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Supplementary Information for

Inflammatory platelet production stimulated by tyrosyl-tRNA synthetase mimicking viral infection

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SI Materials and Methods

Reagents. TruStain FcX (anti-mouse CD16/32), APC anti-mouse Ly-6A/E (Sca-1) [E13-161.7], FITC anti-mouse Ly-6A/E (Sca-1) [E13-161.7], Brilliant Violet 421 anti-mouse CD41 [MWReg30], PE/Cyanine7 anti-mouse CD41 [MWReg30], APC anti-mouse CD41 [MWReg30], PE anti-mouse F4/80 [BM8], PE/Cy7 anti-mouse F4/80, BM8, Brilliant Violet 510 anti-mouse/human CD11b, M1/70, PE anti-mouse CD117, 2B8, PerCP/Cyanine5.5 anti-mouse CD34, HM34, APC/Fire 750 anti-mouse CD150 [TC15-12F12.2], Alexa Fluor 555 Goat anti-rat IgG, and DRAQ5 were from BioLegend (San Diego, CA). PE Anti mouse Integrin αIIb [MWReg30], and apixaban were from Santa Cruz Biotechnology (Dallas, TX). Alexa Fluor 647 anti-mouse GpIbα antibody [5A7](1), Alexa Fluor 405 anti-mouse GPIbα antibody [5A7], Alexa Fluor 488 anti-Fibrin antibody, [64C5], and Alexa Fluor 647 Anti-Fibrin antibody [64C5] were from MERU-VasImmune (San Diego, CA). Dylight649 anti-mouse CD42b Antibody [Xia.G5] was from Emfret Analytics (Eibelstadt, Germany). Anti-GFP polyclonal antibody, Alexa Fluor 488 Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 555 Goat anti-Rabbit IgG (H+L) Cross-Adsorbed secondary antibody, Alexa Fluor 647 Goat anti-Rat IgG (H+L) Cross-Adsorbed secondary antibody, TRIzol™ Reagent, and Alexa Fluor 647 labeled Fibrinogen were from Invitrogen (Carlsbad, CA). PE Rat Anti-Mouse CD144 [11D4.1] was from BD Biosciences (San Jose, CA). Gardiquimod was from AdipoGen Life Sciences (San Diego, CA). Lipopolysaccharide was from Oakwood Chemical (Estill, SC). Propidium iodide and ADP were from Sigma-Aldrich (St Louis, MO). Fc receptor blocking reagent was from INNOVEX biosciences (Richmond, CA). Recombinant YRS^{ACT} protein was prepared as previously reported (2).

High-dimensional Mass cytometry. To analyze the surface marker of B6 WT mouse BM cells, we employed t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis and high-dimensional mass cytometry as previously described (3). BM cells were harvested from femurs of WT mice by flushing with PBS containing 0.5% BSA and 2 mM EDTA. After centrifugation, cells were fixed in 1% paraformal-

dehyde at 4°C for 2 hours. The fixe BM cells were then washed and resuspended in PBS containing 2 mM EDTA. After blocking Fc receptors with TruStain FcX, cells were stained with a panel of the antibodies; CD41, CD11b, Sca1, CD34, c-Kit, F4/80, GPIbα, and CD150. The nuclei of cells were counter-stained with Propidium Iodide (PI). Total of $2 \cdot 10^5$ cell events were acquired using Aurora spectral analytical flow cytometer (CYTEK, Fremont, CA). We performed t-SNE analysis using FlowJo v.10.7.1, t-SNE plugin. t-SNE plots were generated with the following parameters: 1000 iterations, 45 perplexity, 8183 learning rate, Exact (vantage point tree) KNN algorithm and Barnes-Hut gradient algorithm. Prior to the analysis, we excluded doublet cells and multi-nucleated matured MKs (> 4N), focusing on 2N-4N singlet cells including immature MKs. For determination of 2N-4N region, we used peripheral blood mono-nuclear cells (PBMC) as the reference.

