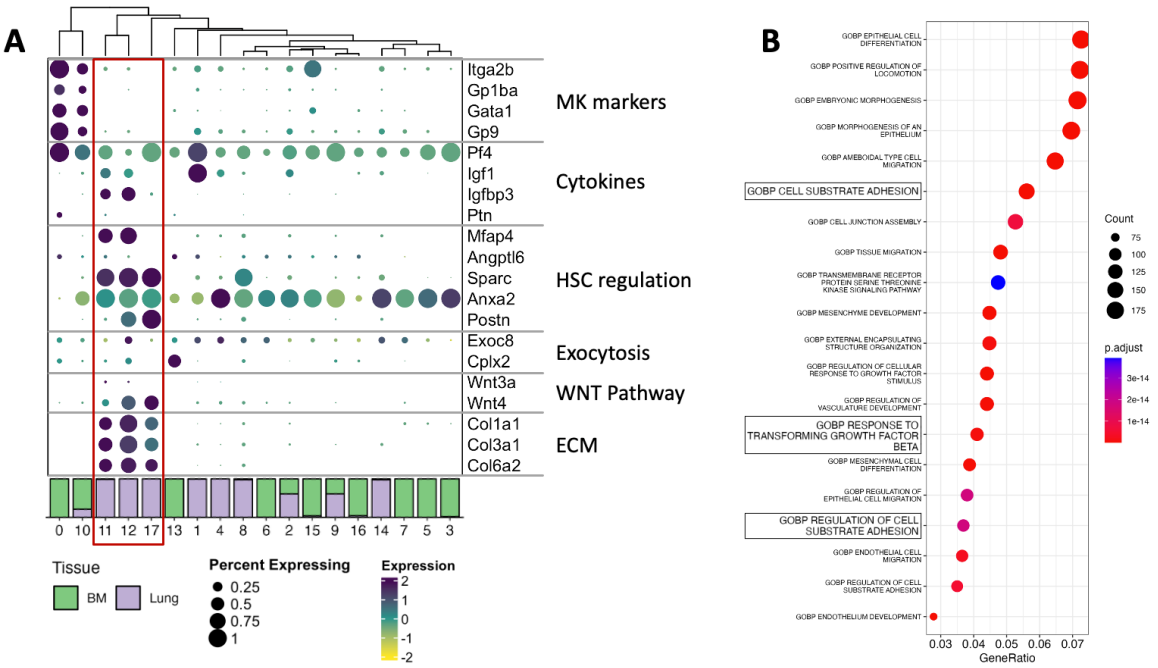
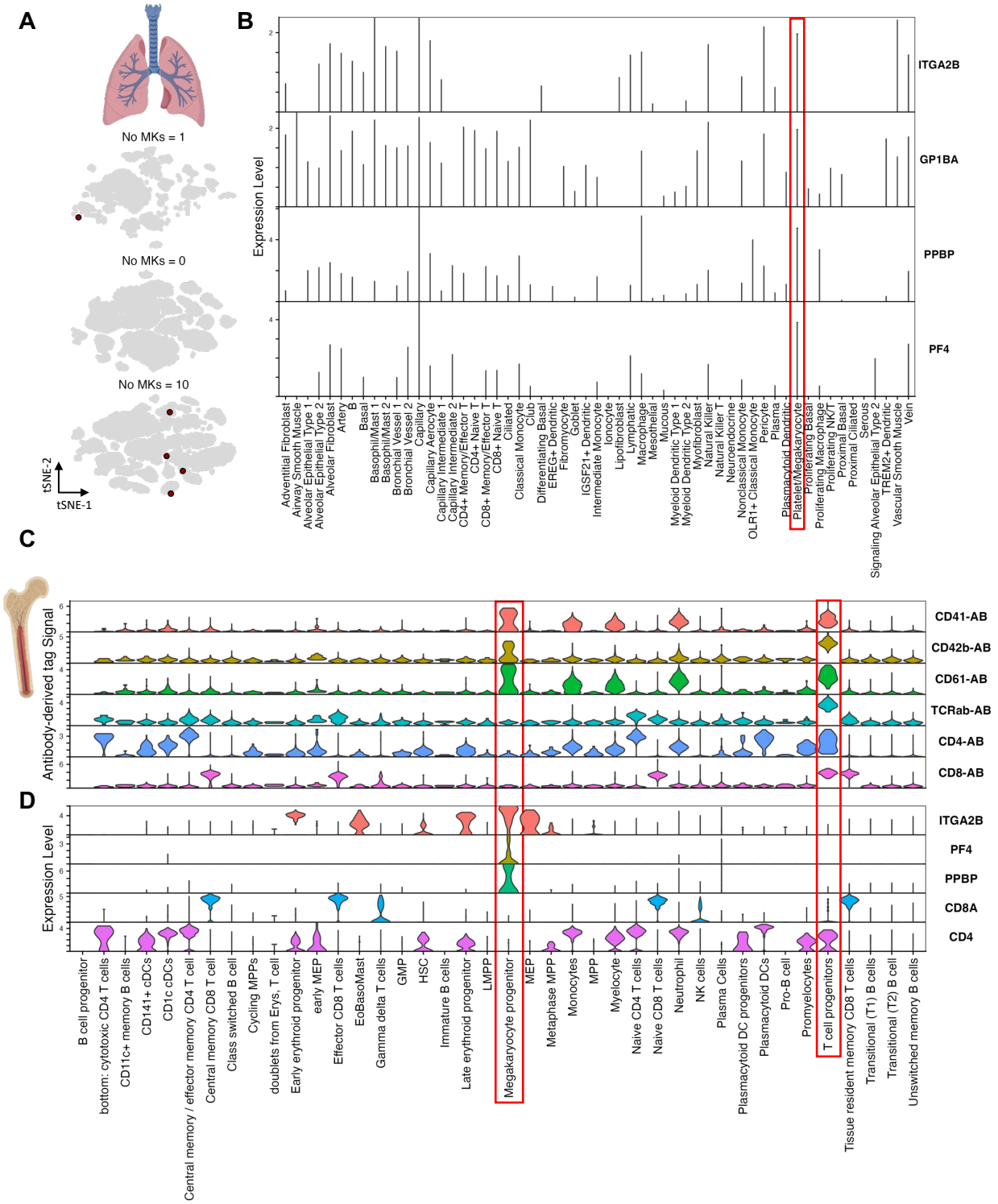
Supplementary material
Figure 1



