Supplementary Figures

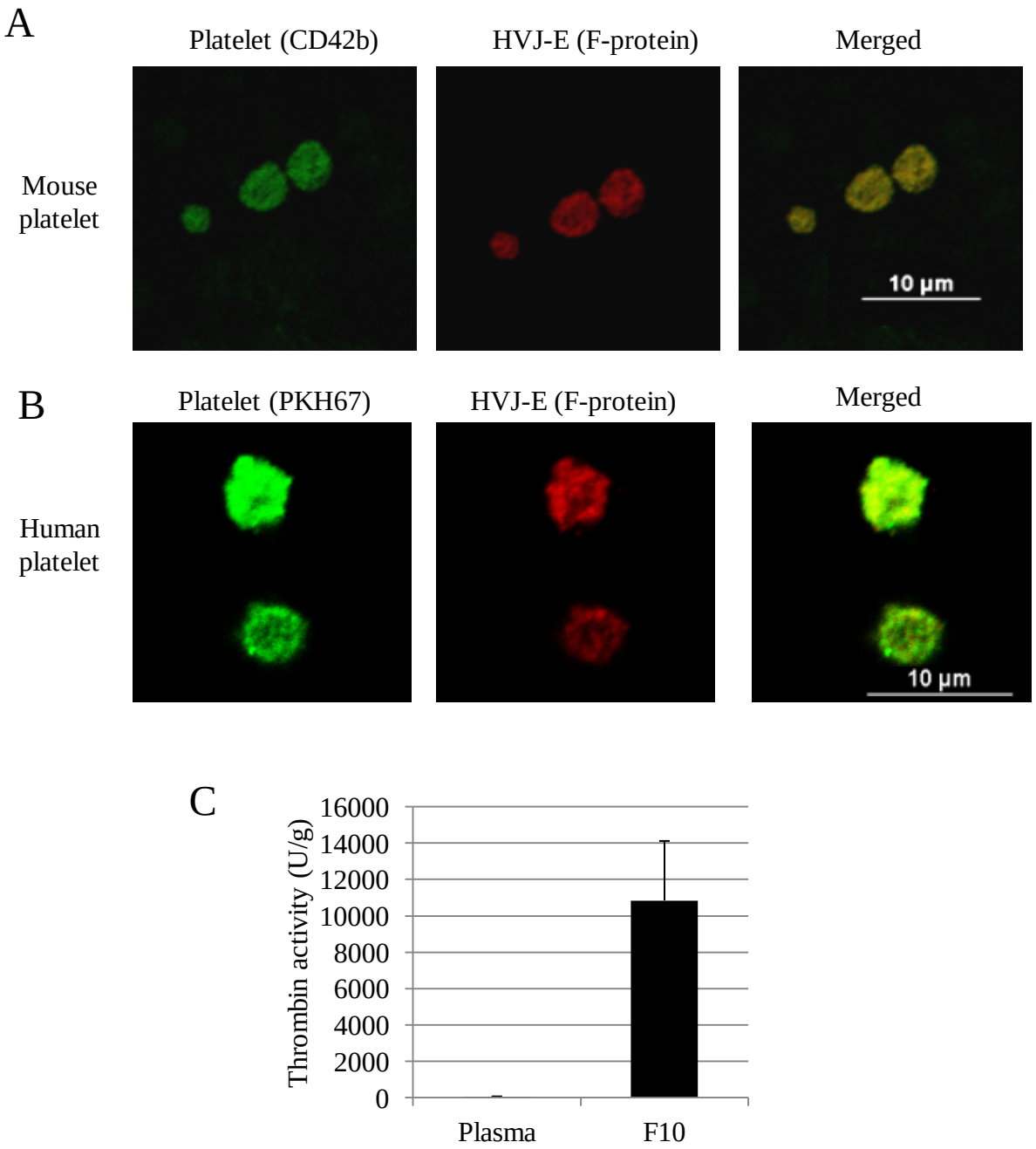


Fig. S1. Infused HVJ-E F protein (red) and platelets (green). The PH complexes were treated with Triton solution to detect HVJ-E inside the platelets. (A) Mouse platelet infusing HVJ-E particles. (B) Human platelet infusing HVJ-E particles. (C) Thrombin activity in B16F10 tumor and plasma from B16F10-bearing mouse.

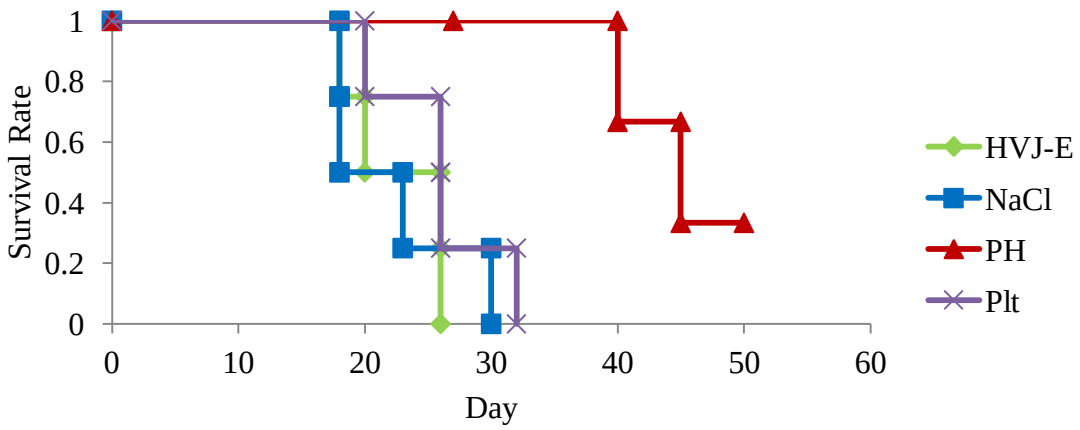


Fig. S2. Survival rate of Platelet/HVJ-E complex (PH), HVJ-E, Platelet (Plt) or NaCl solution (NaCl) treated B16F10-bearing mouse.

