Supplemental Information for

Human podoplanin-positive monocytes and platelets enhance lymphangiogenesis through the activation of the podoplanin/CLEC-2 axis

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Supplementary Methods

Fluorescent Activated Cell Sorter (FACS) analysis

PBMCs and hematospheres were treated with Accutase (StemCell Technologies) for 3 min at 37°C to dissociate into single cells and washed with PBS. Cells with FACS buffer (PBS containing 1% FBS and 0.1% BSA) were stained with antibodies specific for cell surface markers and analyzed by flow cytometry using a BD Canto II ^{TM} (BD Biosciences) and were sorted using a BD Aria (BD Biosciences). Antibodies used were; Podoplanin-PE (eBioscience) and Podoplanin-APC (Biolegend) CD14-FITC (eBioscience). The appropriate isotype antibodies served as negative controls.

Western blot

Cells were lysed in lysis buffer (Cell signaling) containing protease inhibitors (Roche). Total protein (20μg) was immunoblotted with primary antibodies against podoplanin (Sigma), VEGFR-3 (Sigma), total Akt, phospho Akt, total Erk and phospho Erk (Cell signaling) followed by incubation with appropriate HRP-labeled second-step Abs. Beta-actin (Santa Cruz Biotechnology) was used as an internal control.

Immunofluorescence staining

Cultured monocytes and platelets were fixed in 2% cold paraformaldehyde for 10 min on ice. After washed with PBS, cells blocked with 1% BSA and 0.3% Triton X-100 were stained with Podoplanin (Sigma), VEGFR-3 (Sigma), Phalloidin (Sigma), IL-1b (Santa Cruz Biotechnology), CLEC-2 (R&D), and CD41 (eBioscience) primary antibodies, followed by incubation with secondary antibodies. DAPI was used for nuclear staining. For immunohistochemistry, samples frozen in OCT embedding

medium (Sakura) or blocked in paraffin were sectioned and stained with antibodies. Primary antibodies used were as followed: rabbit anti-mouse LYVE-1 (AngioBio), anti-human VEGFR-3 (Sigma) and anti-human HLA-ABC (Millipore).

Gene expression analysis

Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instruction. mRNA was reverse transcribed with a Primescript 1st strand cDNA synthesis kit (TAKARA) and oligo-dT primer. Semi-quantative PCR was performed with Maxime PCR Pre-Mix (Intron) according to manufacturer's instructions and Real-time PCR was performed with Sybr Green I Mastermix (Roche) using an ABI PRISM TM 7500 Sequence Detection System (Applied Biosystems). Experiments were conducted to contrast relative levels of each transcript and endogenous control GAPDH in every sample. Information of corresponding primers is provided in Supplementary Table S2.

Supplementary Figure Legends

Supplementary Figure S1. Flow cytometry analysis of hematospheres cultured for 5 days in various culture media. Hematospheres cultured in StemSpan or mTeSR have more podoplanin-positive cells than those cultured in EBM 5%.

Supplementary Figure S2. Flow cytometry analysis of hematospheres using different clones of podoplanin antibodies. Hematospheres cultured for 5 days were stained with podoplanin NZ-1.3 or podoplanin NC-08 antibodies. Appropriate isotype antibodies were used in each analysis as negative control. Both figures show similar percentage of podoplanin-positive cells in hematospheres.

Supplementary Figure S3. (a) Flow cytometry analysis of fresh PBMCs and cultured hematospheres at day 3 and 5 was performed with podoplanin and VEGFR-3 antibody. Bar graph represented the percentage of VEGFR-3 in CD14 and podoplanin-positive cells. (**P<0.01; n=3) (b) Flow cytometry analysis of fresh PBMCs and cultured hematospheres at day 3 and 5 were performed with podoplanin and LYVE-1 antibody. Bar graph represented the percentage of LYVE-1 in CD14 and podoplanin-positive cells. (**P<0.01; n=3)

Supplementary Figure S4. (a) Fluorescence-associated cell-sorting strategy of day 5 cultured hematospheres. Two populations exist, $CD14^{+}/Pod^{+}$ and $CD14^{+}/Pod^{-}$ cells. Right panels show the purity of sorted cells. (b) Representative images of podoplanin-positive or podoplanin-negative sorted cells cultured in EBM 5% media. Scale bar= 200μm

Supplementary Figure S5. Flow cytometry-sorted $CD14^+$ Pod^+ cells and $CD14^+$ Pod^- cells from day 5 hematospheres were subjected to immunofluorescence staining for LYVE-1. Scale bar= 10μm.

Supplementary Figure S6. (a) Microscopic images of hematospheres or single-cells unincorporated into hematospheres (non-hematosphere) on suspension and attach culture condition. After attachment culture, hematospheres exhibited spindle-shape morphology. Single cells that were unincorporated into hematospheres remained in suspension after attachment and were washed away after media change. Scale bar= 200μm (b) Dissociated single cells from hematospheres were seeded on 1.5% gelatin coated dish and cultured for 24 hours. Attached cells at 24 hours were subjected to immunofluorescence staining for podoplanin, VEGFR-3 and LYVE-1. Scale bar= 20μm.

Supplementary Figure S7. (a) Phalloidin (green), polymerized actin, staining in freshly isolated platelets and platelets that have adhered and spread on immobilized fibrinogen (200μg/ml) in the presence of rhPodoplanin (20μg/ml) and thrombin (0.01U/ml) for 4 hours. Scale bar=10μm (b) Bar graph representing the average area of Platelet. (**P<0.01, *P<0.05; n=3)

Supplementary Figure S8. (a) Representative figure of Matrigel tube formation using hLEC in various conditioned media. hLEC showed most tube formation in the presence of the conditioned medium from co-culture of PPMs and platelet, while the formation significantly decreased when treated with neutralizing antibody against VEGF-A or HGF. Scale bar=200μm (b) Bar graph representing the number of the

hLEC branching point (**P<0.01, *P<0.05; n=3). (c) Bar graph representing the total tube length of the hLEC. (**P<0.01, *P<0.05; n=3)

Supplementary Figure S9. (a) Experimental scheme for FACS sorting with hematosphere using CD14 and podoplanin antibody and then co-culture of sorted cells and platelets. After 2 days culture, supernatants were harvested. (b) Enzyme-linked immunosorbent assay (ELISA) for lymphangiogenesis-related cytokines (VEGF-A, HGF, and PDFG-BB) in each conditioned medium.

Supplementary Figure S10. (a) Gross appearances of the wounds at the back of nude mice injected with the different cells at the indicated time points. Scale bar= 1mm. (b) Diagram of the kinetics of wound closure in each group. Four mice were analyzed at each time point. (c) Whole-mount immunostaining of ear skin stained with antibodies against LYVE-1. Scale bar= 100μm. (d) Bar graph represented the number of lymphatic vessels branching points. (**P<0.01, *P<0.05; n=4)

Supplementary Figure S11. (a) Confocal microscopy images of back skin from nude mice of indicated group. Scale bar= 100μm. Red fluorescence indicates CD31 and blue indicates DAPI. (b) Bar graph representing the quantification of blood vessel area in the back skin which was determined by scoring CD31 positive vessels. (**P<0.01, *P<0.05; n=4)

Supplementary Tables

Supplementary Table S1. Blood donor information

	Age	Sex	Mobilizing condition		Blood vol.	PBMNC ($x10^7$)	Hematosphere formation	in vivo experiment
			Hx.†	Sys/P.E.‡				
Donor 1	30	M	none	none	200	23.8	yes	o
Donor 2	25	M	none	none	200	21.9	yes	o
Donor 3	24	M	none	none	200	24.2	yes	o
Donor 4	28	M	none	none	200	26.3	yes	o
Donor 5	29	M	none	none	200	29.2	yes	o
Donor 6	29	M	none	none	200	25.4	yes	o
Donor 7	23	M	none	none	130	17.3	yes	-
Donor 8	23	M	none	none	150	18.5	yes	-
Donor 9	27	M	none	none	160	20.7	yes	-
Donor 10	29	M	none	none	130	18.3	yes	-
Donor 11	30	M	none	none	150	19.1	yes	-
Donor 12	28	M	none	none	150	21.4	yes	-
Donor 13	28	M	none	none	130	16.3	yes	-
Donor 14	29	M	none	none	150	20.2	yes	-
Donor 15	26	M	none	none	150	20.4	yes	-

† Excluded if one of the following is present on donor's medical history

1. Malignancy and/or related chemotherapy
2. Ischemic stroke or myocardial infarct
3. Unresolved infection or local inflammatory disease
4. Significant active CNS disease or seizures within the past year.
5. Blood donation within past 90 days
6. Leukapheresis or lymphopheresis within 180days
7. Prior medication medical treatment
    1) G-CSF or GM-CSF within the past 6 months
    2) Treatment for infection within the past 14 days
    3) Any kind of endoscopic procedure within the past 7 days
    4) Other medication which can possibly affect stem cell mobilization
8. Other medical conditions which can possibly affect stem cell mobilization
9. Other medical conditions which contraindicate blood donation.