Imaging flow cytometry. Blood samples collected from naive WT mice were diluted in PBS with 2 mM EDTA, treated with TruStain FcX, followed by staining with FITC-anti-Sca1, PE-anti-CD11b, PECy7-anti-CD41, and Hoechst 33342. Images of the cells were acquired using Amnis ImageStream XMk II (Luminex). The data was analyzed using Amnis Image Data Exploration and Analysis Software (IDEAS; Luminex).

Platelet count analysis. Complete blood count was obtained using the Procyte Dx (IDEXX Laboratories, Westbrook, ME).

Fibrinogen binding analysis. Blood samples collected from retro-orbital plexus using sodium citrate as anticoagulant were diluted in modified Tyrode's buffer, pH 7.4 containing 2 mM EDTA or 0.2 mM Ca^{2+}. AlexaFluor 647-labeled fibrinogen was added at final concentration of 15 µg/mL into diluted whole blood (1.5 µL in the total volume of 100 µL, resulting in approximately ~18-36 µg/mL plasma fibrinogen and 10-12 $\cdot 10^3$/µL platelets) and was incubated in the presence of 10 µM ADP. After incubation at room temperature for 10 minutes, AlexaFluor 405 labeled anti-mouse GPIbα antibody 5A7 was added.

Samples were analyzed using a Novocyte flow cytometer (ACEA Biosciences). The results were analyzed with FlowJo v.10.7.1.

Annexin V binding analysis. Blood samples collected from retro-orbital plexus using sodium citrate as anticoagulant were diluted in modified Tyrode's buffer, pH 7.4 containing 3 mM Ca^{2+} with 200 nM Apixaban. Diluted whole blood (1.5 µl in the total volume of 100 µL) was incubated with AlexaFluor 647-labeled Annexin V in the presence of 2.5 µg/mL Alborhagin. After incubating at room temperature for 30 minutes, AlexaFluor 405 labeled anti-mouse GPIbα antibody 5A7 was added. Samples were analyzed using a Novocyte flow cytometer. The results were analyzed with FlowJo v.10.7.1.

MK ploidy analysis. BM cells either freshly harvested from femurs or ex vivo cultured for 3 days were fixed with 1% paraformaldehyde at 4°C for 2 hours. After washing, BM cells were resuspended in PBS containing 2 mM EDTA. Fc receptors were blocked with TruStain FcX (Biolegend), and then, the cells were stained for 10 minutes at room temperature with Brilliant Violet 421 labeled anti-mouse CD41 antibody (1 µg/mL) (Biolegend) and propidium iodide (100 µg/mL) (Sigma-Aldrich, MO) in the presence of RNase A (50 µg/mL) (Qiagen). The total volume of each sample was adjusted to 300 µL and 100 µL was acquired for analysis by NovoCyte flow cytometer. The results were analyzed with FlowJo v.10.7.1.

***Ex vivo* MK culture.** BM cells were isolated from Sca1/EGFP Tg mice and $IFNAR^{-/-}$Sca1/EGFP Tg mice by flushing femurs and tibias. After washing, BM cells were resuspended at a density of $1.5 \cdot 10^7$ cells in 10 mL of Iscove's modified Dulbecco's medium (Thermo Fisher Scientific) supplemented with 15% fetal bovine serum (Thermo Fisher Scientific). BM cells were cultured for 3 days and analyzed by flow cytometry or immunofluorescence microscopy (2). For the flow cytometry analysis, cells were detached with Trypsin-EDTA (ThermoFisher Scientific), and collected cells were processed for MK ploidy analysis. For the microscopic analysis, the cover slips were sterilized and coated with PEI (25 µg/mL). The BM cells were cultured on the cover slip for 3 days. The cells grown on the cover slip were