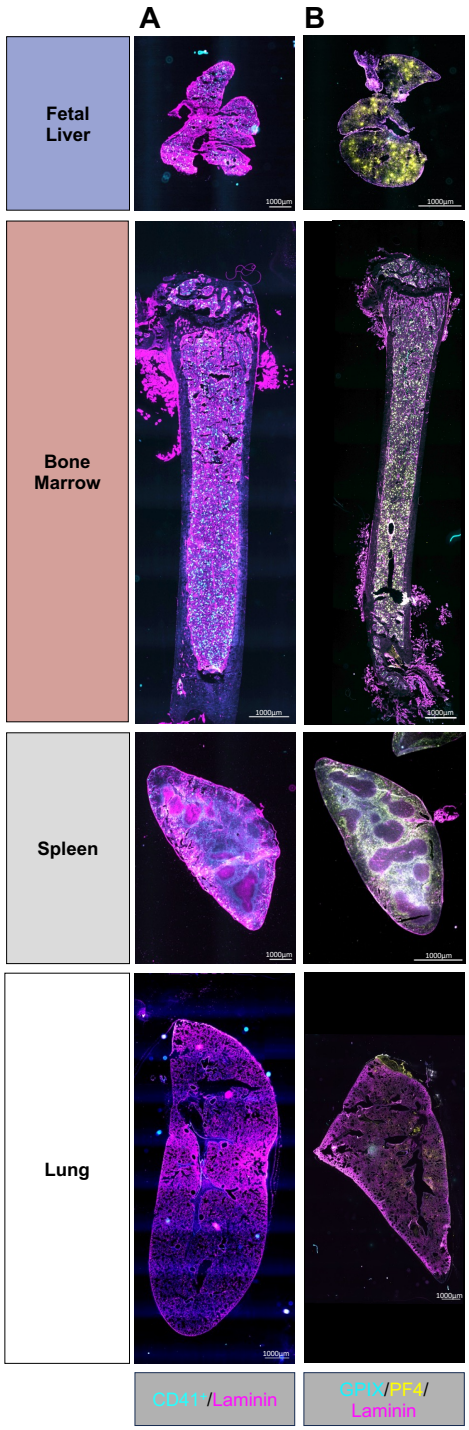
<u>Supplementary Figure 1:</u> Niche MKs were not found in the murine lung : (A) Bubble plot showing gene expression of Niche-MK-related genes in Yeung et al. (Blood Adv 2020)'s dataset. Clusters showing the highest expression for niche-MK genes are highlighted with a red box. (B) Bubble plot showing the results of the Gene Ontology (Biological Process) overrepresentation analysis on differentially upregulated genes in clusters 11, 12, and 17. Gene ontologies previously related to niche MKs are highlighted.