‡ Excluded if one of the following sign is present on systemic review and physical examination of the donor

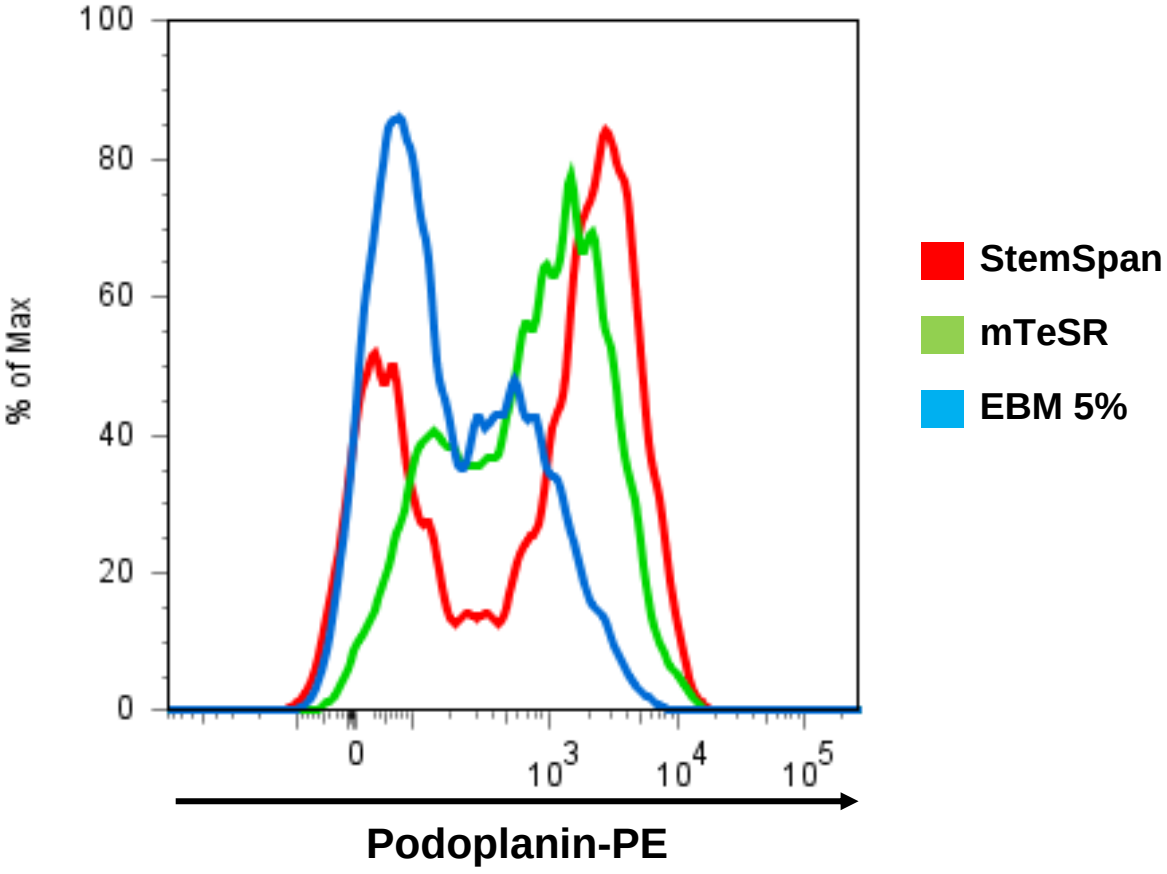
1. Systemic inflammation (i.e. fever or chill, severe hyper/hypothermia)
2. Local infection or inflammation
3. Active bleeding
4. Moderate or severe anemia
5. Other medical conditions which can possibly affect stem cell mobilization
6. Other medical conditions which contraindicate blood donation.

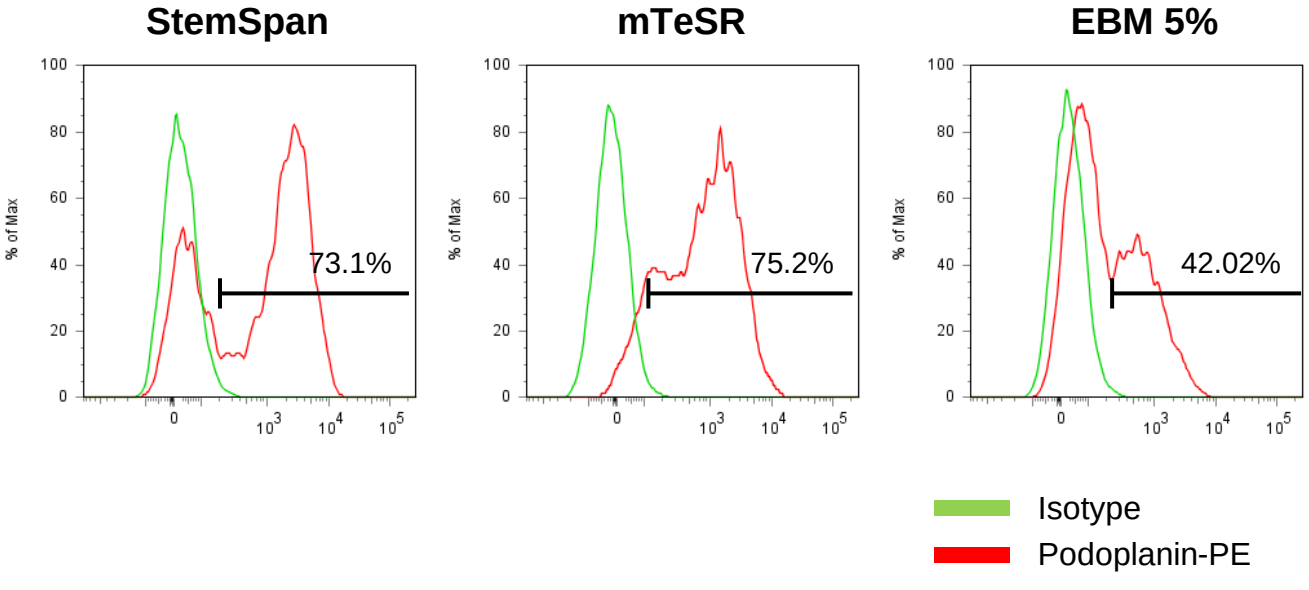
Supplementary Table S2. Information of primers

Primer	Sequence	TM	Access Number	Product size(bp)
Podoplanin	Forward 5′-GCCACCAGTCACTCCACGGAGAA-3′ Reverse5′- AGGGCGAGTACCTTCCCGACA -3′	60	NM_001006624.1	175
VEGFR-3	Forward 5′- GAGACAAGGACAGCGAGGA-3′ Reverse 5′- TGATGAATGGCTGCTCAAAG-3′	60	NM_182925.4	210
Ephrin-B2	Forward 5′- CGTGTTTGAGTCAAGCCAGA-3′ Reverse 5′- GCTGCAATGGTATCCACCTT-3′	58	NM_004442.6	240
Prox-1	Forward 5′- CCACTGACCAGACAGAAGCA-3′ Reverse 5′- TGGGCTCTGAAATGGATAGG-3′	60	NM_001270616.1	194
Sox-18	Forward 5′- CCTAGGAGCCAGTGATGCTC-3′ Reverse 5′- AGGAGCAGGTGCTTCAAAAA-3′	60	NM_018419.2	199
FoxC-2	Forward 5′- AGTTCATCATGGACCGCTTC-3′ Reverse 5′- TCCTTGGACACGTCCTTCTT-3′	60	NM_005251.2	229
Ang-1	Forward 5′- GAAGGGAACCGAGCCTATTC-3′ Reverse 5′- GGGCACATTTGCACATACAG-3′	58	NM_001199859.1	181
Ang-2	Forward 5′- GGGACTCTGGACGTGTGTTT-3′ Reverse 5′- CTCTGCACCGAGTCATCGTA-3′	60	NM_001118888.1	299
TGF-b1	Forward 5′- CACGTGGAGCTGTACCAGAA-3′ Reverse 5′- GAACCCGTTGATGTCCACTT-3′	60	NM_000660.4	219
VEGF-A	Forward 5’-GGGCAGAATCATCACGAAGT-3’ Reverse 5’-TGGTGATGTTGGACTCCTCA -3’	58	NM_001204384.1	211
VEGF-C	Forward 5′- GGAAAGAAGTTCCACCACCA-3′ Reverse 5’- TTTGTTAGCATGGACCCACA-3′	58	NM_005429.2	249

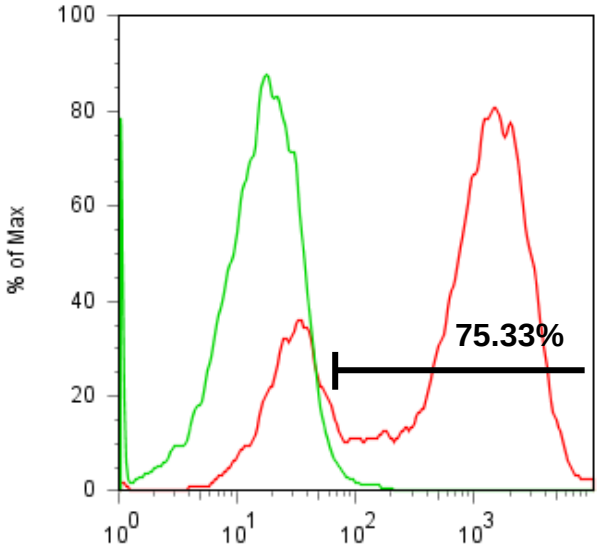
VEGF-D	Forward 5′- AGGACTGGAAGCTGTGGAGA-3′ Reverse 5’- ATCGGAACACGTTCACACA-3′	60	NM_004469.4	246
IFN-gamma	Forward 5′- TTTGGGTTCTCTTGGCTGTT-3′ Reverse 5’- TCTTTTGGATGCTCTGGTCA-3′	58	NM_000619.2	230
IL-1b	Forward 5′- AAACCTCTTCGAGGCACAAG-3′ Reverse 5’- GTTTAGGGCCATCAGCTTCA-3′	60	NM_000576.2	175
CLEC-2	Forward 5′- TGCAGCCCCTGTGACACAAACT-3′ Reverse 5’- ACCGAGCCATCCTCCCACTTCC-3′	60	NM_001099431.1	236
GAPDH	Forward 5′- GAGTCAACGGATTTGGTCGT -3′ Reverse 5′- GACAAGCTTCCCGTTCTCAG-3′	60	NM_002046.4	185

