Supplement Methods

Preparation of human platelets, platelet releasate and plasma

Blood was drawn from healthy volunteers devoid of any medication for at least 10 days using a 21 G needle from the antecubital vein and anticoagulated with citrate or CTAD (BD vacutainer). Platelet-rich plasma (PRP) was generated from citrated blood by centrifugation for 20 min at 120 g. PRP was centrifuged again for 90 sec at 3000 g in the presence of 0.1 µg/ml PGI_2 to obtain platelet-poor plasma and platelet pellets which were resuspended in PBS before incubation for 10 min with protein G dynabeads (150 µg/ml; Life technologies) to scavenge residual IgG. To generate platelet releasate (PLTR), washed platelets were centrifuged for 3 min at 13,000 g and the supernatant was re-centrifuged (3 min, 13,000 g) to clear cellular debris. Platelet-free plasma was obtained by centrifuging platelet-poor plasma for 2 min at 3,000 g, followed by 10 min at 10,000 g. In order to obtain plasma directly from whole blood, CTAD-anticoagulated blood was centrifuged for 10 min at 2,000 g and the supernatant re-centrifuged for 20 min at 2000 g to clear cellular debris. For comparison of PLTR and plasma IgG levels, washed platelets were resuspended in the initial PRP volume, e.g. platelets of 1 ml PRP in 1 ml PBS.

IgG depletion and isolation from human platelet releasate

Human PLTR was depleted of IgG using Nab Protein G Spin Kit (Thermo Scientific) according to manufacturer's instructions. IgG were recovered by eluting the columns.

Preparation of murine platelets, platelet releasate and plasma

Blood was drawn from isoflurane-anesthetized mice via the retroorbital plexus using heparinized microhaematocrit capillaries and immediately anticoagulated with ACD (1:10) and heparin (25 U/ml). Washed platelets were isolated as previously published[1]. Briefly, whole blood was diluted with Tyrode's HEPES buffer and sequentially centrifuged (1x 5 min at 200 g, 2x 6 min at 100 g). Platelets were pelleted (90 sec at 1,000 g) in the presence of 1/25 ACD, 0.5 U/ml Apyrase and 0.1 µg/ml PGI_2 and resuspended in Tyrode's HEPES buffer before incubation for 10 min with protein G dynabeads (150 µg/ml) to scavenge residual IgG. For transfusion experiments platelets of multiple mice were pooled, pelleted and resuspended in saline before removing residual IgG with protein G dynabeads (350 µg/ml, 10 min) and labelling platelets with anti-GPIbβ (X488, 1:200; Emfret Analytics). To generate PLTR isolated platelets were centrifuged for 3 min at 13,000 g and the supernatant was re-centrifuged (1 min, 13,000 g) to clear cellular debris. For plasma preparation, whole blood was sequentially centrifuged for 5 min at 3,000 g and 1 min at 13,000 g. Supernatant plasma was spun again

for 1 min at 13,000 g to clear any cellular debris. For comparison of PLTR and plasma IgG levels, isolated platelets were resuspended in ½ volume of original whole blood.

In vitro virus neutralisation

Human umbilical vein endothelial cells (HUVEC) were infected for 5 h with cytomegalovirus (CMV; clinical isolate VR1814) before incubation with washed platelets, PLTR or plasma (30 min). Viral infection was quantified 72 h after infection via qPCR or immunofluorescence (IF). For a murine setup, Madin-Darby Canine Kidney (MDCK) cells were infected for 2 h with attenuated influenza A virus (IAV; strain A/PR/8/34 H1N1) and cultured for 4 h before incubation with washed platelets, PLTR or plasma (30 min). Viral infection was also quantified at 72 h after infection via qPCR. Optionally, platelets or PLTR was treated for 20 min with 2.5 µM ticagrelor (Sequoia Research Products), 1 mM aspirin (Sigma), 100 µM compstatin (MedChemExpress), 10 µg/ml anti-CD62P blocking antibody (human: clone AK4, BioLegend; mouse: clone RB40.34, BD Biosciences) or vector/isotype before incubation with infected cells. MDCK cells and attenuated IAV were a kind gift of Niklas Arnberg, Umeå University.