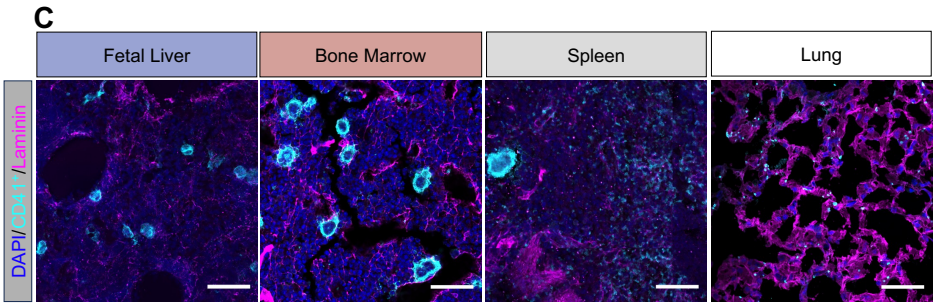
Supplementary material
Figure 2

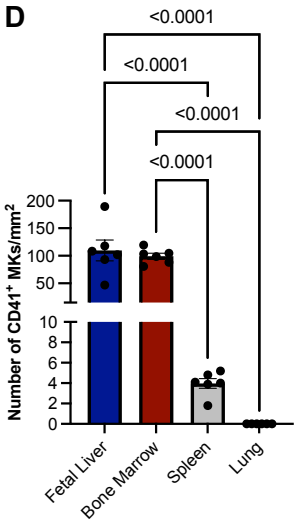


Supplementary Figure 2. scRNAseq and CITE-seq atlases of human Lung and Bone Marrow show that bone marrow is the primary site for thrombopoiesis. (A) tSNE plots of the scRNAseq human lung atlas from three patients. Cells classified as megakaryocytes are labeled in red. (B) Violin plots showing RNA expression of MK markers across clusters on the lung atlas, n = 3 donors. (C) Violin plots showing antibody-derived tag (ADT) signal of MK and T cell markers across clusters on the human Bone Marrow expression atlas. (D) Violin plots showing RNA expression of MK markers across clusters on the BM expression atlas.

Supplementary material
Figure 3

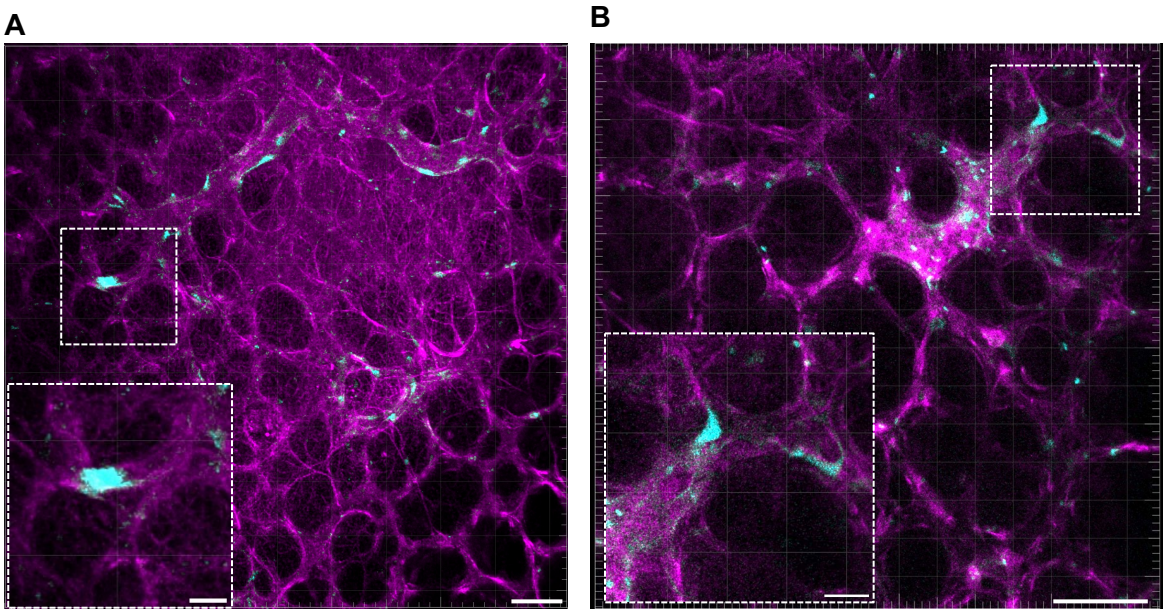






Supplementary Figure 3. In situ, labeling, and ex vivo culturing methods show that murine adult bone marrow is the primary site for megakaryopoiesis. (A) CD41⁺ and laminin composite, representative widefield micrographs of complete cryosections obtained from mouse fetal liver, bone marrow, spleen, and lung. Scale bars represent 1000µm. (B) GPIX⁺, PF4⁺, and laminin composite, representative widefield micrographs of complete cryosections obtained from mouse fetal liver, bone marrow, spleen, and lung. Scale bars represent 1000µm. (C) Representative structured illumination micrographs of mouse fetal liver, spleen, and lung cryosections. Blue = DAPI, cyan = CD41⁺, magenta = laminin. Scale bars are 50µm. (D) Quantitative analysis of megakaryocyte count (CD41⁺ and >16u diameter) within whole organ cryosections MK/mm^2 blue = fetal liver, red= bone marrow, grey = spleen, and white = lung. Data is shown as mean ± SEM; graphs represent data from six mice. Statistics were performed with 1-way ANOVA with Tukey's multiple comparisons test at 95% CI; $p < 0.05$.