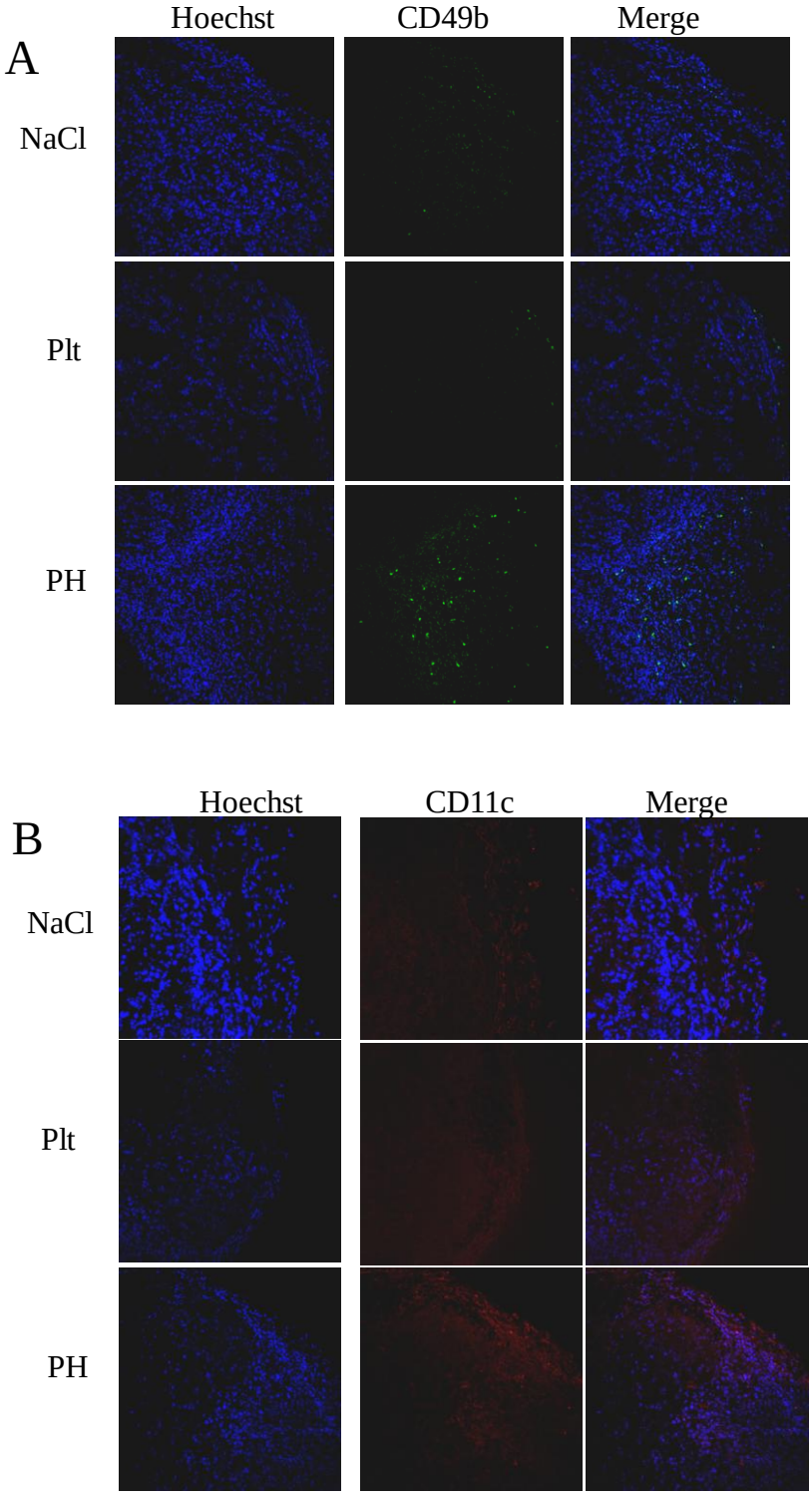


Fig. S3. (A) and (B) B16F10 tumor tissue sections from tumor-bearing mice treated with PH complexes, platelets or NaCl solution were stained with anti-CD49b (green) and anti-CD11c (red) antibodies to visualize the localization of NK cells and DCs, respectively, in the tumor tissues.