In vitro virus neutralisation under flow

HUVEC were seeded into ibiTreat µ-Slide VI$^{0.4}$ channel slides (ibidi) and infected for 5 h with cytomegalovirus (CMV) before perfusion with washed platelets or plasma (10 min) under capillary shear stress (50 dynes/cm²). The number of infected cells was quantified 72 h after infection by IF.

Quantitative PCR (qPCR)

Cells were harvested in TriFast reagent (PeqGold, VWR International GmbH), RNA isolated and transcribed into cDNA (High-Capacity cDNA Reverse Transcription kit) according to the manufacturers' instructions and gene expression levels determined using SybrGreen reagent and StepOne Real-Time PCR System (all Applied Biosystems). HPRT was used as housekeeping gene to analyse expression levels using ΔC_T values. Data were further normalized to infected control cells as indicated. Primers used: hHPRT fwd: TGACACTGGCAAAACAATGCA, rev: GGTCCTTTTCACCAGCAAGCT; CMV immediate early (IE) fwd: AGATGTCCTGGCAGAACTCGTC, rev: TTCTATGCCGCACCATGTCCAC; mHPRT fwd: CGCAGTCCCAGCGTCGTG, rev: CCATCTCCTTCATGACATCTCGAG; IAV hemagglutinin (HA) fwd: TTGCTAAAACCCGGAGACAC, rev: CCTGACGTATTTTGGGCACT; mIRF5 fwd: CAGGTGAACAGCTGCCAGTA, rev: TCCCTGTCTCTTTAGCCCAG; mIFNγ fwd: TGAGCTCATTGAATGCTTGG, rev: ACAGCAAGGCGAAAAAGGAT.

Immunofluorescence microscopy

HUVEC were fixed for 10 min in 1 % formaldehyde, permeabilised with ice-cold methanol (10 min), blocked for 1 h (Protein Block Serum-Free, Dako) and stained for 2 h with anti-CD61-AlexaFluor647 (1:100, Biolegend) and anti-IE-AlexaFluor488 (1:400, Millipore). Slides were sealed with VECTASHIELD DAPI mounting medium (Vector laboratories). All antibodies were diluted in PBS containing 0.3 % triton and 0.03 % BSA and slides were washed three times with PBS in-between individual incubation steps.

Excised murine lungs were fixed in 1 % formaldehyde (1 h), incubated overnight in 30% sucrose and re-incubated overnight in 50 % OCT compound (Tissue-Tek) and 15 % sucrose. Lungs were embedded in OCT compound and frozen at -80°C. Sections (8 µm) were permeabilised with 0.5 % TritonX-100 (30 min), blocked with 1.5 % BSA and 10 % FCS (30 min) and stained overnight with anti-CD41-AlexaFluor488 and anti-CD144-AlexaFluor647 (both 1:50, Biolegend). Tissues were counterstained with Hoechst 33342 (5 µg/ml, 10 min; Invitrogen) and mounted with ProLong Gold antifade reagent (Invitrogen). Images were taken with Nikon A1 plus confocal laser-scanning microscope at indicated magnifications and evaluated using ImageJ 1.52p.

Flow cytometry

Human PRP or isolated murine platelets were fixed in 1 % formaldehyde (15 min). For detection of surface IgG fixed platelets were washed in PBS (5 min, 600 g), stained for 30 min with anti-IgG-AlexaFluor647 (1:50, Biolegend) and washed again. For intracellular staining, platelets were washed in PBS and in permeabilisation buffer (Invitrogen), stained for 30 min with anti-IgG-AlexaFluor647 (1:50) and washed again in permeabilisation buffer and PBS. Cells were measured on a CytoflexS cytometer and analysed by CytExpert 2.3 software (Beckman Coulter).