Supplementary material
Figure 4



Supplementary Figure 4. Live two-photon in-vivo imaging of lung tissue reveals rare intravascular MKs. (A-B) Representative two-photon micrographs of eGFP^{+}, >16μm diameter events passing through the adult murine lung vasculature, depicting potential MK and proplatelet-like fragments. Scale bars represent 50μm and 15μm in the enlargement.

Supplementary Methods

Materials

DMEM complete (Complete Dulbecco's Modification of Eagle's Medium) consisted of 1x DMEM containing 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning) with the supplementation of 10 % v:v fetal bovine serum (Sigma) and 100 U/mL Penicillin-Streptomycin (Gibco).

Isolation of organs and processing of cells

Bones (Femurs, tibias, iliac crests), spleens, and lungs were harvested from mice on a C57BL/6J background. Primary mouse fetal liver megakaryocytes (FLMKs) were generated by creating a single-cell suspension of Day 13.5 CD-1 mouse fetal livers, as described previously [1]. The bones of each mouse were individually harvested and cleaned of muscle and tissue. The bone marrow was spun and isolated at 2500 g for 40 seconds, resuspended into 100 μl of PBS, and kept on ice until all organs were processed. Spleens were crushed and simultaneously filtered through a 100 μm strainer in 5 mL of PBS; the resulting cells were pelleted at 200 g for 5 minutes and kept on ice until further processing. Lungs were perfused with PBS, followed by a cocktail of 2 mg/mL collagenase-dispase solution (cat no# 10269638001) and 0.025 mg/mL DNAse (cat no #D4527-10KU); lungs were then transferred to an Eppendorf tube on ice containing the collagenase-dispase-DNAse cocktail and minced using surgical scissors and allowed to digest for 1 hour at 37°C in a rotating incubator (Benchmark Scientific, NJ, USA). ACK lysing buffer (cat no #A1049201, Thermofisher, MA, USA) was used to lyse red blood cells for all bone, spleen, and lung samples for 5 minutes at RT, followed by a PBS wash. HSPCs harvested from each organ were differentiated into megakaryocytes using DMEM-complete supplemented with in-house conditioned medium containing thrombopoietin (TPO) produced by a modified fibroblast cell line as previously described [2]. Following four days of culture, megakaryocytes differentiated from all organ types were imaged using a Nikon TS-2 microscope equipped with a Plan Achromat NA: 0.4, a working distance of 3.1 mm. Megakaryocytes were enriched by applying the cell media supernatant to bovine serum albumin (BSA) density gradient (d = 1.5 – 3.0%) for 25 minutes at 37°C, 5% CO_2. The resulting sedimented cell fraction was collected and resuspended in 1 mL of DMEM complete.

Flow cytometry

Single-cell suspensions from bone marrow, fetal liver, spleen, and lung were analyzed using the Cytek Aurora spectral flow cytometer four laser system (16V-14B-10YG-8R). Diva software (BD Biosciences) and FlowJo (Tree Star Inc.) were used for data acquisition and analysis. Megakaryocytes were defined as follows ($CD45^+Lin^-Cd41^+CD42d$) HSPCs were defined as follows: Megakaryocyte Progenitors ($CD45^+Lin^-Sca\text{-}1^-cKit^+$ $CD150^+CD41^+$), LT-HSC ($CD45^+Lin^-Sca\text{-}1^+cKit^+Flt3^-$ $CD150^+CD48^-$), short-term HSCs ($CD45^+$ST-HSC; $Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-$ $CD150^-CD48^-$), and multipotent progenitors (MPP4; $CD45^+Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^+$) (MPP3; $CD45^+Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-CD48^+CD150^-$) (MPP2; $CD45^+Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-$ $CD48^+CD150^+$). Data are shown as mean ± SD; graphs represent data from at least three independent mice. Statistics were performed with 1-way ANOVA with Tukey's multiple comparisons test at 95% CI; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