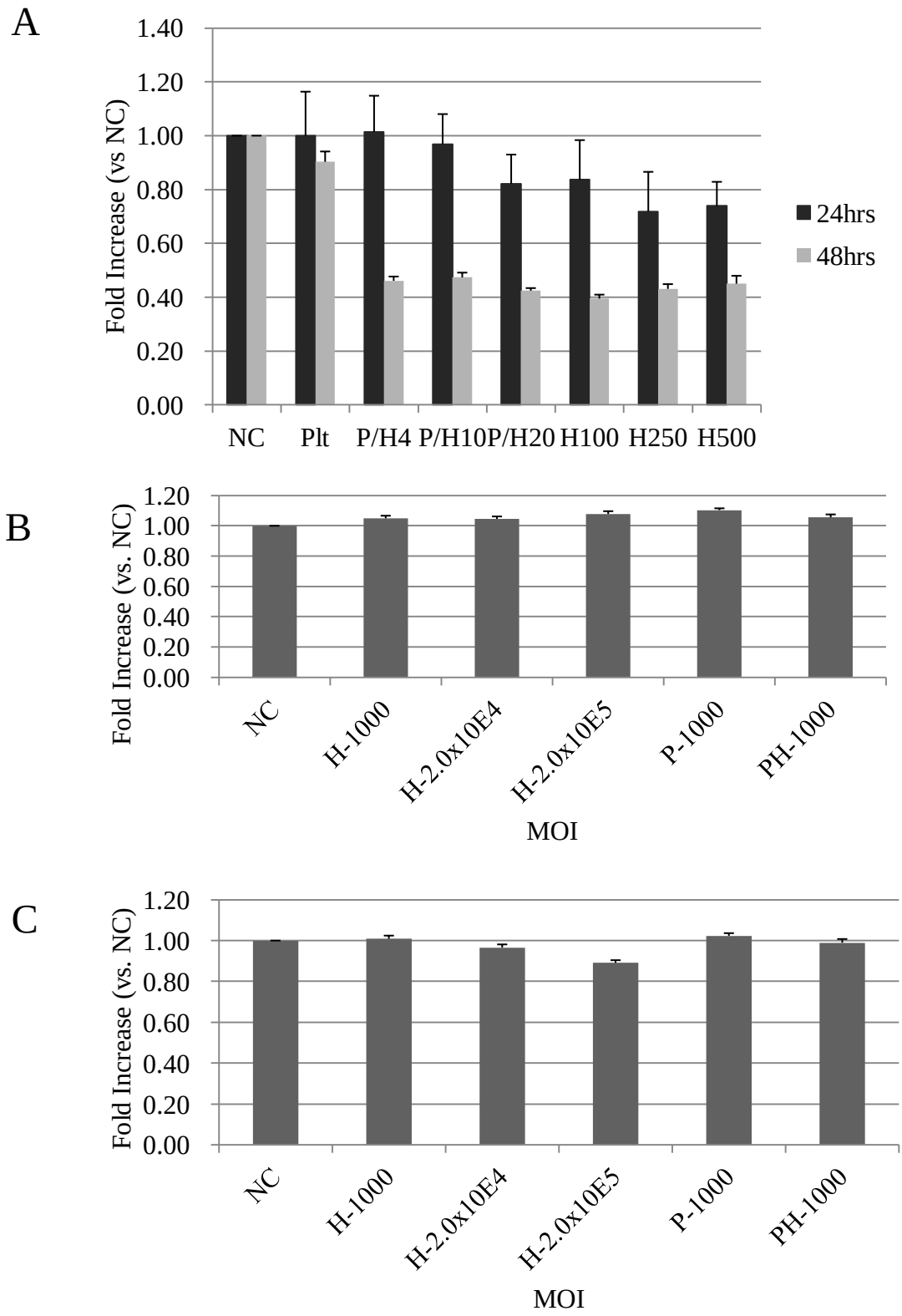


Fig. S4. Proliferation assay of PC3 and human noramal endothelial cells. Different concentrations of HVJ-E (from 4 to 500 HAU or 1,000 MOI) were infused into the same number of platelets (5.0 x 10^6 platelets) to construct various PH complexes. The PH complexes or different concentration of HVJ-Es were added to the cells and survival of cells were measured. 24 or 48 h after the treatment. The data are shown as the mean ± SE (n = 4). (n = 4). (A) PC3s. (B) HUVECs. (C) HAECs.

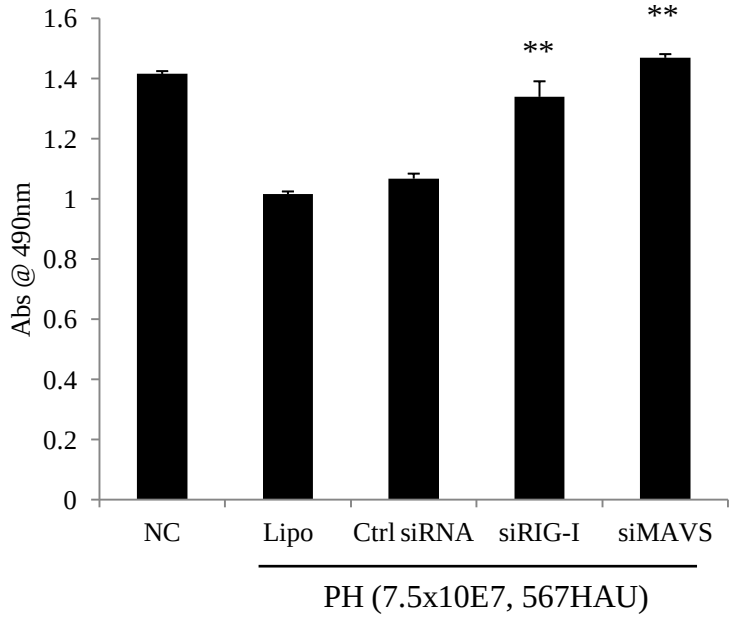


Fig. S5. Proliferation assay of PH complex + RIG-I siRNA (siRIG-I) or MAVS siRNA (siMAVS) transfected B16F10 cells. B16F10 cells were treated with Lipofectamine RNA iMax only (Lipo), control siRNA (Ctrl siRNA), RIG-I siRNA (siRIG-I) or MAVS siRNA (siMAVS), and PH complex ($7.5x10^7$ platelets infusing 567 HAU HVJ-E particles) was added to medium. The data are shown as the mean ± SE (n = 4). **P < 0.01, vs Lipo and Ctrl siRNA.