ELISA

Commercially available kits were used to quantify CXCL4 (Mouse CXCL4/PF4 DuoSet ELISA, R&D Systems) or total IgG (Human/Mouse IgG total ready-set-GO, eBioscience) in plasma and PLTR according to the manufacturers' protocols. For detection of anti-hemagglutinin (HA) antibodies an in-house ELISA was established. Briefly, plates were coated with 100 ng/ml HA (Influenza A/PR/8/34 H1N1; Sino Biological Inc.) in PBS (2 h), washed with PBS containing 0.1 % Tween (PBST) and coated with 2 % bovine serum albumin (BSA) in PBST (2 h). Plates were washed again and incubated for 2 h with samples diluted in sample buffer (PBST containing 1 % BSA). As relative standard plasma of an IAV-immunized and boosted mouse was used. Plates were washed, incubated with anti-IgG-HRP (1:2,000 in sample buffer, 2 h), washed and signals were detected using TMB substrate (eBioscience). Absorbance at 450 nm

was measured on a plate reader (Tecan) and analysed using 4-parameter logistic standard curve fit.

Mice

Wildtype C57BL/6J mice and mice with inducible diphtheria toxin (DT) receptor (iDTR) in the megakaryocyte lineage (stop$^{fl/fl}$-iDTR PF4 iCre$^{+/-}$, referred to as iDTR^{PLT}) were bred at the Centre of Physiology, Medical University of Vienna, under specific-pathogen-free conditions with 12 h light / 12 h dark cycle. Experiments were performed with 8-20 week-old littermates.

Anaesthesia

Mice were anesthetised by isoflurane (Forane; Abott Laboratories Ltd.) or by intraperitoneally injecting a mixture of ketamine (50 µg/g, Ketaminol; Intervet International) and xylazine (5 µg/g, Xylasol; Dr. E. Gräub AG). Animals were euthanized by isoflurane overdose.

Anti-platelet drug treatment

Wildtype mice were injected intraperitoneally with 2.5 µg/g body weight of ticagrelor (Sequuoia Research Products) or vector control (1.6 % DMSO, 8.4 % propylene glycol). Blood was drawn retroorbitally after 1.5 h.

Ex vivo platelet activation

Platelets were isolated from ticagrelor- or control-treated mice as described above and adjusted to equal cell density. Platelets were stimulated with IAV (1x10^3 egg-infectious dose (EID)/µl) or convulxin (CVX; 220 ng/ml) for 15 min in the presence of protein G dynabeads (600 µg/ml). Beads were separated from platelets by magnetic force and bound IgG eluted using elution and neutralization buffer of Nab Protein G Spin Kit as described in the manufacturer's instructions (Thermo Fisher). Platelets were centrifuged for 2 min at 1,000 g in the presence of 1 µg/ml PGI_2 and the supernatant PLTR cleared of debris as described above.

Generation of seropositive donor mice

Naïve wildtype mice of both sexes were infected with IAV (1x10^4 EID in 25 µl sterile saline) intranasally (i.n.). Seropositivity of mice was confirmed by ELISA. Donor mice were boosted with 100 EID IAV i.n. 7 days before isolation of platelets or plasma.

Measurement of platelet count in murine blood

Whole blood was taken retroorbitally using heparinised microhaematocrit capillaries and anticoagulated with EDTA. Whole blood was stained for 20 min with anti-CD41-FITC (1:200, Biolegend), fixed in 1 % formaldehyde and measured on a CytoflexS cytometer. Platelet count

per µl blood was calculated by measuring CD41-positive events in a fixed volume of cell suspension.