fixed with 4% paraformaldehyde, blocked and permeabilized with 0.1% Triton X-100 PBS containing 5% normal goat serum (Thermo Fisher Scientific). The expression of CD41 was visualized by staining with rat anti-mouse integrin αIIb (MWReg30; Santa Cruz Biotechnology, CA) followed by AlexaFluor 647 labeled goat anti-rat IgG secondary antibody (Thermo Fisher Scientific). The expression of EGFP was detected by staining with chicken anti-GFP polyclonal antibody (A10262, Thermo Fisher Scientific) followed by AlexaFluor 488 labeled goat anti-chicken IgG secondary antibody (A11039, Thermo Fisher Scientific). F4/80 was stained with rabbit anti-F4/80 antibody (CI:A3-1, Absolute Antibody) followed by AlexaFluor 555 labeled goat anti-rabbit IgG secondary antibody (A21422, Thermo Fisher Scientific). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Dojindo Molecular Technologies, Kumamoto, Japan). The images were observed with LSM880 laser scanning confocal microscope (Zeiss).

Quantitative PCR. Total RNA was extracted from homogenized mouse BM by using TRIzol reagent (Thermo Fisher Scientific) and purified using Monarch Total RNA Miniprep Kit (New England BioLabs, Ipswich, MA). Complementary DNAs were synthesized using ProtoScript II (New England BioLabs). Relative gene expressions were calculated according to the comparative Ct method using Ubiquitin C (UBC) as an internal control (4). qPCR was carried out on an ABI StepOnePlus System (Applied Biosystems) using the following primers (4).

