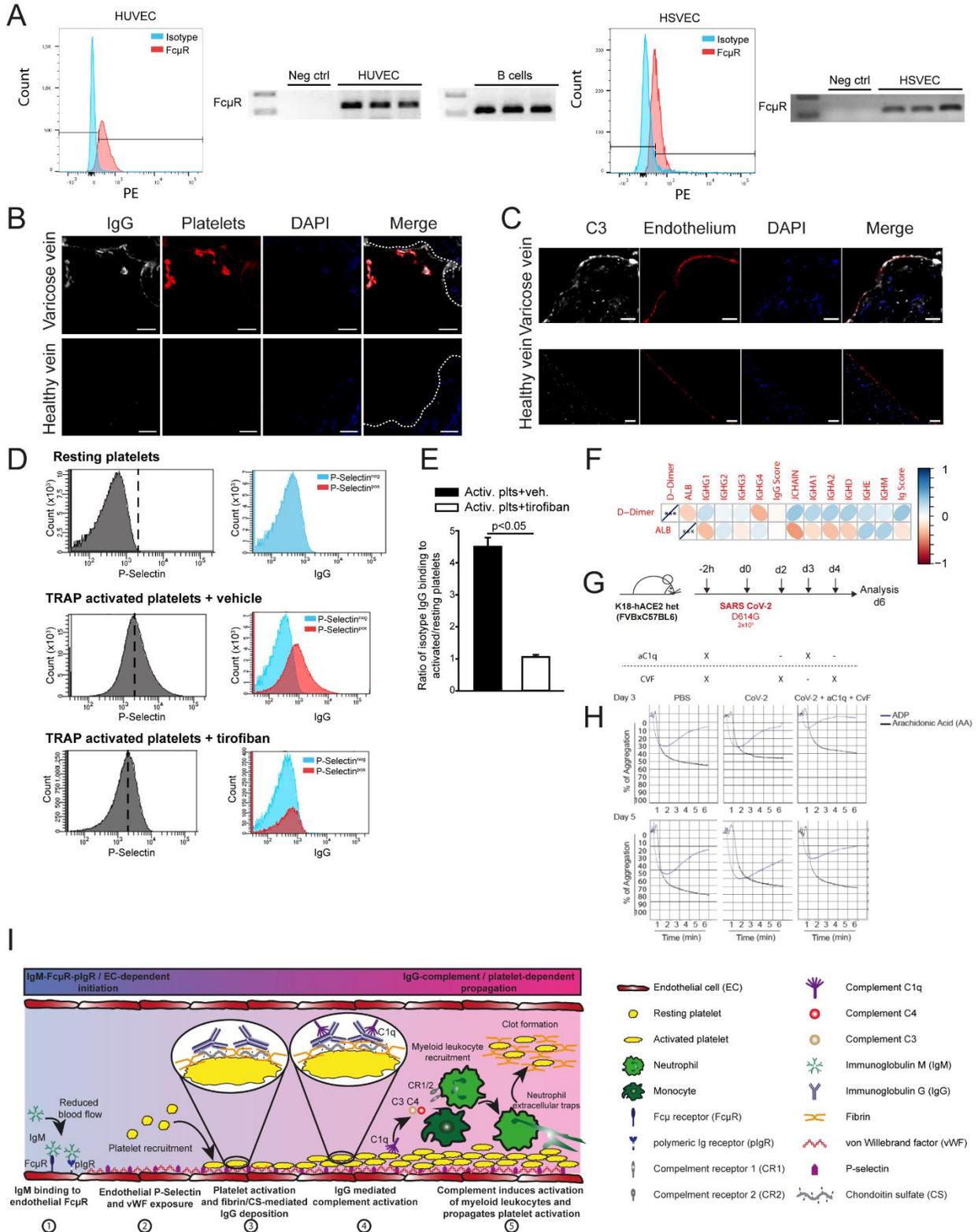


Figure S5.