In vivo virus neutralisation

Female iDTR^{PLT} mice were injected subcutaneously 3x per week with DT (week 1: 100 ng; week 2: 250 ng), resulting in >90 % platelet depletion within 5 days[2]. Thrombocytopenic recipient mice (6 days DT) were transfused with 5.9x10^8 platelets of seronegative or seropositive donor mice before intranasal infection with mouse-adapted 1x10^4 EID IAV (A/PR/8/34 H1N1) in 25 µl sterile saline. Platelet transfusion was repeated after 4 days and organs were harvested after 7 days. The largest lobes were excised, fixed in formaldehyde and prepared for histologic examination. The other lobes were snap-frozen in liquid nitrogen and stored at -80 °C. A defined lung lobe was homogenized in TriFast reagent before preparation of RNA, cDNA and mRNA expression analysis via qPCR.

Histologic evaluation of lung pathology

Lungs were fixed in 4 % formaldehyde overnight, dehydrated and embedded in paraffin. Sections (3 µm) were stained by haematoxylin (Mayer's Haematoxylin solution, Sigma-Aldrich) and eosin (Eosin Y solution alcoholic, Sigma-Aldrich) according to standard protocol and evaluated by a pathologist blinded to the experimental setup for edema formation, alveolar haemorrhage and alveolar exudates. Scores were applied as 0=none, 1=mild, 2=moderate and 3=severe. For each mouse two sections were evaluated independently to obtain the mean score. Images were taken with TissueFaxs (TissueGnostics) using 20x and 40x objectives.

Statistical analysis

Statistical analyses were performed by GraphPad Prism Software 6.01. Bar graphs represent mean ± S.D., box plots span minimum to maximum with horizontal lines indicating the median. Data were tested for normal distribution by D'Agostino & Person omnibus test and further evaluated accordingly, using paired analyses where applicable. Fold-control expression data were analysed by one-sample t-test/Wilcoxon Signed Rank test. Differences between two treatment datasets were analysed by (un-)paired t-test with Welch's correction for normally distributed data or by Mann-Whitney test/Wilcoxon matched-pairs signed rank test. Effects of inhibitors relative to untreated platelets were evaluated by one-way ANOVA and weight curves were analysed by two-way ANOVA. Biological replicates, representing number of mice or donors, are indicated in the figure legends as n. A p-value $p<0.05$ was considered significant. Different p-values $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ are indicated as *, **, *** and ****, respectively, or as $^{\#}$, $^{\#\#}$, $^{\#\#\#}$ and $^{\#\#\#\#}$ when referring to fold-control analyses.

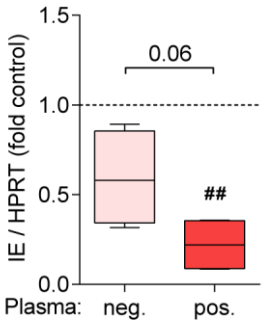
Study approval

All experiments and animal studies were conducted according to institutional guidelines. The Animal Care and Use Committee of the Medical University of Vienna and the Austrian Ministry of Sciences approved all performed animal experiments (BMWFW-66.009/0337-WF/V/3b/2016). All volunteers signed informed consent in accordance with approval of the Human Ethics Committee of the Medical University of Vienna (EK237/2004) and the Declaration of Helsinki.

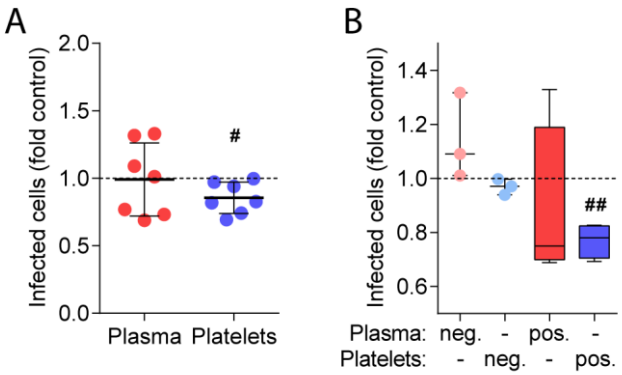
REFERENCES

1. Kral-Pointner JB, Schrottmaier WC, Salzmann M, et al. Platelet PI3K Modulates Innate Leukocyte Extravasation during Acid-Induced Acute Lung Inflammation. *Thromb Haemost.* 2019;19(10):1642-1654.
2. Salzmann M, Schrottmaier WC, Kral-Pointner JB, et al. Genetic platelet depletion is superior in platelet transfusion compared to current models. *Haematologica.* 2020;105(6):1738-1749.