IFNa2; ATCCAGAAGGCTCAAGCCATCC, GGAGGGTTGTATTCCAAGCAGC

IFNa7; TCCTGCCTGAAGGACAGAAAGG, GGTCAGCTCATGCAGAACACAG

IFNb1; CTCCACCACAGCCCTCTC, CATCTTCTCCGTCATCTCCATAG

Sca1; CCTACCCTGATGGAGTCTGTGT, CACGTTGACCTTAGTACCCAGG

F3; GCACCGAGCAATGGAAGAGTTTC, CTTTCTGTCCCGCTCGGTTCTT

UBC; CCCAGTGTTACCACCAAGAAG, CCCCATCACACCCAAGAACA

Supplemental Figures

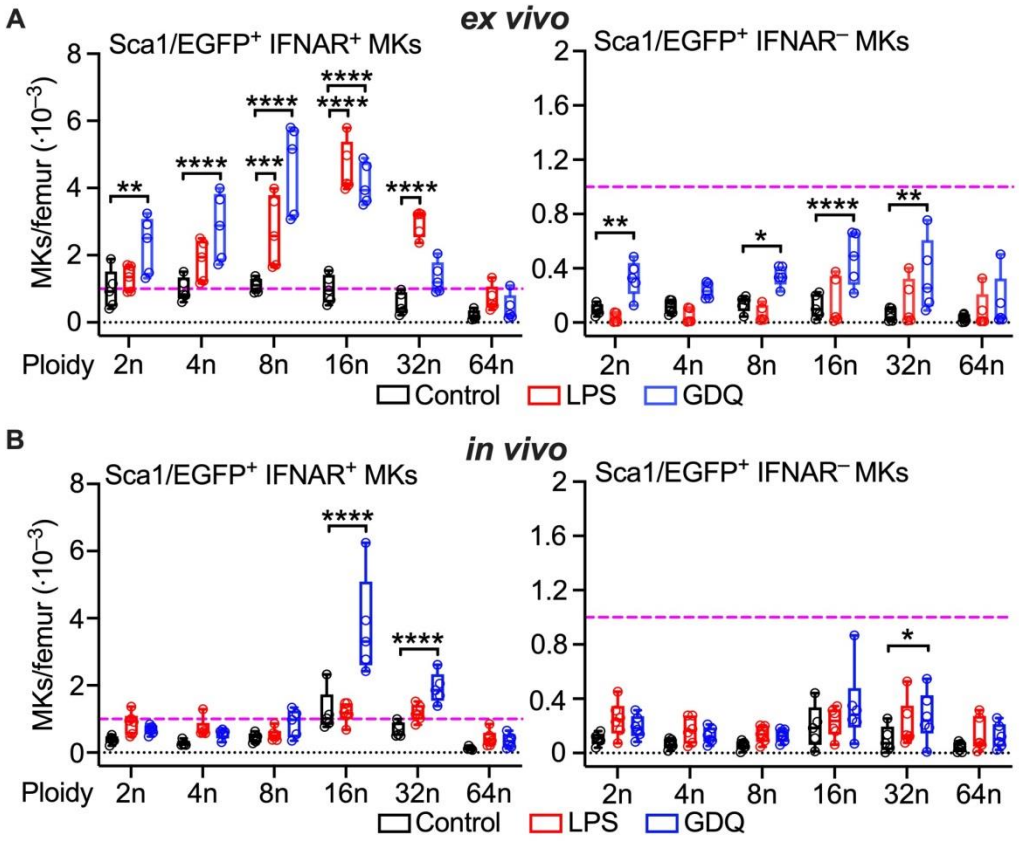


Fig. S1. Effect of TLR4 or TLR7 activation on MK ploidy. (**A**) Ploidy distribution of Sca1/EGFP$^+$ MKs in BM cells from IFNAR+ (left panel) or IFNAR− (right panel) Sca1/EGFP Tg mice, analyzed by flow cytometry after 3 days in culture with GDQ (0.5 μg/mL), LPS (1 μg/mL) or vehicle control (CON); n = 5 each. (**B**) Analysis of BM cells harvested from IFNAR+ (left panel) or IFNAR− (right panel) Sca1/EGFP Tg mice 2 days after i.p. injection of GDQ (1 mg/kg, n = 5), LPS (2 mg/kg, n = 6) or CON (n = 5). Data, shown as 25th-75th percentile boxes with min-to-max whiskers, were analyzed by repeated measures (RM) two-way ANOVA without assuming sphericity (Geisser-Greenhouse correction) with Dunnett's post-test for multiple paired comparisons. Note the 4 times difference in the ordinate scale for results obtained with IFNAR$^{-/-}$ mice due to the markedly reduced number of Sca1/EGFP$^+$ MKs; to facilitate comparisons, a broken blue line is set at 1,000 in each panel. *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001.