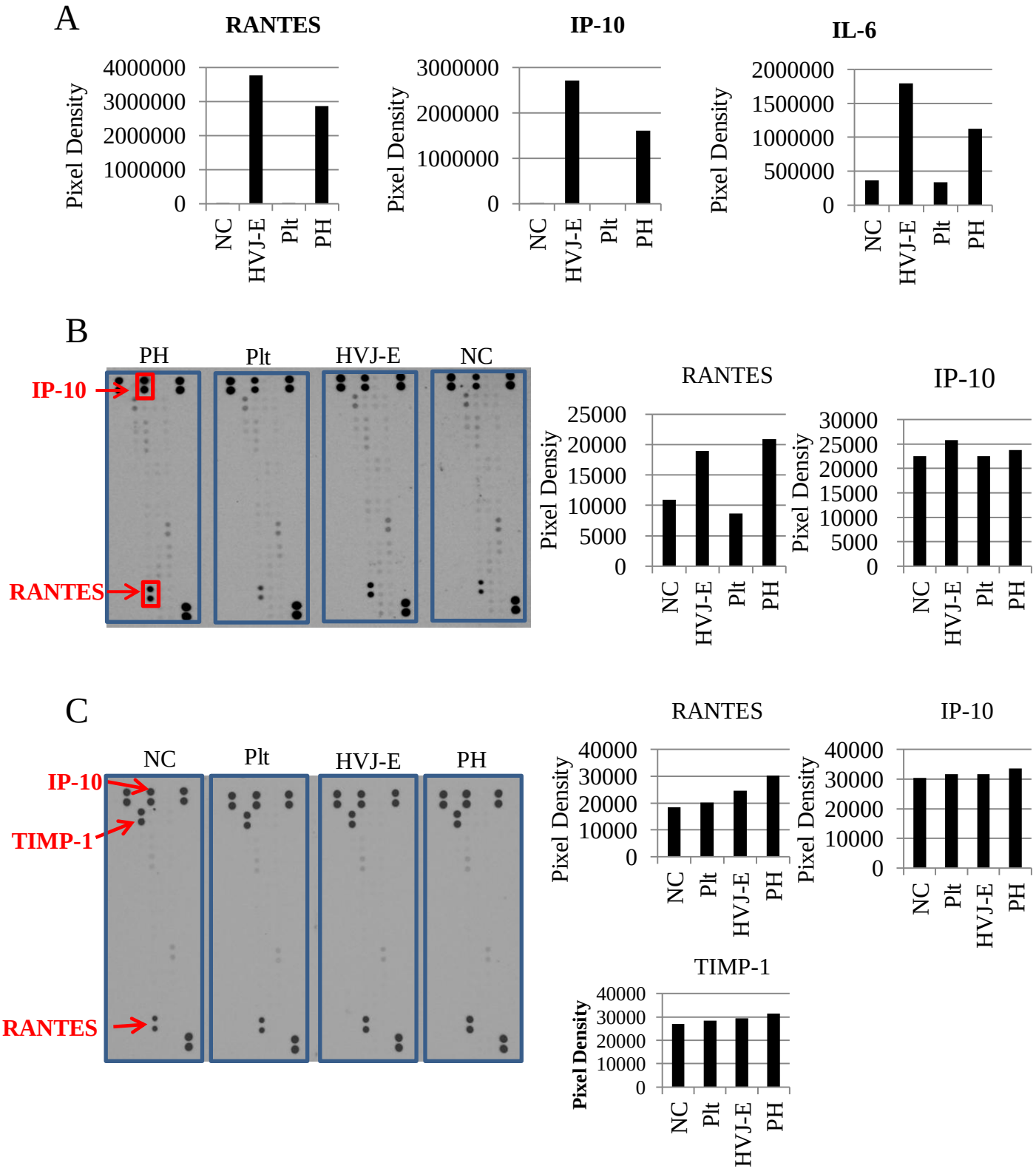


Fig. S6. Cemokine and cytokine array of PH stimulated cells. (A)Human Cytokine and Chemokine Array of PH complex stimulated HUVEC. Production of RANTES, IP-10 and IL-6 from HUVEC stimulated with PH complexes (PH), platelet (Plt) or HVJ-E were mesaured by Human Cytokine and Chemokine Array. (B) Mouse cytokine and chemokine array of stimulated TECs (isolated tumor endothelial cells). (C) Mouse chemokine and cytokine array of stimulated B16F10 cells.

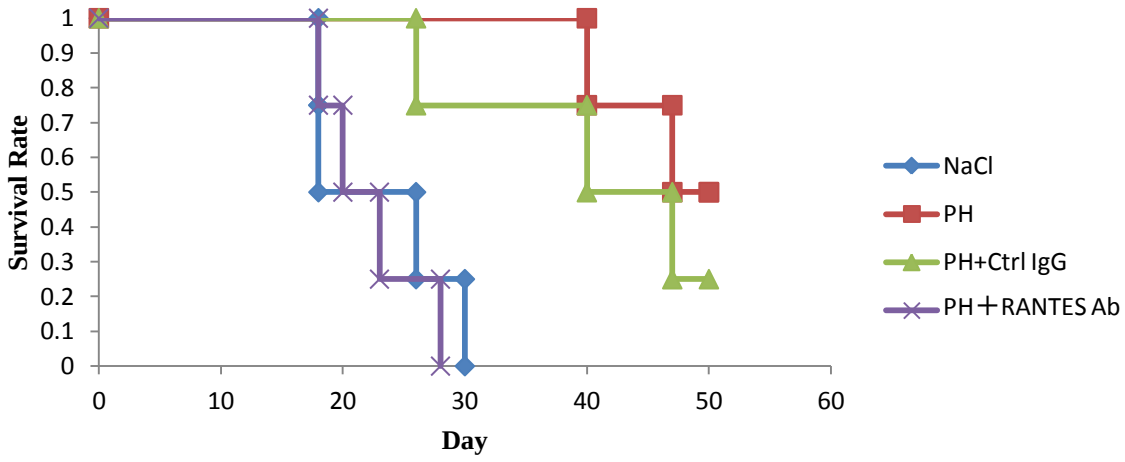


Fig. S7. Survival rate of Platelet/HVJ-E complex (PH), HVJ-E, PH complex + rabbit control IgG (PH+Ctrl IgG), PH complex + anti-RANTES neutralizing antibody (PH+RANTES ab) or NaCl solution (NaCl) treated B16F10-bearing mouse.