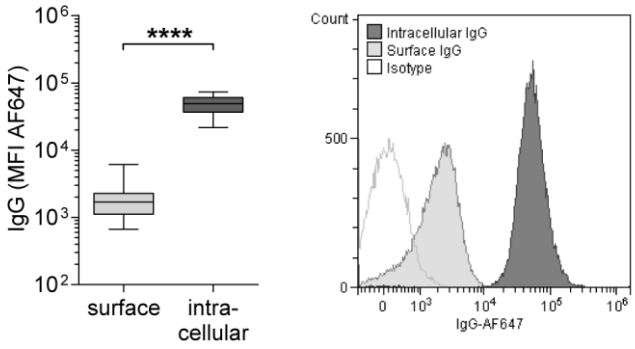
Supplement Figures



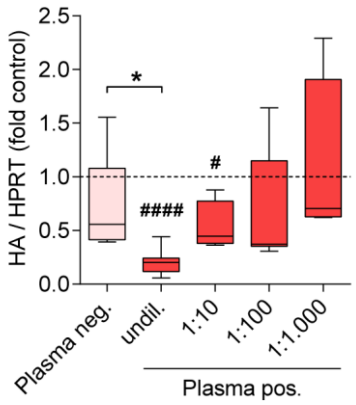
Supplement Figure 1: Seropositive plasma diminished endothelial CMV infection upon static incubation. HUVEC were infected with CMV (5 h) and incubated with buffer (control) or plasma from anti-CMV IgG seronegative (neg.) or seropositive (pos.) donors for 30 min. Viral load was quantified 72 h after infection via qPCR by determining relative CMV immediate early (IE) expression. n=4. $^{\#\#}p<0.01$ relative to control.



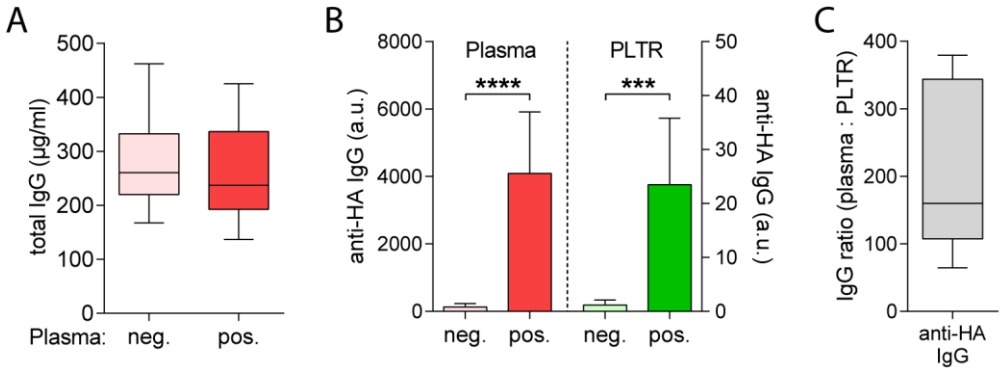
Supplement Figure 2: Under capillary shear stress seropositive platelets reduce endothelial CMV infection more potently than plasma. (A) HUVEC were infected with CMV (5 h) and perfused with buffer (control), plasma or washed platelets under capillary shear stress (10 min, 50 dynes/cm²). The relative number of infected cells was quantified 72 h after infection by immunofluorescence. n=7. (B) Donors of (A) were stratified according to their anti-CMV IgG serostatus in seronegative (neg.) and seropositive (pos.). n=3-4. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ relative to control.