Supplementary Figure S1

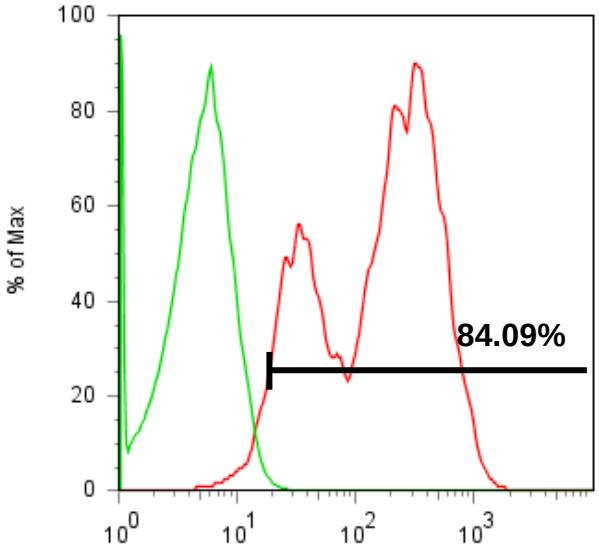




Supplementary Figure S2



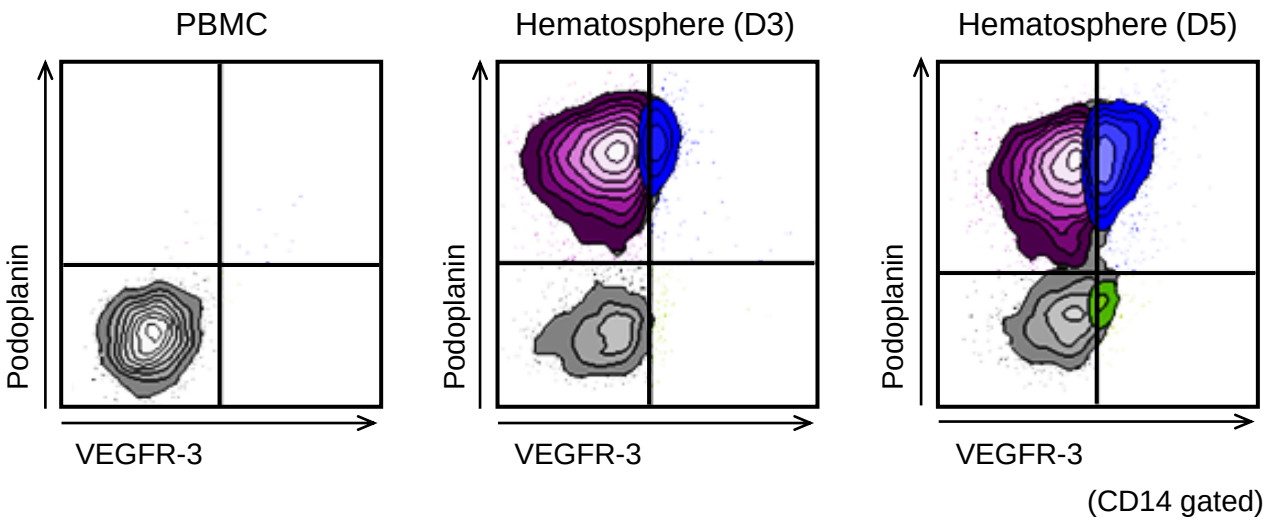
Podoplanin (NZ-1.3)
Isotype control



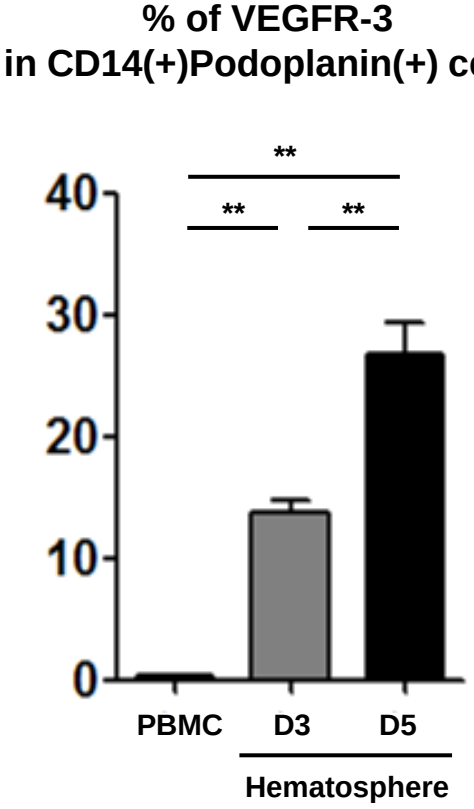
Podoplanin (NC-08)
Isotype control

Supplementary Figure S3

a



(CD14 gated)



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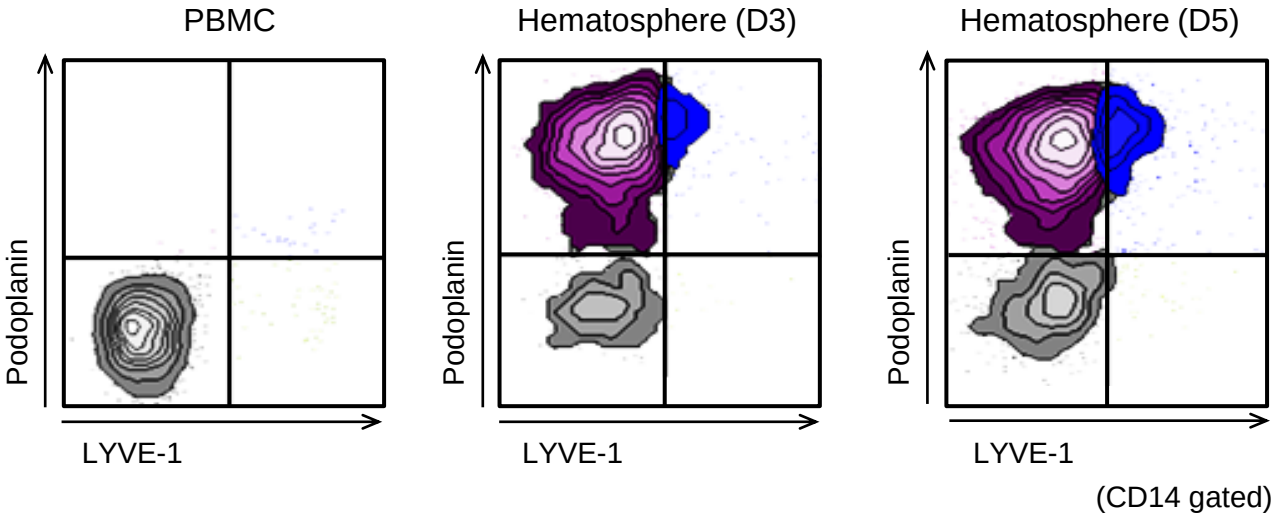