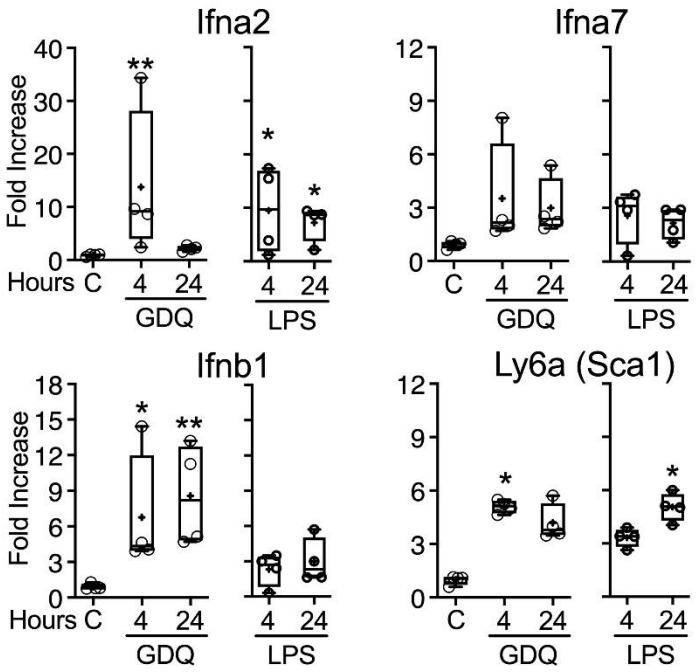


Fig. S2. Effect of TLR4 or TLR7 activation on select IFN-I gene expression. Total RNA was extracted from BM cells of Sca1/EGFP Tg mice treated with GDQ, LPS or CON (see Fig. S1) and changes in expression of Ifna2, Ifna7, Ifnb1 and Ly6a (Sca1) were measured by real-time PCR. Relative gene expression is shown as fold increase of treated over control according to the comparative Ct method (ΔCt) using Ubiquitin C (UBC) as internal control. Data (n = 4) are shown as 25^{th}-75^{th} percentile boxes with min-to-max whiskers and individual values (open circles); means are indicated by a cross. Statistical analysis was performed by non-parametric repeated measures one-way ANOVA (Friedman test) with Dunn's post-test for multiple comparisons; *P <0.05, **P <0.01.

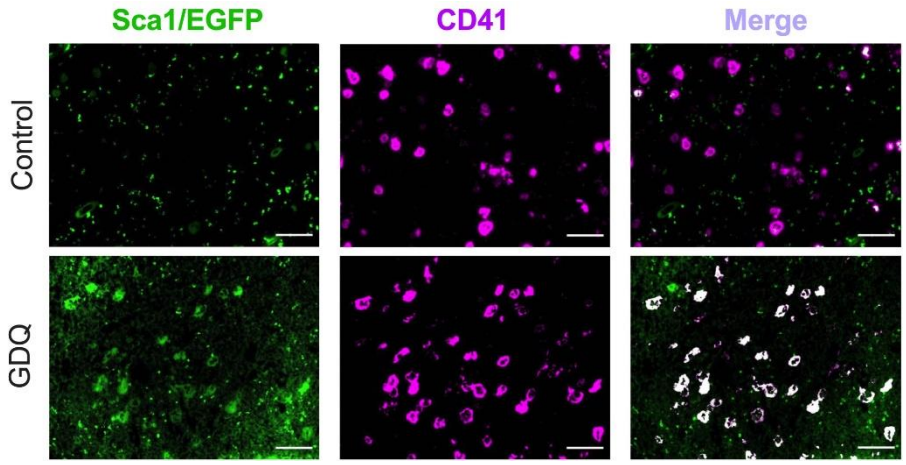
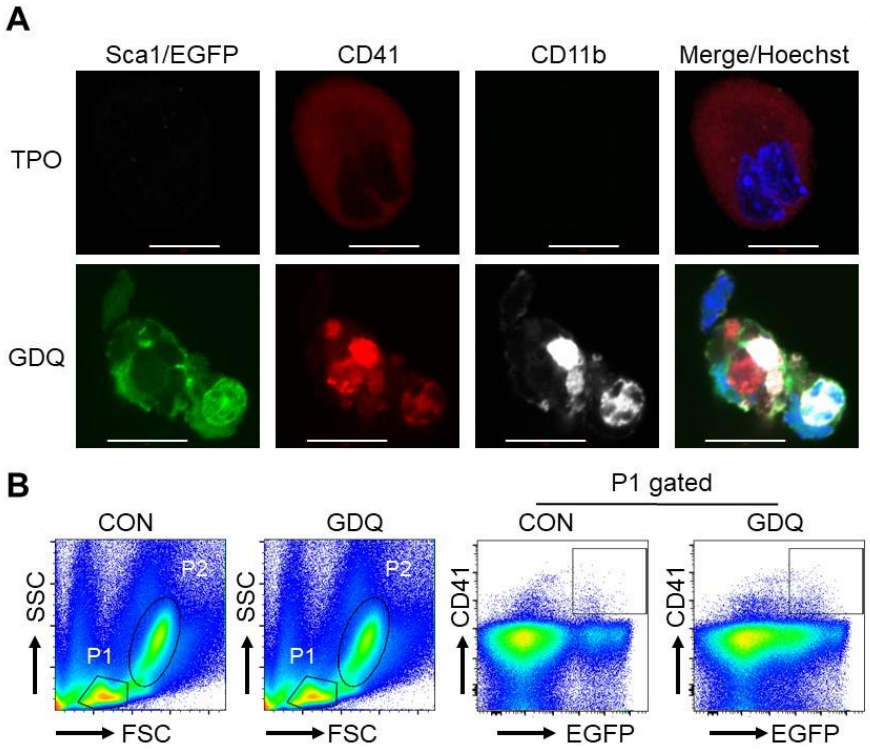