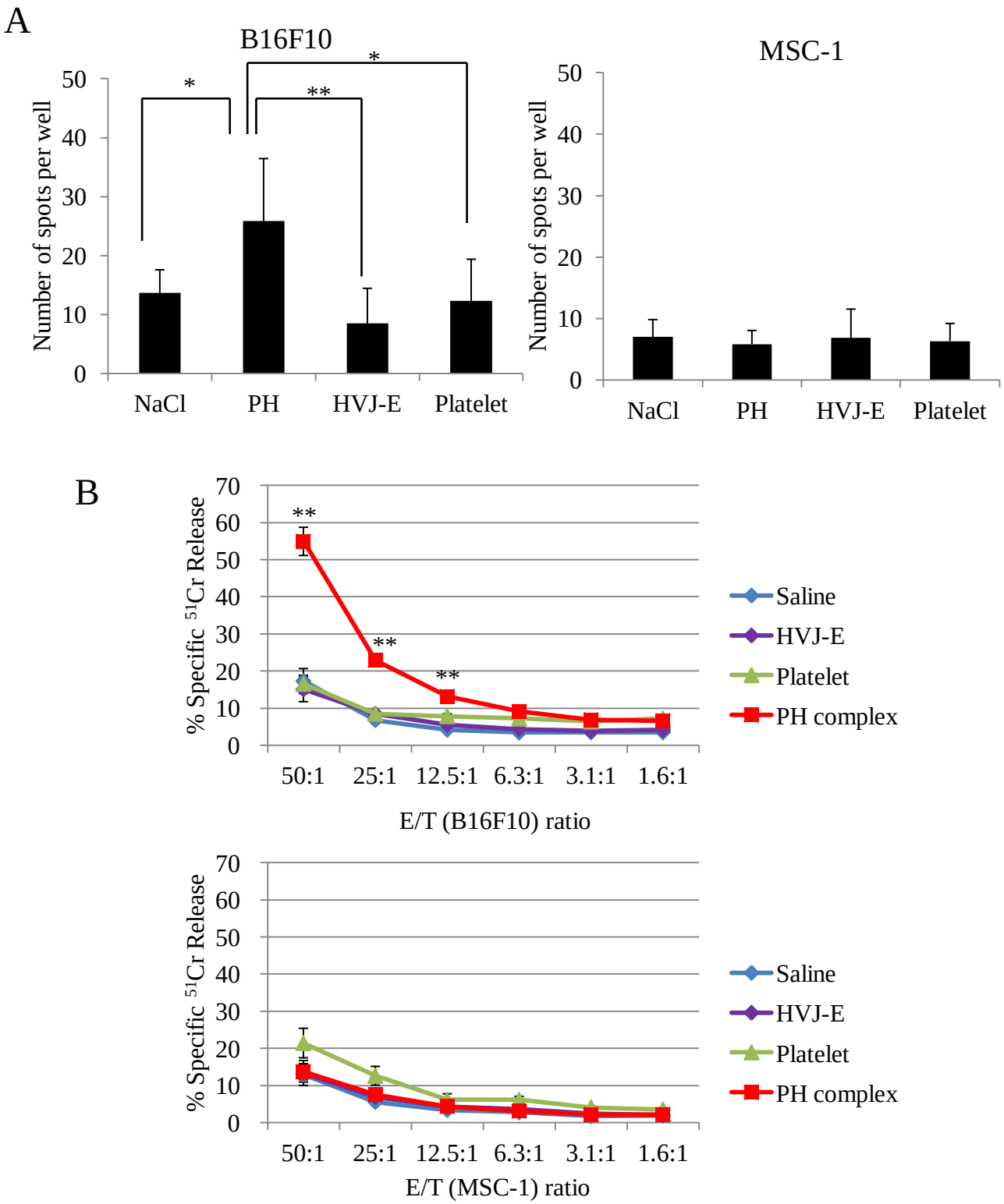
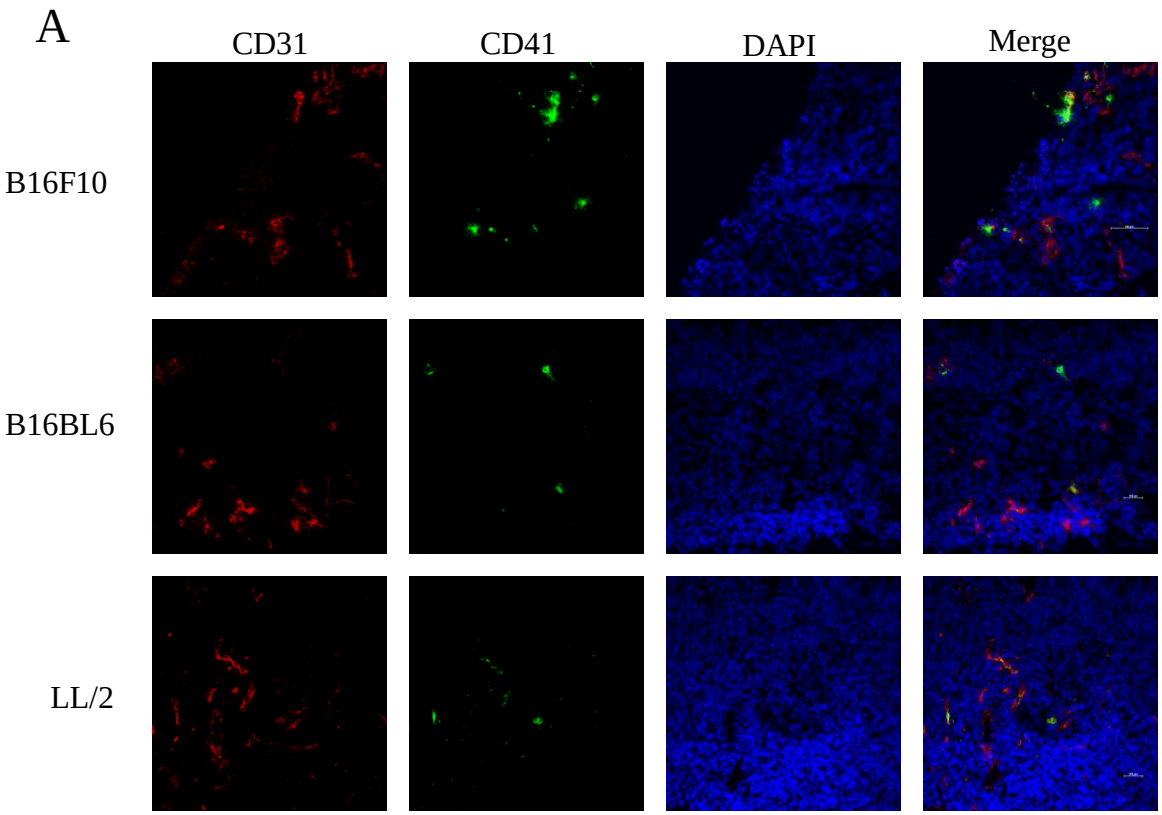