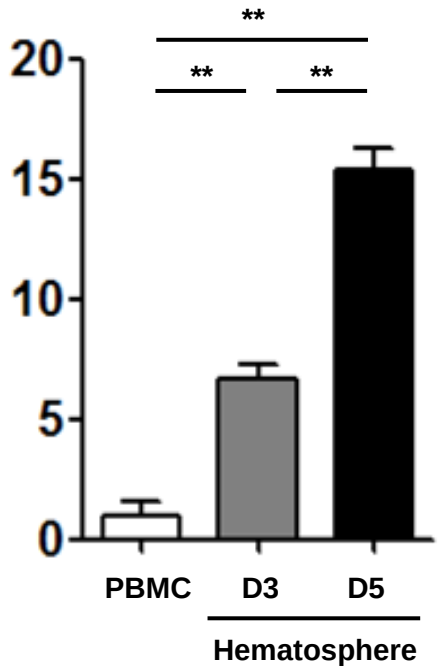

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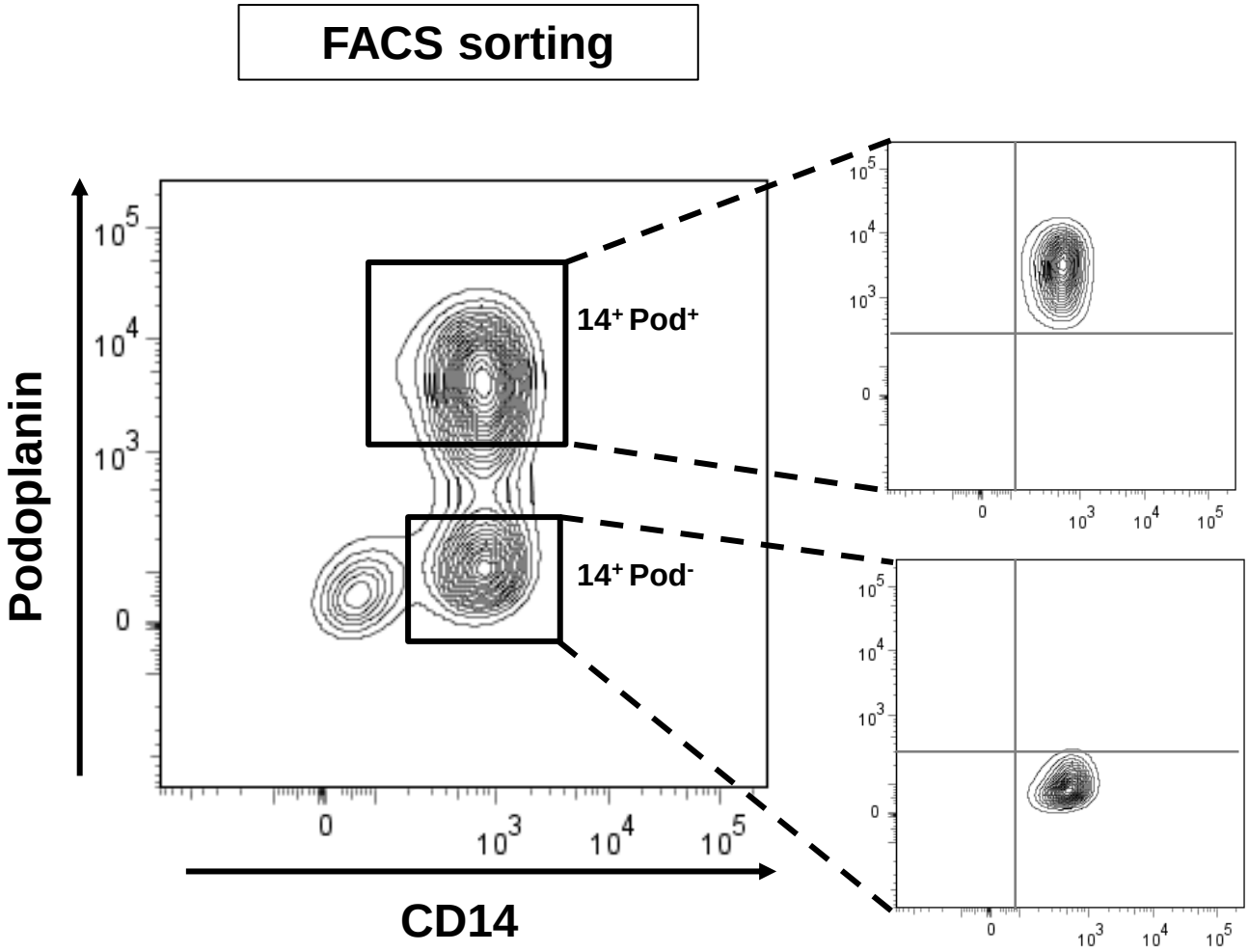

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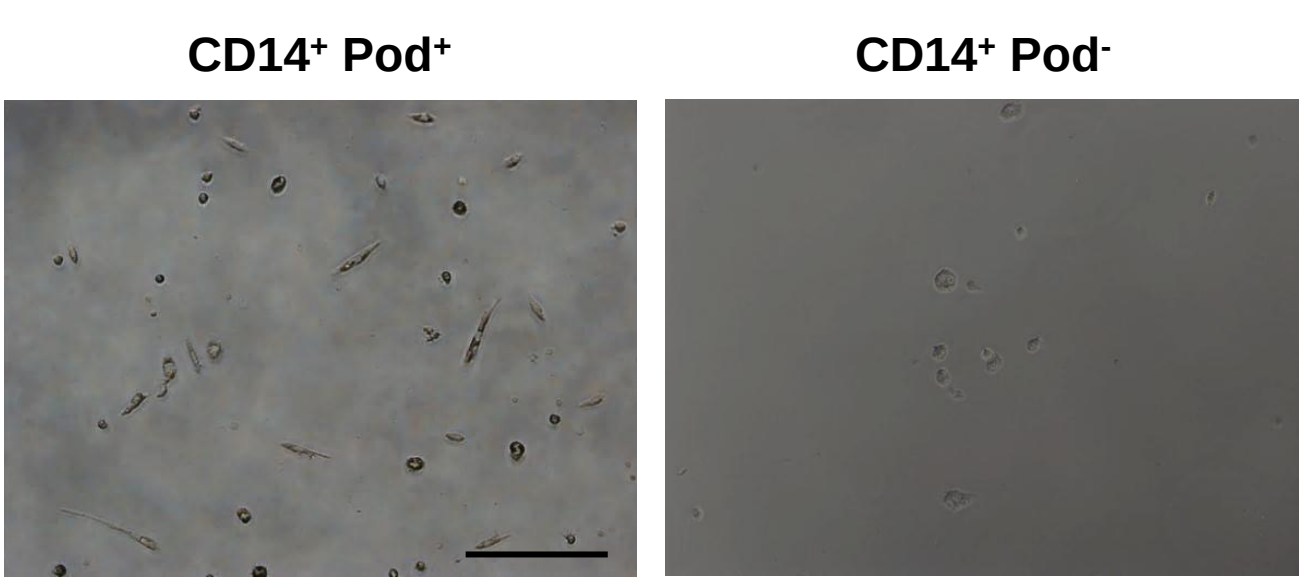

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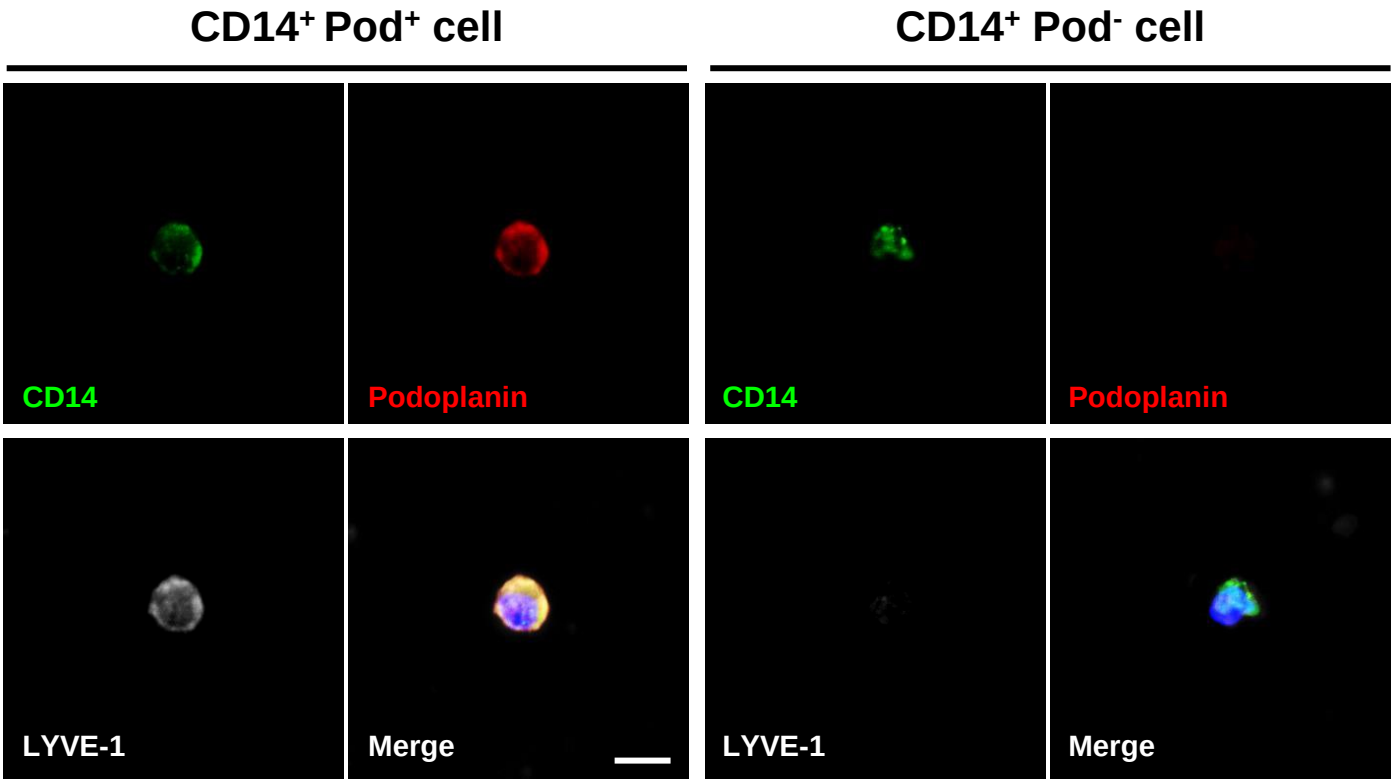