Fig. S3. Immunofluorescence microscopy analysis of BM cells from Sca1/EGFP Tg mice treated with GDQ. Femurs of Sca1/EGFP Tg mice 3 days after GDQ or CON injection were harvested, cryo-sectioned and incubated with anti-CD41 antibody to stain MKs and MK progenitors. Images collected in the green (EGFP) and magenta (CD41) fluorescence channels are shown along with the merged (white) image. Scale bars =100 μm.

Categories	Genes	TPM	
		Sca1/EGFP^{+} platelets	Sca1/EGFP^{-} platelets
MK/Platelet	Gp5	188918	111231
	Gp9	165597	106521
	Gp6	39379	18895
	Vwf	136532	68718
	Pecam1	44876	20910
Stem cell / IFN-related gene	Ly6a	1074	0
Cytoskeletal proteins	Myl9	909589	553664
	Myh9	10402	1518
	Flna	179203	93991
	Tuba1b	96	2
	Tubb1	516159	248862
	Tubb6	1555	0
Integrin signaling	Itga2b	1149052	684469
	Itgb3	201814	128418
	Tln1	100301	43081
IFN-related gene	Ifitm2	6531	3682
	Ifit2	11505	5333
Others	F5	57135	27241
	Rhoa	8175	1022
	Rhoc	298	0
	Rhoh	1381	5720
	F2r	591	0
	Stat5b	5825	0

Fig. S4. Different transcriptional profiles of Sca1/EGFP +/− platelets. Blood samples (each pooled from 4 individuals) were collected from Sca1/EGFP Tg mice 3 days after i.p. injection of GDQ or CON. Platelets were sorted on the basis of Sca1/EGFP expression and analyzed by RNA-Seq. Values are shown as Transcripts Per kilobase Million (TPM). Selected differentially expressed genes (>1.5 times up or down difference in two independent experiments) are shown.



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Categories	Genes	CON	GDQ
MK/Platelet	Gp1ba	2	72
	Gp5	8749	2576
	Gp9	74	1090
	Gp6	0	0
	Vwf	200	2184
	Pecam1	44876	20910
Stem cell	Slamf1 (CD150)	2297	255
	Cd34	1345	11
	Kit	106	34
	Ly6a	54	17093
Myeloid	Adgre1 (F4/80)	124	404
	Itgam (CD11b)	1713	3281
	Mpo	12056	21098
	Ms4a3	504	2376
Erythroid	Slc4a11 (Band3)	0	91
	Tfrc	0	3128
Cytoskeletal proteins	Myl9	947	2199
	Myh9	7928	5227
	Flna	27327	10147
	Tubb1	0	385
	Tubb6	0	1336
Integrin signaling	Itga2b (CD41)	15251	3474
	Itgb3	4379	1251
IFN-related gene	Ifitm3	8279	39372
	Ifitm2	3242	5889
	Ifit2	52	894
Others	F5	0	86
	Rhoc	6	58
	Rhoh	1260	863
	F2r	800	181