In-situ quantification of megakaryocyte number

Intact organs were extracted as described above from three independent mice were washed in PBS, and were fixed with 4% Paraformaldehyde (cat no. #158127-500G, Millipore Sigma, MA, USA) for 24 hours, followed by a sucrose gradient (10-30%) increasing daily. Organs were then embedded in OCT compound (cat no. #23-730-571) or SCEM embedding medium (cat no. #R031006, SECTION-LAB Co. Japan) and snap frozen on a layer of super-chilled ethanol and stored at -80°C. For each organ, a Cryostat CM3050 S (Leica Biosystems) was used to create ~10μm thick sections of each organ and mounted onto a charged microscopy

slide (cat no. #12-550-16, Fisher Scientific, MA, USA). Cryosections were blocked in 1% BSA, followed by primary antibody labeling overnight at 4°C with CD41 1:200 (Biolegend, Rat mAb #133902) or laminin 1:200 (Millipore Sigma, rabbit pAb #L9393). Secondary antibody staining was performed for 2 hours at room temperature using a combination of AlexaFluor® antibodies (488-647nm, ThermoFisher) raised against the relevant primary antibody human, mouse, or rat epitopes and DAPI. Cryosections were washed with PBS, mounted onto coverslips, and stored at -20°C until imaged.

Labeled cryosections were then imaged using a Lionheart FX automated microscope using a Phase objective, 4x Olympus Plan Fluorite NA: 0.13, working distance 17 mm in conjunction with a GFP imaging filter cube, EX 469/35 nm, EM 525/39 nm, dichroic mirror 497 nm, and CY5 Imaging filter cube, EX 628/40 nm, EM 685/40 nm, dichroic mirror 660 nm. Whole organ sections were imaged and stitched automatically within Gen 5.10 software (Agilent, CA, USA). The raw data sets for each channel were exported individually and analyzed in ImageJ. A median filter with a radius of 1 pixel was used to perform background subtraction. The criteria for MK quantification were as follows: objects that were 16-50μm in diameter and reported a $CD41^+$ or $GPIX^+$ (depending on the panel analyzed) fluorescence intensity value between 20,000 and 65525. A custom polygon was created around the organ outline to calculate the total organ area, and MK number/mm^2 was calculated for each organ. A One-way ANOVA with multiple comparisons was used to determine the statistical significance of MK number/mm^2 between the fetal liver, bone marrow, spleen, and lung cryosections.

Additionally, structured illumination laser scanning confocal microscopy was used to create a high-resolution, representative image of each organ type using the same cryosections used for the quantified Lionheart FX data. The Zeiss laser scanning confocal microscope (LSCM) equipped with a Plan-Apochromat 20x/0.8 Oil DIC M27 objective and Airyscan detector (chA) was set to super-resolution mode with a digital magnification of 1.8x. Images were taken in a singular plane with a total X: Y pixel density of 7323 x 7418 (2x2 centered grid with 5% overlap). The following settings were used. Lasers; 405, 488, and 633 with beam splitters, FW:1 Rear, DBS1: Plate, MBS-Invis: 405, MBS-vis: MBS- 488/561/633. Filters, chA 405: BP 420-480 + BP 495-550, chA 488: BP 420-480 + BP 495-550, chA 647: 495-550 + LP 570. Deconvolution of the Airyscan images was performed using the ZEN black 2.3 SP1 software (Carl Zeiss, CA, USA). Final Images (X:Y 4200x 4200 pixels) were stitched, and scale bars were added using Zen Blue 2.3 software (Zeiss, CA, USA).

Light-sheet fluorescence microscopy (LSFM)

Mice

Animal experiments related to light-sheet fluorescence microscopy (LSFM) were approved by the district government of Lower Frankonia (Bezirksregierung Unterfranken). C57BL/6JRj mice, obtained from Janvier Labs, were used at 8-12 weeks of age.

Antibodies and reagents

Anesthetic drugs (medetomidine (Pfizer, Karlsruhe, Germany), midazolam (Roche, Grenzach-Wyhlen, Germany) and fentanyl (Janssen – Cilag, Neuss, Germany)) were used according to the local regulations. Mice received anti-CD105 Alexa647 (0.4 µg/g body weight; BioLegend, San Diego, CA, USA) and anti-GPIX Alexa750 derivative (0.6 µg/g body weight; lab-made [3] to label blood vessels and megakaryocytes, respectively.

Tissue preparation for LSFM

Platelets/MKs and BM vasculature were stained by injecting Alexa Fluor 750-labeled anti-GPIX derivative (0.6 µg/g body weight) and anti-CD105 Alexa Fluor 647 (0.4 µg/g body weight), respectively. 30 min following injection, the mice were transcardially perfused with ice-cold PBS to wash out the blood and ice-cold 4% paraformaldehyde (PFA, Sigma-Aldrich,

Schnelldorf, Germany (pH 7.2) to fix the tissues. Lung, spleen, and femora were harvested and stored in 4% PFA for 30 minutes. Bone samples were washed in PBS and decalcified in 10% ethylenediaminetetraacetic acid (EDTA, AppliChem, Darmstadt, Germany) for 96 hours at 4°C on a shaker. Samples were washed in PBS, followed by dehydration in a graded methanol (Sigma-Aldrich) series (50%, 70%, 95%, 100% for 30 minutes each) at RT and stored at 4°C overnight. The methanol was replaced stepwise by a clearing solution consisting of 1 part benzyl alcohol to 2 parts benzyl benzoate (BABB, Sigma-Aldrich). After incubation in the clearing solution for at least 2 h at RT, tissue specimens became optically transparent and were used for LSFM imaging the following day.