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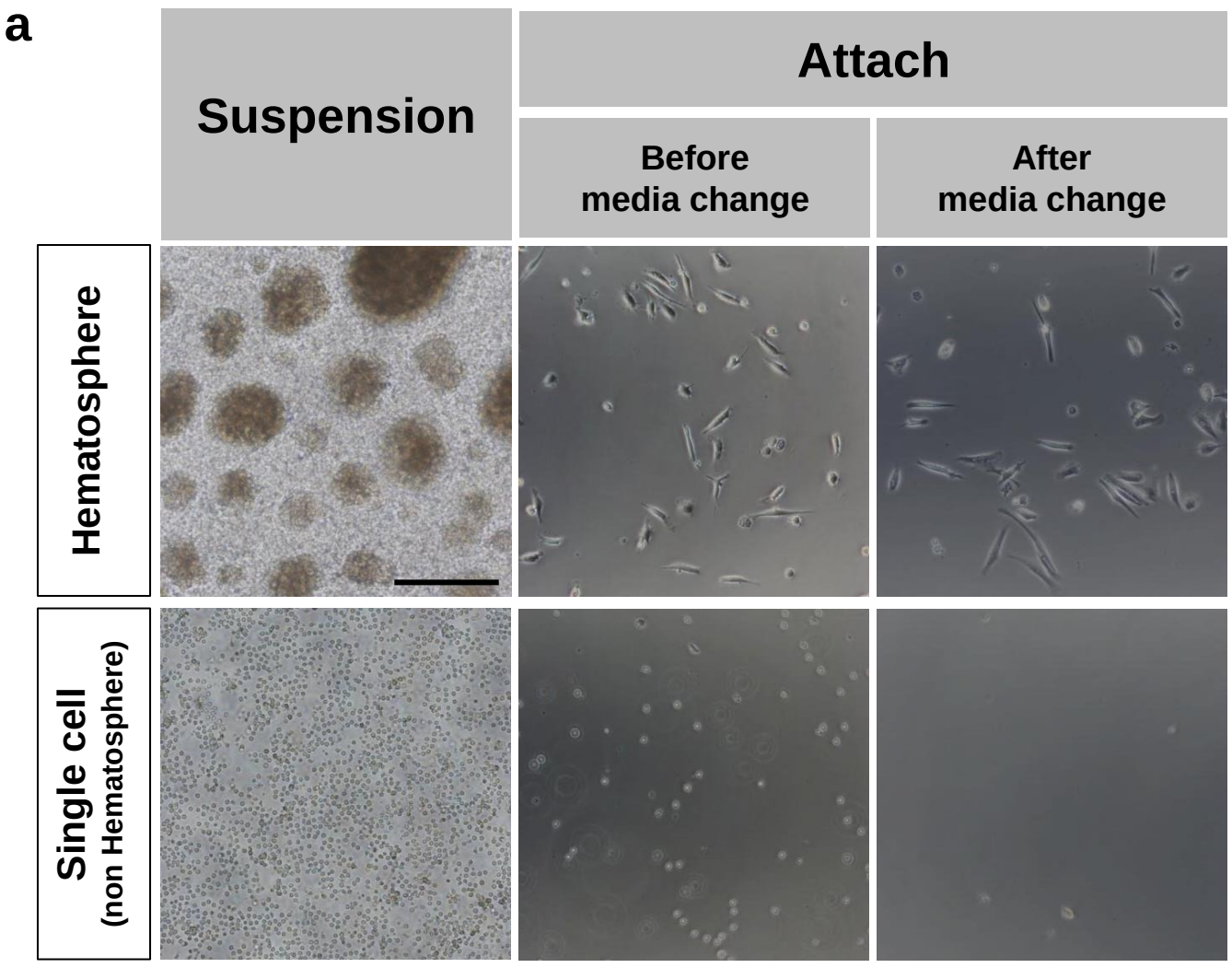

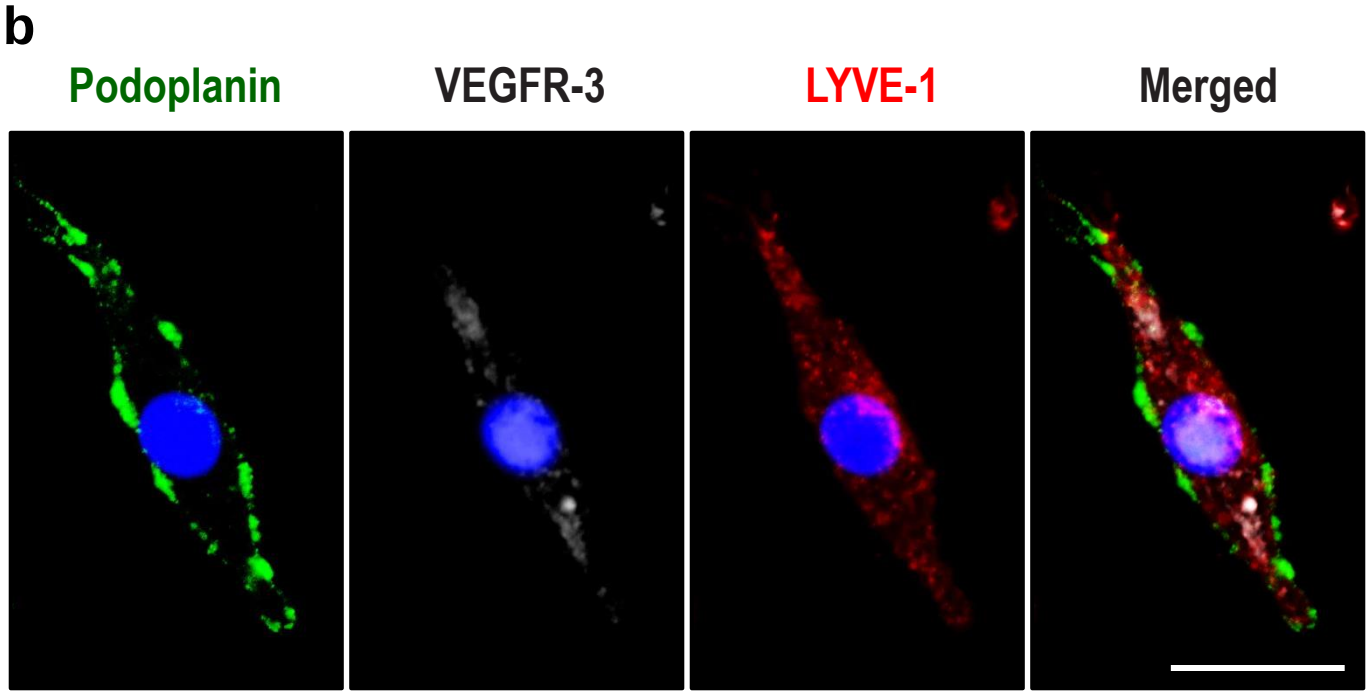

Supplementary Figure S7

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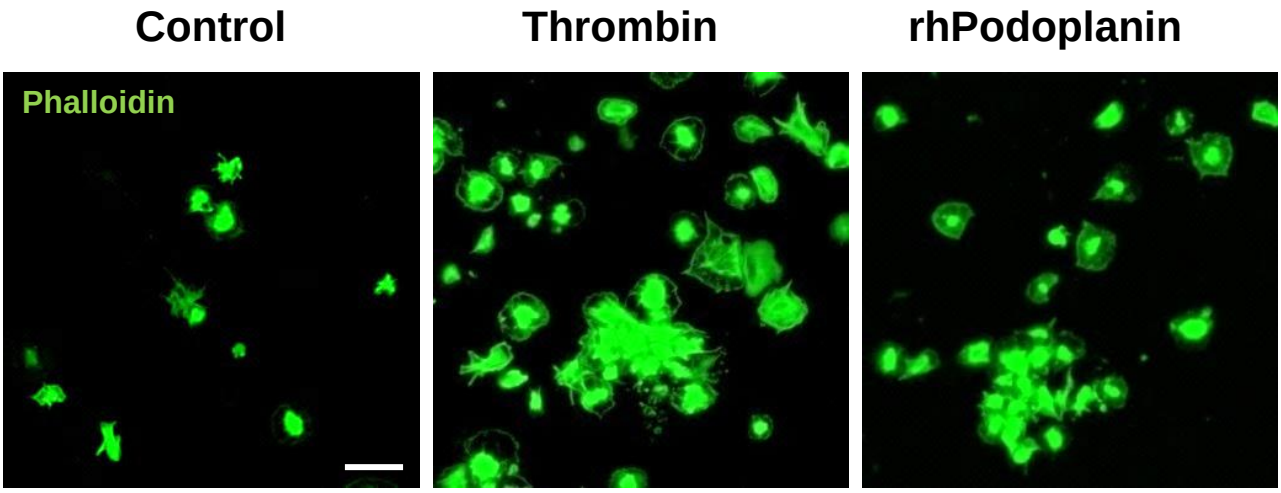