Fig. S8. Specific antitumor effects induced by PH complex injection to B16F10-bearing mouse. (A) B16F10 specific IFN-γ secretion from splenocytes was measured by ELISPOT assay. The isolated splenocytes were stimulated with B16F10 or MSC-1. The data are shown as the mean ± SE (n = 4). *, $P < 0.05$, **, $P < 0.01$. (B) CTL activity against B16F10s was measured by ^{51}Cr-release assay. B16F10 or MSC-1 cells were used as target cells. The data are shown as the mean ± SE (n = 14). **$P < 0.01$, vs Saline, HVJ-E and Platelet.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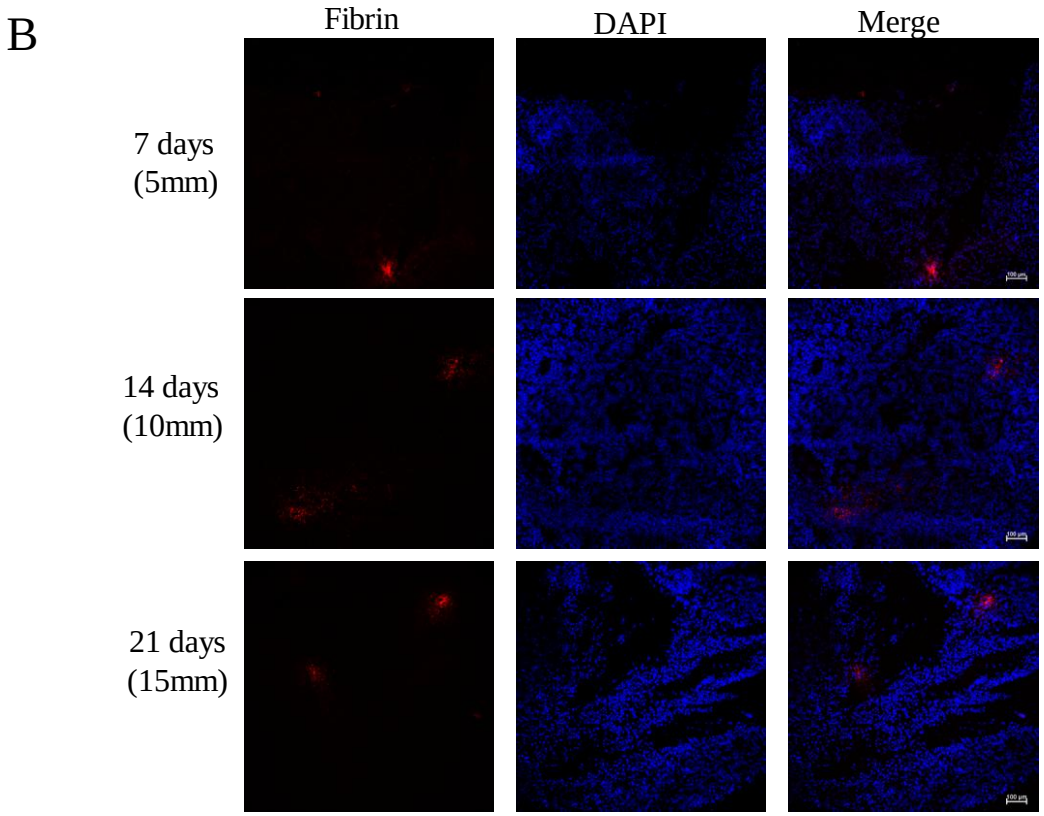
CD31
CD41
DAPI
Merge
B16F10
B16BL6
LL/2