LSFM data acquisition and image analysis

Image acquisition of cleared samples was performed in a home-built scanning light-sheet fluorescence microscope as described previously [3]. The specimen was mounted on a motorized (8CMA06-25/15 Standa, Vilnius, Lithuania) four-axis stage (XYZ translation (Newport, Darmstadt, Germany) in a custom-built coverglass chamber filled with BABB solution allowed imaging of the cleared sample immersed in clearing solution. A custom fiber coupled laser combiner (BFI OPTiLAS GmbH, Groebenzell, Germany) with two red laser lines (642 nm and 730 nm) was used for fluorescence excitation. For collimation, an objective lens (A10/0.25 Hund, Wetzlar, Germany) providing a beam diameter of roughly 3 mm was used. All laser lines were joined via a dichroic beamsplitter (DCLP 660, AHF Analysentechnik, Tübingen, Germany). A galvanometric scanner (6210H, Cambridge Technology, Bedford, MA, USA) elongated the resulting laser beam with a frequency of 600 Hz before being focused via a theta lens (VISIR f. TCS-MR II, Leica, Mannheim, Germany) to create a virtual light sheet. The focused beam was relayed with a tube lens and an objective lens (EC Plan-Neofluar 5x/0.16 M27, Zeiss, Göttingen, Germany) to the sample. For detection, a HCX APO L20x/0.95 IMM objective (Leica, Wetzlar, Germany) was mounted on a translation stage (Newport) perpendicular to the light sheet. The fluorescence emission was spectrally filtered using a motorized filter wheel (MAC 6000 Filter Wheel Emission TV 60 C 1.0x (D)) with a MAC 6000 Controller (Zeiss, Göttingen, Germany) equipped with the following filters according to the fluorescence of the used fluorescent labels: 642 nm HQ 697/58 (Alexa Fluor 647) and 730 nm BrightLine HC 785/62 (Alexa Fluor 750) (AHF). The image was generated by an infinity-corrected 1.3x tube lens ∞/240-340 (098.9001.000, Leica, Wetzlar, Germany) and detected by an sCMOS camera Neo 5.5 (2560 x 2160 pixels, 16.6 mm x 14.0 mm sensor size, 6.5 µm pixel size, Andor, Belfast, UK). Synchronization of the laser illumination, image acquisition, focus correction, and z-scanning was controlled by IQ 2.9 software (Andor). Multicolor stacks were generated by imaging the three color channels sequentially in each plane in increments of 2 µm. Images were saved as TIFF stacks. Multicolor LSFM stacks were processed and analyzed by FIJI [4] and Imaris® 9.9.1 (Bitplane AG, Zurich, Switzerland). Data visualization and analysis were performed in three major steps: (i) image preprocessing, (ii) segmentation, (iii) data extraction. Step (i) was performed using either Imaris or FIJI, steps (ii) and (iii) were performed using Imaris® 9.9.1, including the ImarisCell module. Megakaryocytes were counted manually for lung and spleen specimens; MK numbers and volumes were derived from Imaris for femora. Statistical analysis was performed using GraphPad Prism 7.04 (GraphPad Software Inc., Boston, MA, USA). Supplementary movies were generated with Imraris 9.9.1 and Shotcut 23.06.14 (Meltytech, LLC).

Bone Marrow Intravital Imaging

C57BL/6 vWF-eGFP mice were anesthetized using isoflurane in oxygen and placed on a heated stage. The mouse head was immobilized using a head holder with an anesthetic mask (Narishige SGM4). The skin between the parietal and frontal bones was gently removed with scissors. The periosteum covering the calvarial bone was removed, and a custom-made head stabilizer was adapted onto the bone.

Spleen Intravital Imaging

Anesthetized mice were placed on a heated stage. An incision was made below the ribcage on the left lateral position. The spleen was exposed, and an imaging apparatus, consisting of a suction window, was attached to a micromanipulator on the microscope stage, placed into position, and 25–35 mmHg of suction was applied (Amvex Corporation) to immobilize the spleen.