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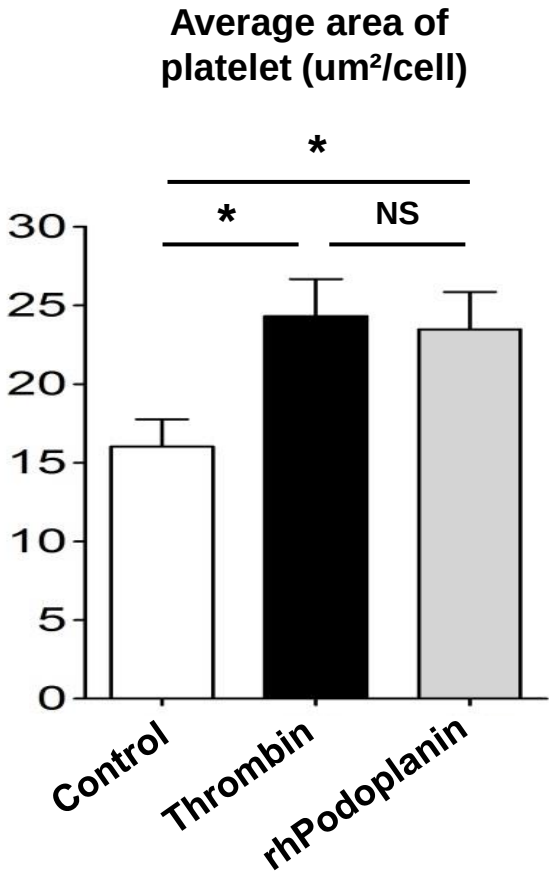
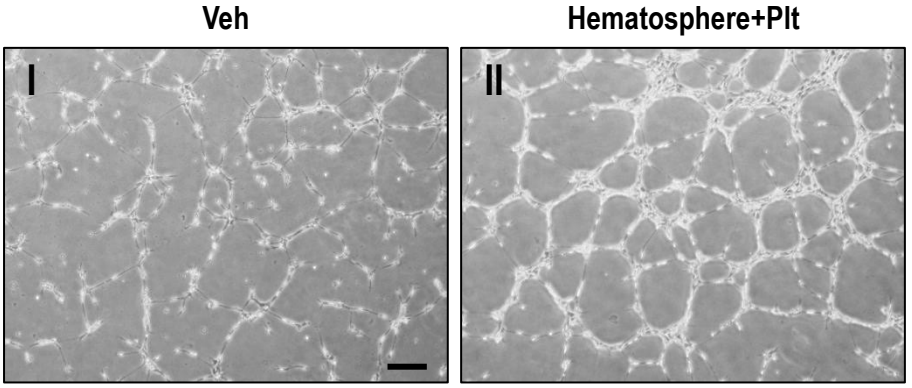
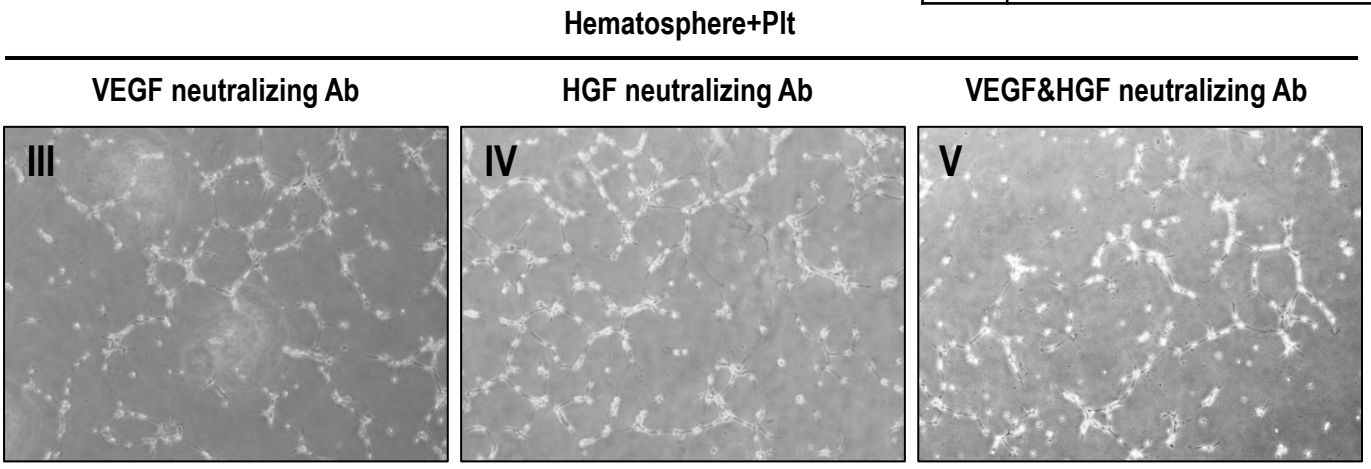


Supplementary Figure S8

a Matrigel tube formation of human LECs using culture supernatant



I	Veh
II	Hematosphere+Plt
III	Neutralizing (VEGF Ab) Hematosphere+Plt
IV	Neutralizing (HGF Ab) Hematosphere+Plt
V	Neutralizing (VEGF&HGF Ab) Hematosphere+Plt



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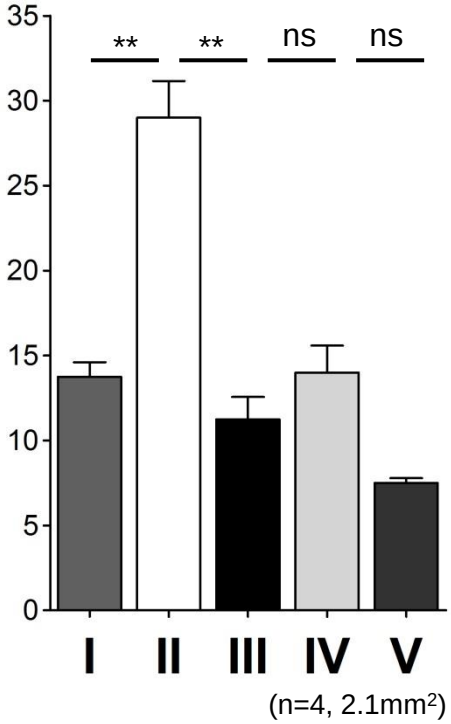
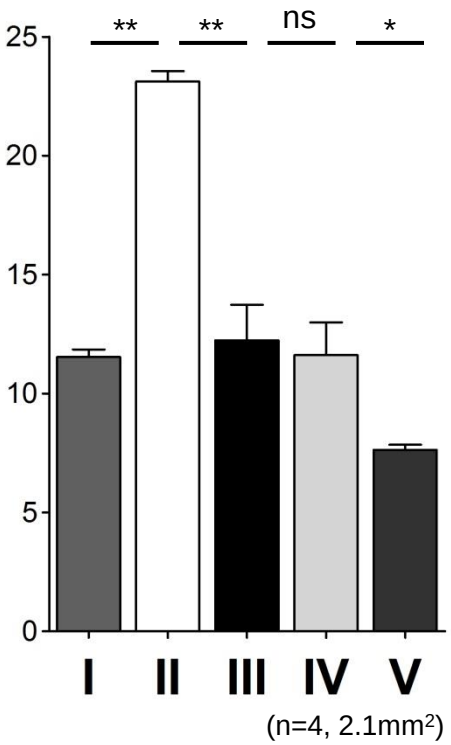


c Tube length (mm)



Supplementary Figure S9

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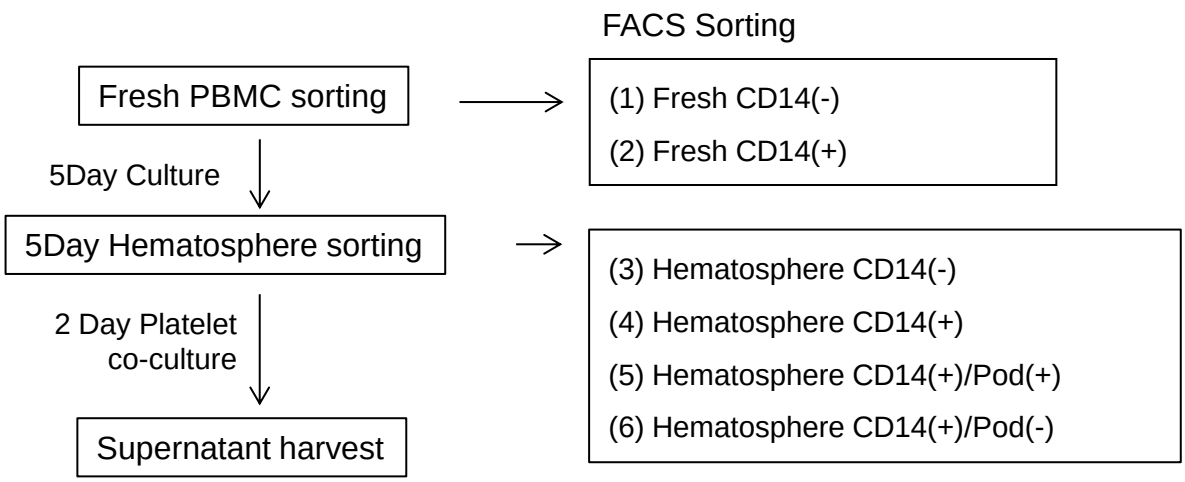