B
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7 days
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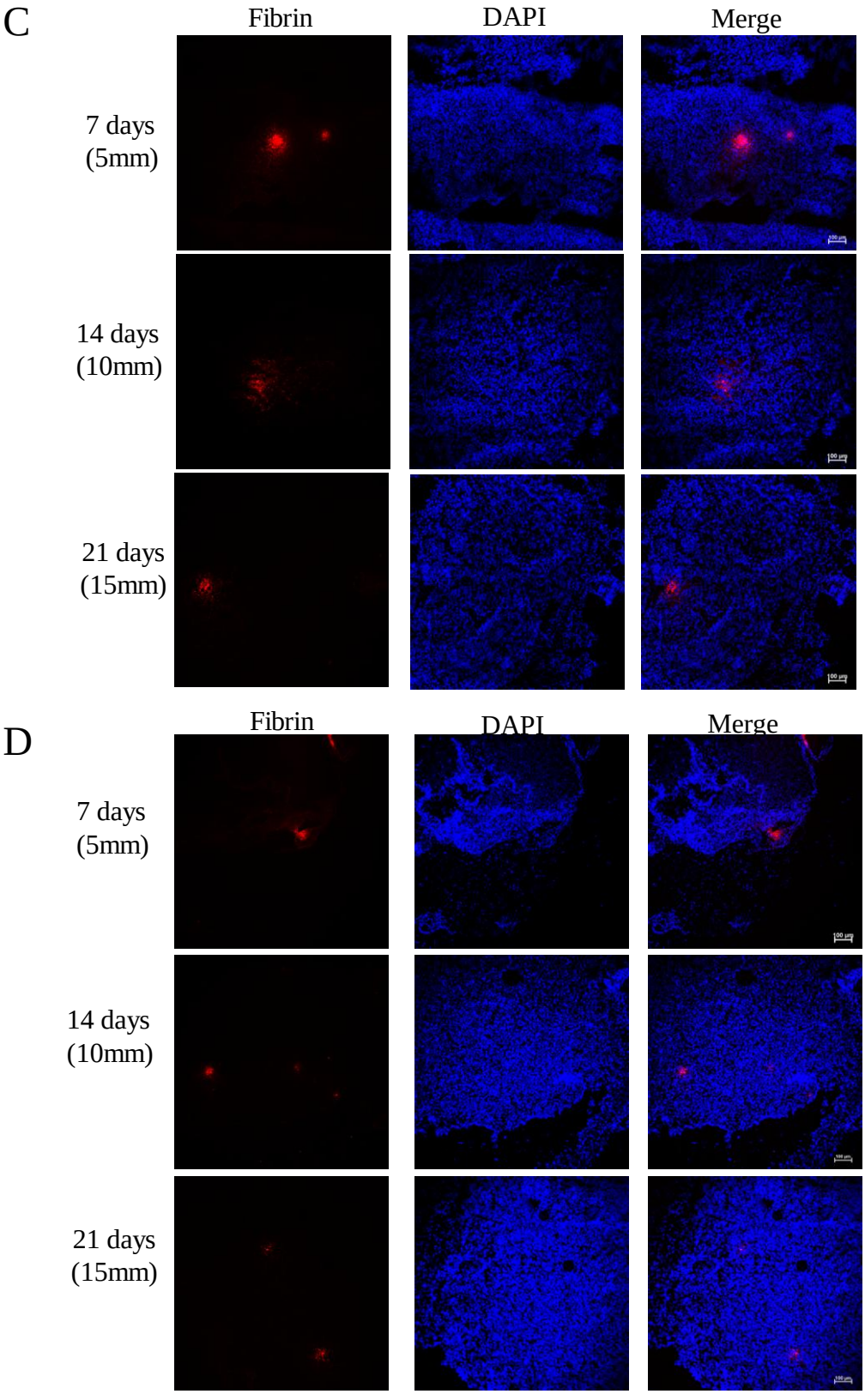

Fig. S9. Tumor microenvironment in various cancer cell lines inoculated C57BL/6N mouse. (A) Tumor blood vessels and thrombus were stained with anti-CD31 antibody (red) and anti-CD41 antibody, respectively, in mouse melanoma tumors (B16F10 and B16BL6) and LL/2 (mouse Lewis lung carcinoma). (B) Threre different time points and sizes (7, 14 and 21 days after cancer cells inoculation) of tumor sections were stained with anti-fibrin antibody to observe fibrin clot loclization in B16F10, (C) B16BL6 or (D) LL/2 tumor tissues.

Supplementary Materials and Methods

Hemagglutination (HA) assay

PH complexes were serially diluted in PBS in a 96-well U-bottom plate. A 50-μl aliquot of a suspension of chicken red blood cells (0.5% RBCs) (Nacalai Tesque Inc., Kyoto Japan) was then added to each well, and the samples were incubated at 4° C for 16 hours. The agglutination titer was determined as the last dilution at which agglutination was detected.

Cell culture and proliferation assay

Human prostate cancer (PC-3) cell line was purchased from the American Type Culture Collection (ATCC). maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque Inc., Kyoto Japan) cells were maintained in RPMI-1640 medium (Nacalai Tesque Inc.), with 10% FBS (Biowest), 100 U/mL penicillin, and 100 μg/mL streptomycin (penicillin–streptomycin mixed solution; Nacalai Tesque Inc.). Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were purchased from KURABO Industries Ltd. (Osaka, Japan) and were maintained in HuMedia-EG2 supplemented with the HuMedia-EG growth factor set (KE6150; KURABO). The cells were incubated at 37° C in a humidified atmosphere of 95% air and 5% CO_2. PC3s, HUVECs and HAECs (1.0×10^3 cells/well) were seeded into 96-well plates, and HVJ-E or PH complexes were added to the wells. After 24 or 48 hours of incubation, 20 ml of Cell Titer 96 AQueous One Solution (Promega, Madison, WI) was added to each well to measure the viability of the cancer cells. The plates were incubated at 37° C for 2 hours, and the absorbance was measured at a wavelength of 490 nm according to the manufacturer's instructions.