Lung Intravital Imaging

Anesthetized mice were placed on a heated microscope stage and underwent a tracheostomy. The tubing was adapted to a rodent ventilator to facilitate mechanical ventilation (model 845 - Harvard Apparatus). Mice were ventilated with pressure control ventilation (12–15 cm H_2O), a respiratory rate of 115 breaths per minute, FiO_2 of 0.5–1.0, and PEEP of 3 cm H_2O. Isoflurane was continuously delivered at 2 % to maintain anesthesia. Mice were then placed on the right-hand side lateral decubitus position, and the thorax was exposed by removing the skin and fatty tissue above the thorax. An incision between two left anterior ribs was performed, and the left lung was carefully exposed. An imaging apparatus (which consists of a thoracic suction window (obtained from Marc Looney at UCSF) was attached to a micromanipulator on the microscope stage, placed into position, and 25–35 mmHg of suction was applied (Amvex Corporation) to immobilize the lung.

Two-photon intravital microscopy (2PIVM)

Following surgery, mice were retro-orbitally injected with Evans Blue (3µg/g of mouse body weight) to visualize the blood circulation. Mouse body temperature was monitored using a rectal probe. Intravital images were obtained with a Thorlabs Bergamo II 2-photon microscope equipped with a water-dipping objective Olympus XLUMPLFLN 20X and an sCOS camera. eGFP and Evans Blue fluorophores were excited with a 960nm laser line, (Chameleon Discovery NX with TPC) and emissions were detected with 525 nm ± 25nm and 647 → 680nm emission filters. Images were acquired in 3D with a minimum total of 512 x 512-pixels (X: Y) representing 290 x 290 µm (X:Y), and z-stacking with 2µm step size for a total minimum z coverage of 30µm was obtained, every 10 seconds per z-stack for a total of 15 minutes per field of view. Five fields of view were imaged for each organ per mouse. Images were analyzed using Imaris 10.0.1 software, and MKs ($eGFP^+$ events >16um in diameter) were detected after applying a threshold on pixel intensity, followed by a maximum z projection. The number of MKs were normalized to MK/mm^3. A One-way ANOVA with multiple comparisons was used to determine any significant differences in total megakaryocyte counts between each organ type. N = 3 mice per organ were imaged.

Ex-vivo quantification of megakaryocyte number

As described above, an enriched fraction of megakaryocytes was obtained from each organ type. Cell imaging chambers (Lab-Tek #155383) were coated with mouse 0.5 µg/ml CD31 (Biolegend, Rat mAb # 102502) at 37°C for 1 hour. The chambers were blocked with 3 % BSA for 1 hour at 37°C and 5 % CO_2. The enriched fraction of either FLMKs, bone marrow, spleen, or lungs were plated onto the cell imaging chamber and incubated overnight at 37°C to allow for proplatelet formation. The supernatant was removed, and the adhered megakaryocyte forming proplatelets were fixed with 4 % PFA – 0.1 % tween cocktail for 20 minutes at 37°C 5 % CO_2, followed by a blocking incubation using 3 % BSA. Primary and secondary antibodies were used to label proplatelet-forming megakaryocytes. Primary antibody staining was performed in 3 % BSA overnight at 4°C with CD41 1:200 (Biolegend, Rat mAb #133902) and α-tubulin 1:200 (Thermofisher, Mouse, mAb, Cat no. #A11126). Secondary antibody staining was performed for 2 hours at room temperature using a combination of AlexaFluor® antibodies raised against the relevant epitopes and DAPI. Cells were washed with PBS and incubated in PBS at 4°C until imaging. The cell imaging chambers were then imaged using Lionheart FX automated microscope using a phase objective, 4x Olympus Plan Fluorite NA: 0.13, working distance 17 mm in conjunction with the GFP imaging filter cube, EX 469/35 nm, EM 525/39 nm, dichroic mirror 497 nm, and CY5 Imaging filter cube, EX 628/40 nm, EM 685/40 nm, dichroic mirror 660 nm. The total megakaryocyte cell count was determined by observing the

total number of structures that were $CD41^+$ and were 20-60μm in size. A One-way ANOVA with multiple comparisons was used to determine any significant differences in total megakaryocyte counts between each organ type. N = 5 animals for the fetal liver, n = 8 animals for the bone and spleen, and n = 8 animals for the lung pooled into two conditions due to minimal cellular material.

Additionally, structured illumination laser scanning confocal microscopy was used to create a super-resolution, representative image of the cultured cells of each organ type using the same cell imaging chamber used for the quantified Lionheart FX data. The Zeiss laser scanning confocal microscope (LSCM) equipped with a Plan-Apochromat 63x/1.4 NA Oil DIC M27 objective and Airyscan detector (chA) was set to super-resolution mode with a digital magnification of 2.3-2.5x. Images were taken in a singular plane with an X: Y pixel density of 2024 x 2024. The following settings were used. Lasers: 405, 488, 561 with beam splitters, FW:1 Rear, DBS1: Plate, MBS-Invis: 405, MBS-vis: MBS- 488/561/633. Filters, chA 405: BP 420-480 + BP 495-550, chA 488: BP 420-480 + BP 495-550, chA 568: BP 495-550 + LP 570, Deconvolution of the airyscan images were processed directly using the ZEN black software (Carl Zeiss, CA, USA) followed by the generation of maximal intensity projections. Scale bars (10μm) were added using Zen Blue software (Carl Zeiss, CA, USA).