Antibody-platelet interactions in humans, related to Figure 5. (A) Representative FACS analysis (from n=5 each) and rtPCR gels (from n=5 each) for Fc μ R from human umbilical vein endothelial cells (HUVECs) and B cells as positive control (left) or human saphenous vein endothelial cells (HSVEC). (B-C) Representative immunofluorescence staining of human varicose veins (top) and healthy veins (bottom) from n=4 independent specimens each for: (B) IgG (white) and platelets (CD42b, red). (C) C3 (white) and endothelium (ICAM-1, red); Dotted line indicated endothelium, DAPI in blue. Bar 50 μ m. Representative of n=8 each. (D) FACS analysis of P-selectin exposure and isotype IgG binding to resting or PAR4 agonist (TRAP) activated washed human P-selectin⁺ (red) or P-selectin⁻ platelets (blue) incubated with PBS (vehicle) or tirofiban. Representative of n=4 experiments. (E) Quantification of isotype IgG binding to resting or PAR4 agonist (TRAP) activated human platelets incubated with PBS (vehicle, n=13) or tirofiban (n=9) compared by two-tailed unpaired two-sample t-test. Data given as mean \pm s.e.m. (F) Proteome analysis for the correlation of D-Dimer and albumin (as control) with antibody subclasses given as individual components or pooled subclasses (IgG score as IGHG1-G4; Ig score as all subclasses). Positive correlations are shown in blue and negative correlations in red. Color intensity and the size of the ellipse are proportional to the correlation coefficient. (G) Experimental setup for complement depletion (with a C1q blocking antibody and cobra venom factor (CVF)) and SARS-CoV-2 infection in K18-hACE2het mice. (H) Representative aggregometry experiments (of n=3 experiments) in non-infected (PBS), SARS-CoV-2 infected and control (isotype antibody + PBS) or C1q blocking and CVF treated mice at day three (top) and five (bottom) after infection. (I) Reduction of venous blood flow enables IgM binding to Fc μ R and pIgR on endothelial cells, triggering the release of P-Selectin and vWF, initiating recruitment of platelets and leukocytes. Platelets amplify thrombus formation beyond the endothelial lining by surface absorption of IgG mediated by fibrin and chondroitin-sulfate initiating classical complement pathway activation. This fosters platelet activation by C1q and C3, recruitment of neutrophils through C3/C4 and CR1/2, and triggers NET release yielding occlusive thrombus formation.

Supplementary Table 1

List of mouse strains used in this article, related to STAR methods.

Mouse Strain	Description	Reference
<i>Aicda</i> ^{-/-}	lack all class-switched Ab (including IgG) / overproduce IgM	1
<i>Aicda</i> ^{-/-} <i>Ighm</i> ^{tm1Ch}	lack all secreted antibodies	2
<i>Alb</i> ^{-/-}	albumin deficient mice	3
<i>B10.D2-Hc1 H2d H2-T18c/nSnJ</i>	C5 sufficient control group for <i>C5</i> ^{-/-} mice	4
<i>C1q</i> ^{-/-}	lack complement component 1q	5
<i>C3</i> ^{-/-}	lack complement component 3	6
<i>C3ar1</i> ^{-/-}	lack complement component 3a receptor	7
<i>C4</i> ^{-/-}	lack complement component 4	8
<i>C5</i> ^{-/-}	lack complement component 5	4
<i>C5ar1</i> ^{-/-}	lack complement component 5a receptor	9
<i>Itgam</i> ^{-/-}	CD11b (Integrin alpha M (ITGAM)) deficient mice, lack complement receptor 3	10
<i>Itgax</i> ^{-/-}	CD11c (Integrin alpha X) deficient mice, lack complement receptor 4	11
<i>Cr2</i> ^{-/-}	lack binding of both C3- and C4-derived opsonin through deficiency of complement receptors 1 and 2	12
<i>Cμ13</i>	express IgM that cannot bind C1q to activate the classical complement pathway	13
<i>Cfb</i> ^{-/-}	Factor B (component of the alternative complement pathway) deficient mice	14
<i>Fcgr</i> ^{-/-} <i>Fcgr2b</i> ^{-/-}	lack all known IgG-specific Fcγ receptors	15,16
<i>Fcμr</i> ^{-/-}	lack Fcμ receptor (IgM receptor)	17
<i>hFcgr2a</i>	express human FcγRIIA on platelets and macrophages	18
<i>KL25</i>	knock-in mice: all their antibodies are specific for the foreign, nonmurine, lymphocytic choriomeningitis virus (LCMV) glycoprotein	19
<i>K18-hACE2</i>	expresses hACE2 predominantly in epithelial cells under the control of the cytokeratin 18 (KRT18) promoter	20
<i>Mbl1/2</i> ^{-/-}	Mannose-binding lectin (MBL) (component of the complement lectin pathway) deficient mice	21
<i>Pf4Cre/Rosa-Yfp</i>	Reporter mice expressing YFP under the control of the PF4 promoter in platelets	22
<i>Rag1</i> ^{-/-}	B cell deficient mice	23
<i>Ighm</i> ^{tm1Ch}	lack secreted IgM antibody	24

<i>VeCdhCrexFcmr^{f/f}</i> <i>f^l</i>	lack FcμR (Toso) receptor specifically in vascular endothelial cells	25,26
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