Thrombin activation in B16F10 tumor tissue and plasama from B16F10-bearing mice

B16F10 tumor were excised from mice after blood withdrawal, weighed and homogenized with the supernatant extracted. Dilutions of samples were prepared, including thrombin standards, and assayed for thrombin activity according to manufacturer's protocol (SensoLyte 520 Thrombin Activity Assay Kit, AnaSpec). In brief, 50ul of samples were equilibrated to room temperature and mixed with 50ul substrate in 1x assay buffer on a 96-well plate. Plate was incubated at room temperature in dark for 45 minutes and read for fluorescent signal. Specific thrombin activity was obtained by converting signals to thrombin units per standards and normalized by weights.

Immunostaining of immune cells in B16F10 tumor tissues, and thrombi and fibrin in tumor tissues

The PH complex-treated and other B16F10, B16BL6 and LL/2 tumor sections were fixed with 4% paraformaldehyde solution and blocked with 3% BSA. The B16F10 sections were stained with an anti-CD11c primary antibody (Sigma-Aldrich Japan Co., Tokyo Japan). The secondary antibodies included an Alexa Fluor 488-conjugated rabbit anti-mouse IgG (Life Technologies Corporation, Carlsbad, CA) and a CD49b FITC-conjugated antibody (BioLegend, San Diego, CA). For tumor vessels, thrombus and fibrin staining of B16F10, B16BL6 and LL/2 tumor sections, anti-CD31(ab28364, abcam, Tokyo, Japan) anti-41 (BioLegend, San Diego, CA) and anti-FGA (Abnova, Taipei, Taiwan) antibody were used respectively. The stained sections were mounted in VECTASHIELD mounting medium (Vector Laboratories, Inc., Burlingame, CA) and imaged with a confocal laser microscope (Radiance 2100; Bio-Rad Laboratories, Inc., Drive Hercules, CA) equipped with the Laser Sharp 2000 software program.

Cytokine and chemokine arrays of HUVEC

HUVECs, TECs or B16F10s were seeded into 6-well plates (1.5 x 10^6 cells/well), and platelets (2.5 x 10^5), HVJ-E (1.89 x 10^9 particles), or PH complexes (1.89 x 10^9 particles) were added to the medium. After 24 hours, the media were collected and used as samples for human or mouse cytokine and chemokine arrays (Proteome Profiler Panel A Array Kit; RD systems, Minneapolis, MN). The pixel densities of the spots on the array membranes were quantified using Image Quant TL software (GE Healthcare Japan, Tokyo, Japan).

RIG-I and MAVS siRNA transfection to cells

SiRNAs (MAVS; Sigma-Aldrich Japan, Inc., RIG-I; Invitrogen) were transferred to cells using and Lipofectamine RNAiMAX (Invitrogen). The siRNA concentration was 20 pmol/well (12 well plate), and the sequences were as follows:

MAVS : 5′-GAAUGCCUCUCCUGUUGCATT
5′-UGCAACAGGAGAGGCAUUCTT

RIG-I : 5′-UAAGGUUGUUCACAAGAAUCUGUGG,
5′-CCACAGAUUCUUGUGAACAACCUUA

siRNAs were tranfected 24 hours before PH complexes (7.5x10E7 platelets infusing 567 HAU HVJ-E particles) were added to the medium of B16F10 cells. MTS assay was performed 48 hours after addition of PH complexes.

^{51}Cr-release CTL assays and ELISpot assays

PH complexes, HVJ-E, platelets or NaCl solution was injected systemically via the tail vein in the mice bearing B16F10 mealnoma. The spleens were isolated from the mice 2 days after the last injection was administered. F10 melanoma cells were treated with mitomycin C (15 mg/mL) for 45 minutes. For the ^{51}Cr release CTL assays, theisolated splenocytes and mitomycin C-treated F10 cells were mixed at a 10:1 ratio and cultured with culture medium that included10 ng/mL recombinant mouse IL-2 (R&D Systems). Three days later, culture medium containing 5 ng/mL recombinant mouse IL-2 was added to the cultured cells, and the cells were cultured for another 3 days. Nonadherent splenocytes were collected, and serial 2-fold dilutions of splenocytes (50, 25, 12.5, 6.3, 3.1, and 1.6 x 2.0 x 10^3/100 μL/well) were made in 96-well plates. As positive and negative controls, 1% NP-40 and culture medium were added to the wells, respectively. The F10 melanoma cells were treated with the chromium-51 radionuclide (^{51}Cr; PerkinElmer Japan Co., Ltd.; 1.85 MBq/250 mL) for 45 minutes at 37C and washed 3 times with RPMI-1640. A 51Cr-labeled BF10 or MSC-1 cells suspension (2 x 10^3 cells/100 μL) was added to each well of the 96-well plate, which contained a 2-fold dilution of splenocytes, and the cells were incubated for 4 hours at 37C. The supernatant of each well was collected after incubation, and the amount of ^{51}Cr-released from the labeled F10 melanoma cells was determined using γ-scintillation counting.

For the ELISpot assay, the splenocytes and mitomycin-C treated B16F10 or MSC-1 cells were mixed at a ratio of 10:1. Forty-eight hours later, nonadherent splenocytes were collected, and the ELISpot assay was conducted using the Mouse IFN-g Development Module (R&D Systems) and the ELISpot Blue Color Module (R&D Systems). The number of IFN-γ–secreting cells was subsequently counted.