Fig. S5. Morphological features of inflammatory MKs and transcriptional profiles of cells enriched in Sca1/EGFP^{+} CD41^{+} progenitors. (**A**) Sca1/EGFP Tg mouse BM cells were cultured in the presence of mouse thrombopoietin (TPO; 50 ng/mL) or Gardiquimod (GDQ; 2 μg/mL) for 2 days. Cells were stained with the indicated antibodies after fixation and permeabilization. Immunofluorescence imaging of Sca1/EGFP (green), CD41 (red), CD11b (white) and Hoechst (blue) were obtained using LSM880 confocal microscope (Zeiss). Scale bars =10 μm. (**B**) Sca1/EGFP Tg mice were treated with or without GDQ (1 mg/kg) for 17 hours and BM cells were collected. FSC^{low} SSC^{low} cells (P1) were gated to exclude the population that contains mature MKs (P2). EGFP^{high} CD41^{high} cells (black rectangles) enriched in Sca1/EGFP^{+} CD41^{+} progenitors were sorted and subjected to bulk RNA-Seq. (**C**) Values are shown as Transcripts Per kilobase Million (TPM). Selected differentially expressed genes (>1.5 times up or down difference in two independent experiments) are shown.

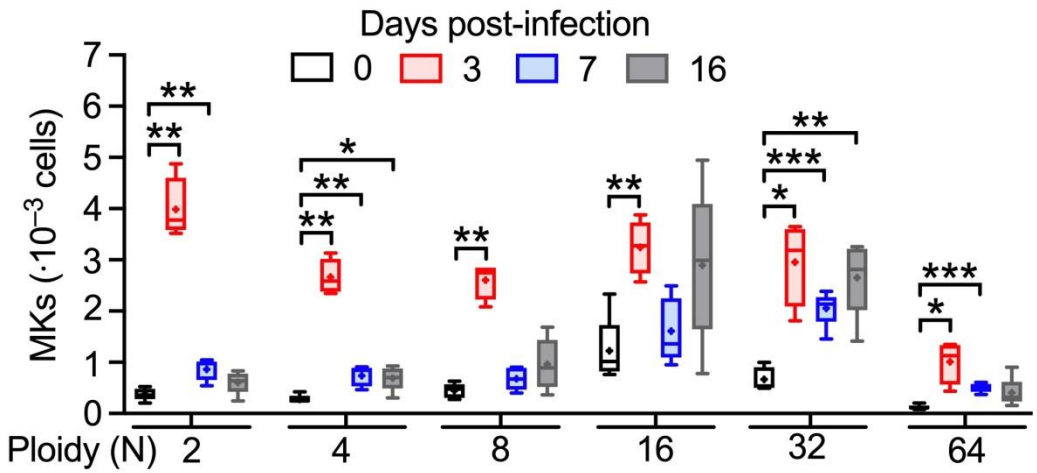

Fig. S6. Ploidy analysis of Sca1/EGFP$^+$ MKs from Sca1/EGFP Tg mice infected with LCMV-A. Ploidy distribution of Sca1/EGFP$^+$ MKs was evaluated in BM cells harvested from femurs of Sca1/EGFP Tg mice before (0) and at the indicated days after LCMV-A infection. MK numbers per femur are shown as 25th-75th percentile boxes with min-to-max whiskers, line at the median and cross at the mean. Data were analyzed by RM two-way ANOVA with no sphericity assumption (Geisser-Greenhouse correction) and Dunnett's post-test for multiple pre-post comparisons. *P < 0.05, **P < 0.01, ***P < 0.001; n = 5 for days 0, 7 and 16; n =4 for day 3.