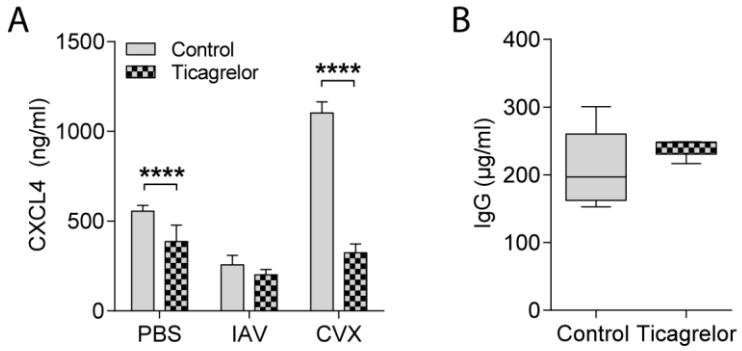
Supplement Figure 3: Murine platelets contain IgG mostly intracellularly. Surface and intracellular IgG levels of murine platelets were determined via flow cytometry. Quantification and representative histogram plot given. n=20. ****p<0.0001 between indicated groups.



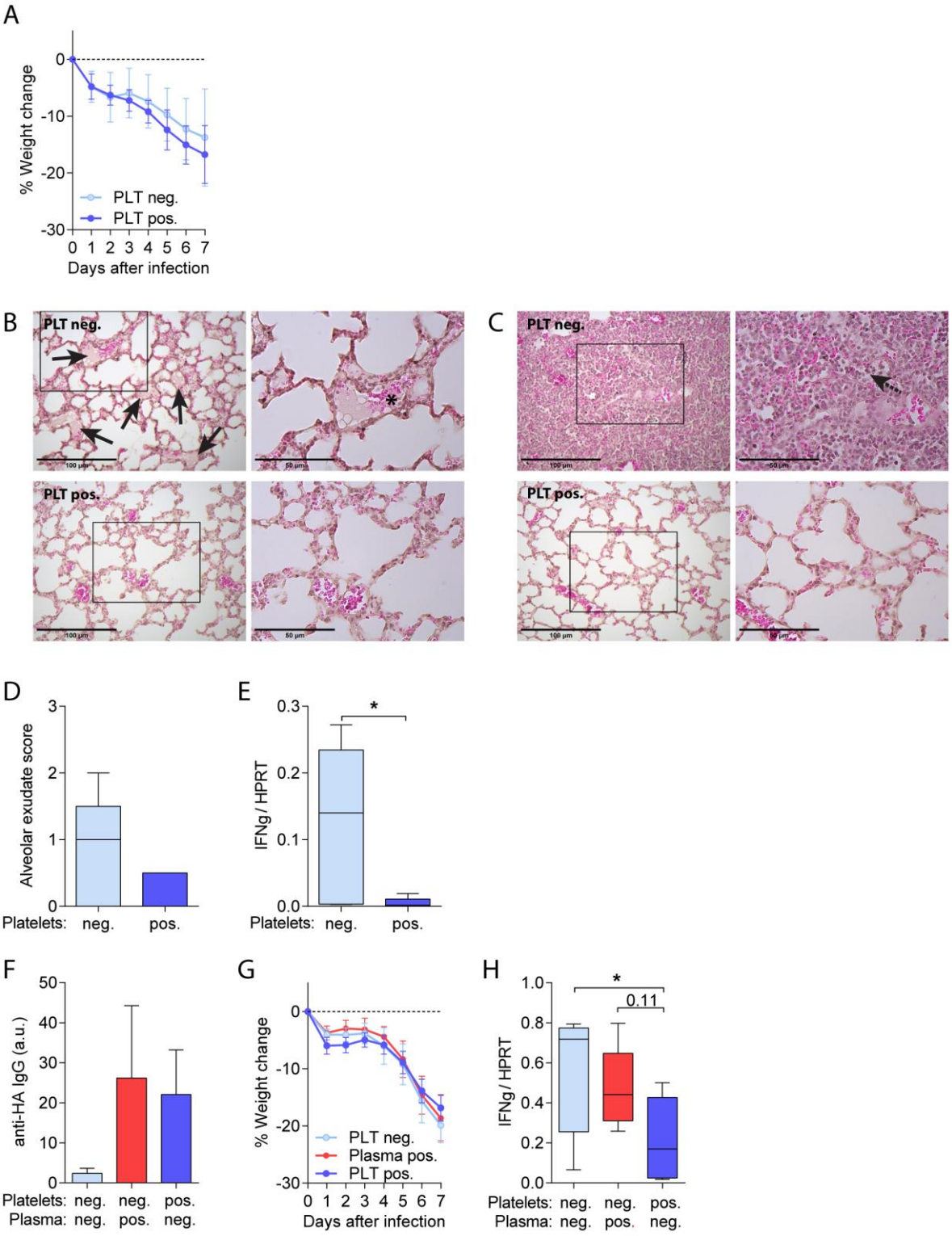
Supplement Figure 4: Murine seropositive plasma reduces IAV infection of MDCK cells in a concentration-depending way. MDCK cells were infected with IAV (2 h) and incubated with buffer (control) or plasma from anti-IAV IgG seronegative (neg.; undiluted) or seropositive (pos.) mice for 30 min. Viral load was quantified 72 h after infection via qPCR by determining relative IAV hemagglutinin (HA) expression. n=4-8. *p<0.05 between indicated groups, $^{\#}$p<0.05 and $^{\#\#\#\#}$p<0.0001 relative to control.