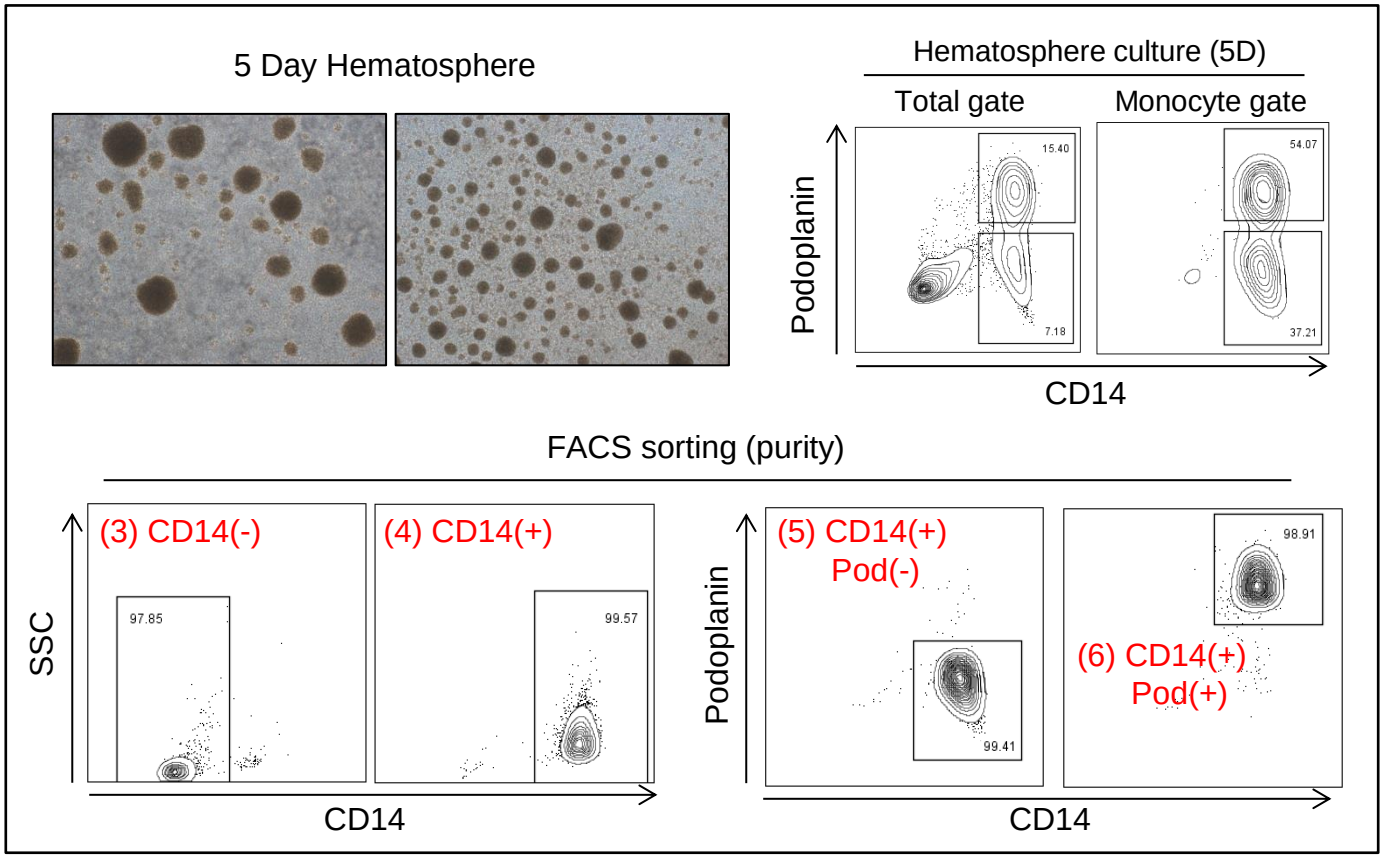

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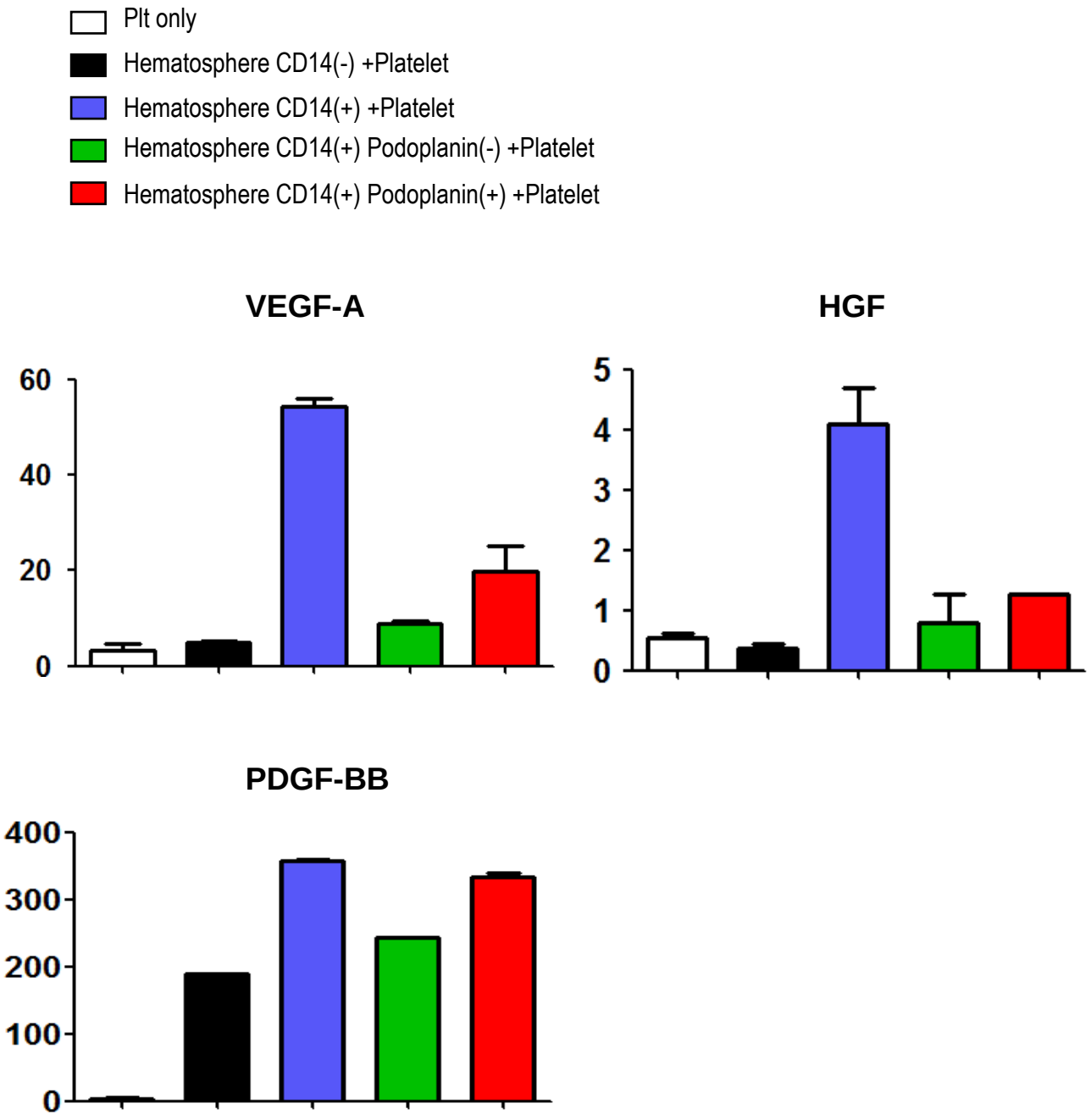
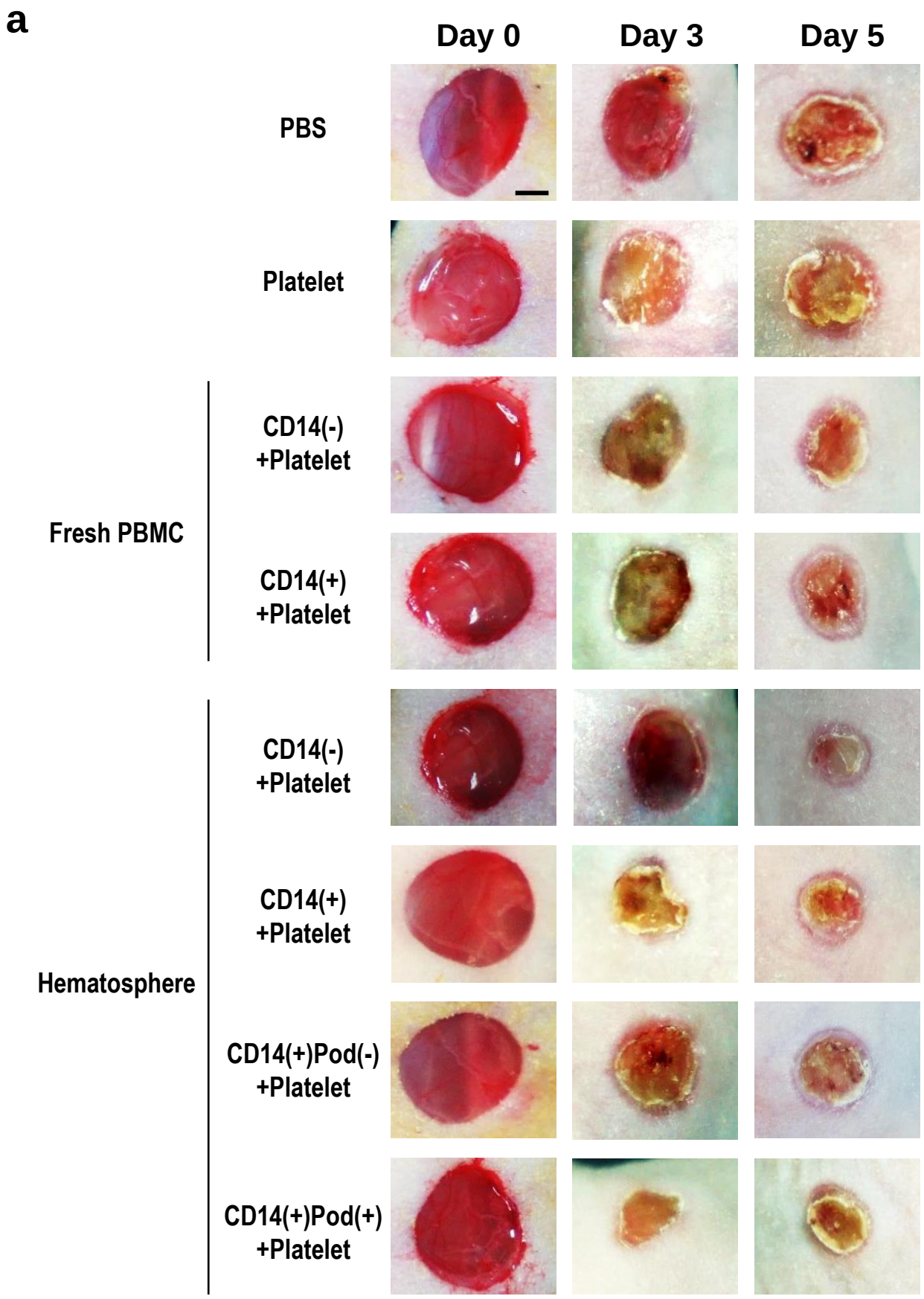