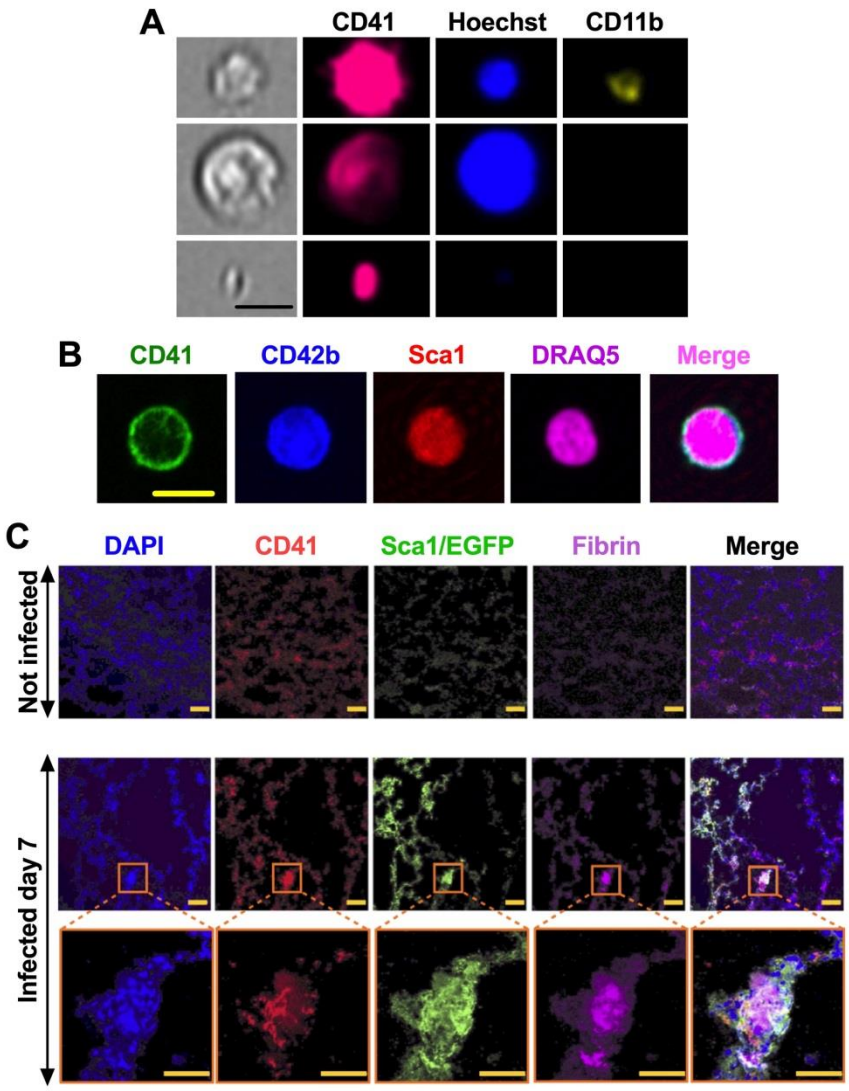

Fig. S7. Immunofluorescence microscopy analysis of CD41$^+$ circulating cells and lung tissue in LCMV-A infected or control (not infected) mice. (**A**) Mouse blood was diluted in PBS with 2 mM EDTA, treated with Fc blocking antibodies and stained with anti-CD41 and anti-CD11b antibodies; nuclei were counterstained with Hoechst. Images were acquired by imaging flow cytometry using the Amnis ImageStream XMk and data analyzed using Amnis Image Data Exploration and Analysis Software (IDEAS; Luminex). (**B**) Blood cells were stained with anti-CD41, anti-CD42b and anti-Sca1 antibodies; nuclei with DRAQ5. Cells were sorted using MoFlo Astrios (Beckman Coulter), fixed with 2% paraformaldehyde and attached to glass slides before imaging. (**C**) Lung tissue from Sca1/EGFP Tg mice not infected (upper row) or 7 days post-infection with LCMV-A (middle and lower rows) was cryosectioned, fixed, permeabilized and stained with antibodies against CD41, EGFP and fibrin; nuclei were counterstained with DAPI. Magnified images of the cropped area enclosed by rectangles in the middle row are shown in the lower row. Images in (B, C) were taken with a LSM880 confocal microscope (Zeiss). Scale bars: A, B = 5 μm; C, upper and middle rows = 100 μm, lower row = 50 μm.

References

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