Supplement Figure 5: Characterization of IgG levels in transfusion donor mice and transfused platelet suspensions. (A) Total plasma IgG levels in naïve (seronegative; neg.) and IAV-immunized (seropositive; pos.) donor mice were examined by ELISA. n=10. (B) Anti-hemagglutinin (HA) IgG levels in plasma (left Y-axis) and platelet releasate (PLTR) (right Y-axis) of seronegative and seropositive donor mice were determined by ELISA. n=7-10 (C) Plasma-to-PLTR ratio of anti-HA IgG in seropositive donor mice. n=7-9. ***p<0.001 and ****p<0.0001 between indicated groups. a.u.: arbitrary units.



Supplement Figure 6: *In vivo* anti-platelet treatment abolishes release of α-granule derived proteins. (A) Platelets isolated from wildtype mice treated with ticagrelor (2.5 µg/g, 1.5 h) or control were stimulated for 15 min with IAV or convulxin (CVX) *ex vivo*. Platelets were pelleted and obtained supernatant examined for levels of CXCL4 by ELISA. (B) Total IgG plasma levels immediately prior to injection of ticagrelor or control were determined by ELISA. n=5. ****p<0.0001 between indicated groups.



Supplement Figure 7: Platelets ameliorate disease severity in viral infection depending on their serostatus. (A-E) Thrombocytopenic iDTR^{PLT} mice were transfused with seronegative or seropositive donor platelets in buffer, infected with IAV and **(A)** monitored for weight loss before evaluation of disease severity after 7 days. n=5. **(B-D)** Lung sections stained with haematoxylin and eosin were evaluated and scored for (B) oedema formation (arrows), alveolar haemorrhage (✱) and (C-D) alveolar exudation (dashed arrow).

(E) Interferon response in lung tissue was quantified by measuring relative mRNA expression of interferon-γ (IFNγ). **(F-H)** Thrombocytopenic iDTR^{PLT} mice were transfused with seronegative platelets in diluted seronegative plasma (PLT neg.), seronegative platelets in diluted seropositive plasma (Plasma pos.) or with seropositive platelets in diluted seronegative plasma (PLT pos.) and infected with IAV. (F) Transfused platelets in diluted plasma were pelleted as for PLTR and obtained supernatant examined for levels of anti-HA IgG by ELISA. n=2. **(G)** Weight loss and **(H)** pulmonary interferon response were assessed as in (A) and (E). n=5-6. *$p<0.05$ between indicated groups.