Supplementary Figure S10



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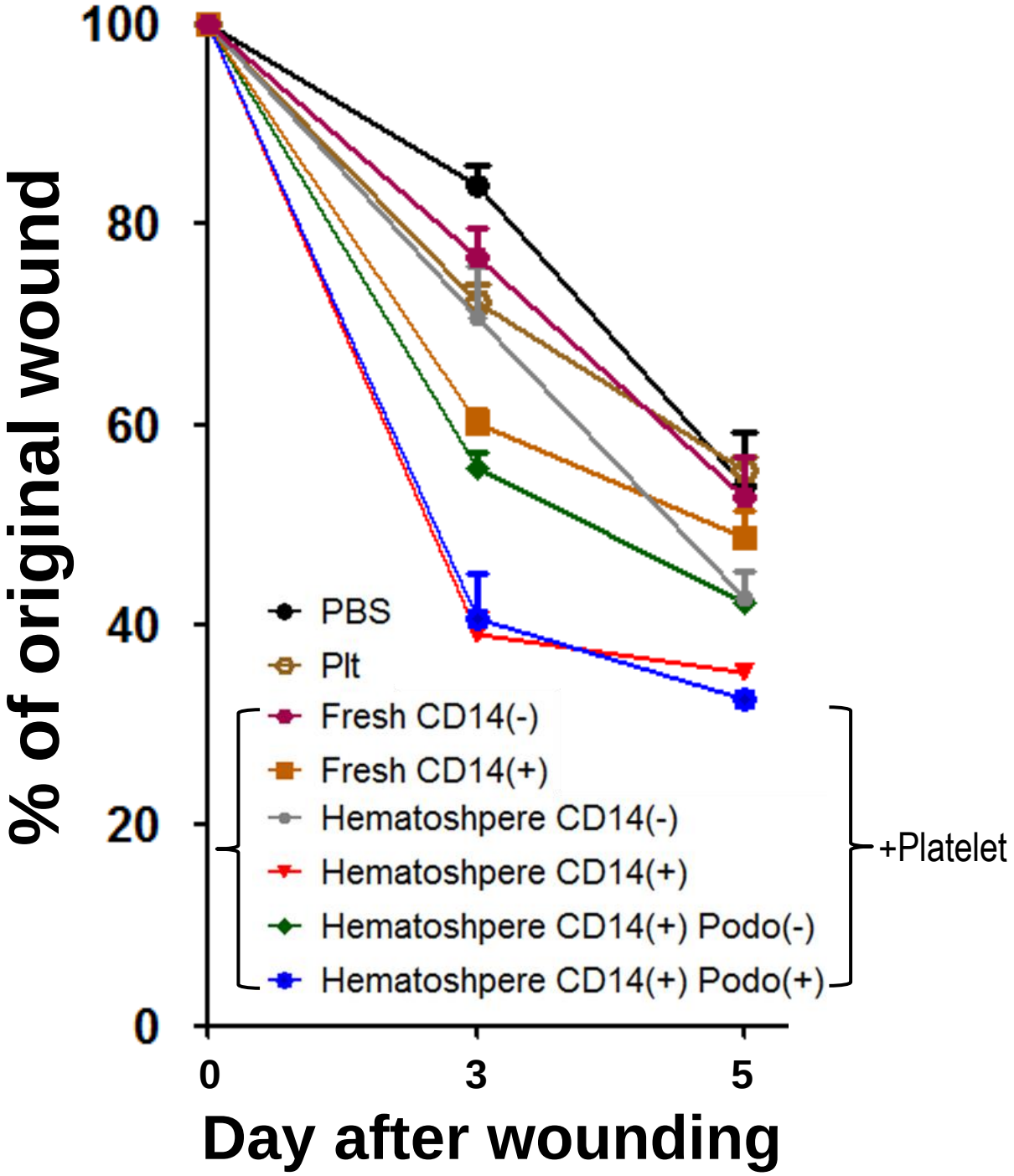

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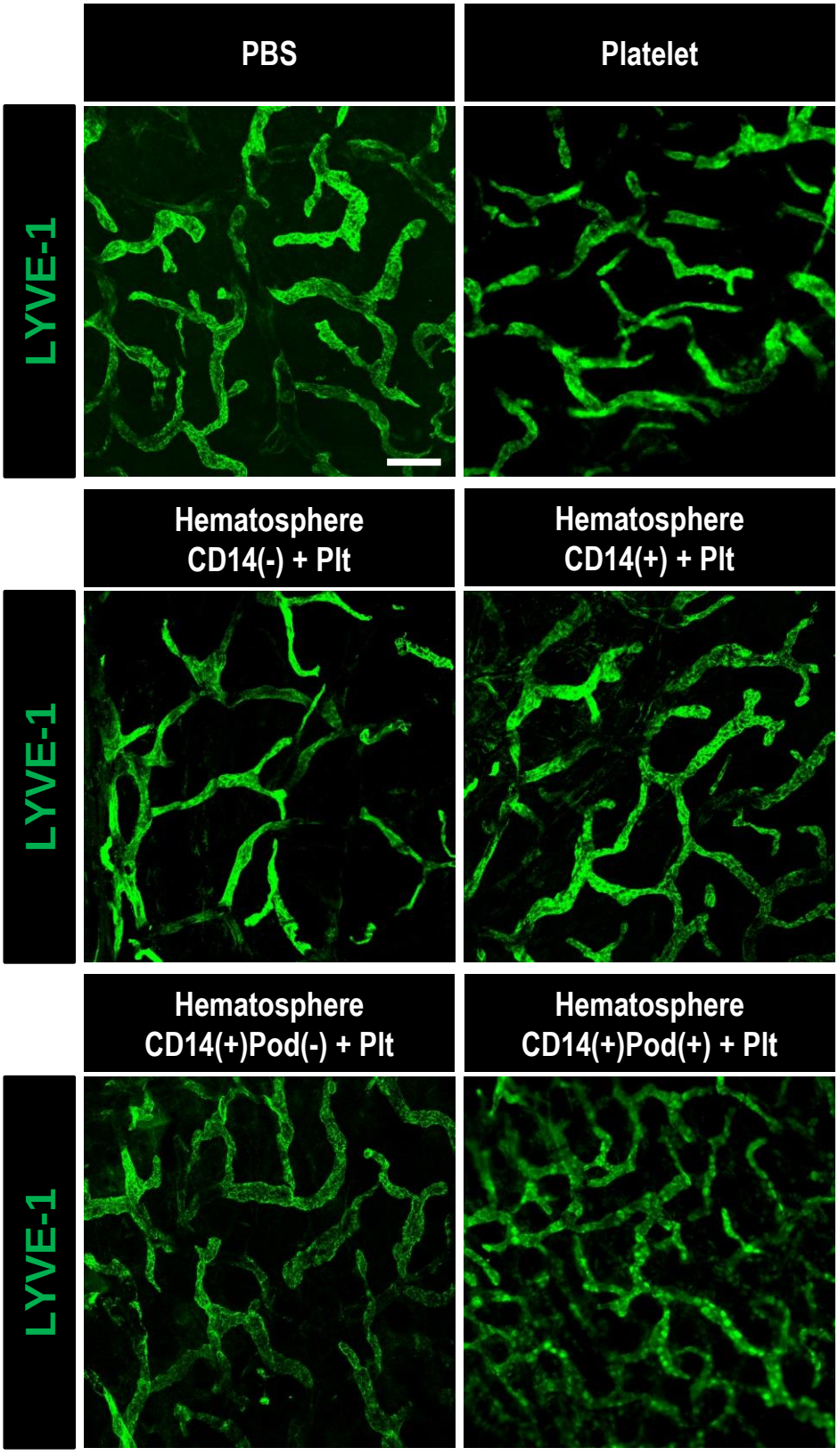

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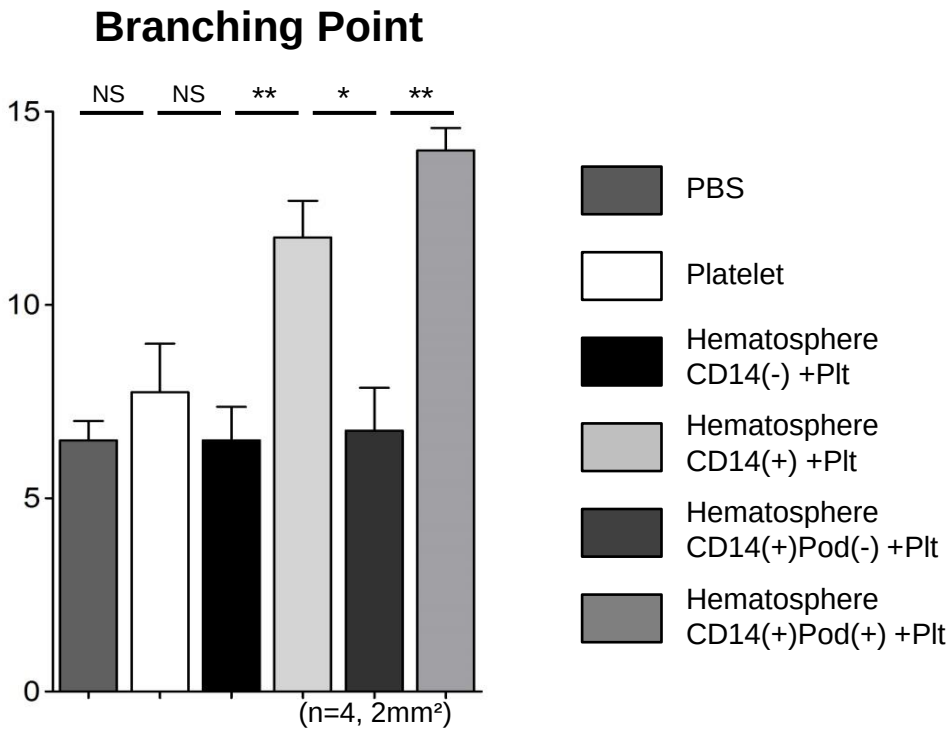

Supplementary Figure S11