Live cell In vitro proplatelet forming assay (Incucyte)

As described above, three independent mice obtained an enriched fraction of megakaryocytes from each organ type. The enriched fractions were then plated in triplicate into a 96-well plate (cat no. #353072 Corning, NY, USA) and imaged in an Incucyte imaging platform (Essen BioScience) at 37ºC and 5% CO_2. Wells were imaged using a Nikon, 10x Plan Fluor NA: 0.3, working distance 16 mm in the phase contrast modality at 1-hour intervals from multiple 950 × 760–μm^2 regions per well. Label-free pixel-based machine learning using Elastik generated probability maps of cellular features followed by a custom cell profiler pipeline that distinguished MKs from proplatelets and allowed the quantification of the percentage of proplatelet formation per MK for each organ. An unpaired t-test was performed to calculate significate differences in percentage proplatelet formation between FLMK and BMK cultures.

Assembly of custom microfluidic devices to assess proplatelet formation

PDMS (polydimethylsiloxane)/glass Microfluidic chips were fabricated using photolithography and soft lithography. The master (serving as a mold) was created by replicating the patterns designed using AutoCAD and printed on a high-resolution Chromium/Glass mask on a 76mm silicon wafer (University Wafers, MA, USA). The microfluidics device consisted of 2 inlet channels. The central channel: H: 30μm x W:240μm x L: 2400μm with two adjacent, parallel side channels that were H:30μm x W:60μm x L: 2400μm. A series of pillars separated the central and side channels (10 μm wide and 90 um long) spaced 2 μm. Photoresist SU8 2025 (Kayaku Advanced materials, MA, USA) was spun on the wafer to achieve rectangular microfluidic channels of ~30 μm deep, followed by baking at 65°C for 2 minutes and 95°C for 5 minutes and exposed to UV light (Mask Aligner SUSS MJB3- 25 mJ cm^2) through the chrome mask. The non-crosslinked SU-8 photoresist was removed by submerging the substrate into propylene glycol monomethyl ether acetate for 2 minutes. PDMS (Sylgard 184, CO, USA) was poured onto the patterned side of the silicon wafer, degassed, and cross-linked at 150°C for 30 minutes. Following curing, the PDMS layer was peeled off the mold, and the inlet/outlet holes were punched with the port creator (CoreSolutions, MA, USA) 0.75 mm diameter punch. The cover slide and the PDMS devices were ultrasonic solvent cleaned. The microchannels were sealed by bonding the PDMS chip to a glass cover slide (#1.5 0.17 x 22 x 50 mm) after treatment with oxygen plasma (Technics Plasma etcher 500-II, CA, USA).

Shear-driven proplatelet forming assay utilizing microfluidics

As described above, an enriched fraction of megakaryocytes was obtained from each organ type. The Harvard Medical School microfluidics core produced custom-built in-house

microfluidic devices. The device consisted of two channels: the central channel: H: 30μm x W:240μm x L: 2400μm with two adjacent, parallel side channels that were H:30 μm x W:60 μm x L: 2400 μm. 2 μm pores at regular 90 μm intervals along the device connected the central channel. Two independently motor-driven syringe pumps (cat no: #70-2209, Harvard Apparatus, MA, USA) were set to flow rates of 0.001mL/min for the central channel and 0.01mL/min for the adjacent side channels and connected to the custom microfluidic device using polyethylene tubing with an internal diameter of 0.01 inch (cat no. #AAD04091, Saint Gobain, Courbevoie, France). Megakaryocytes were fused into the central channel, and DMEM complete was infused into the two adjacent side channels to allow crossflow generation within the device. A Nikon Eclipse TE2000-E microscope (Nikon, NY, USA) equipped with a Plan Fluor 10x objective (Nikon, NY, USA) and ORCA Flash4.OLT camera (Hamamatsu, Shizuoka, Japan) was used to acquire brightfield images every 1 second to capture the proplatelet forming process. Individual TIFF images were compiled into TIFF stacks and converted into AVI files set to 5 fps (frames per second). The device's 1321 μm x 421 μm region was used to calculate the percentage of proplatelet-forming MKs within the fetal liver and bone marrow microfluidic device. A proplatelet forming MK was determined to be a megakaryocyte producing and releasing at least one cytoplasmic extension without the whole MK transmigrating from the central to either side channel. Megakaryocytes were counted within three independent microfluidic device runs for each organ type. A One-way ANOVA was performed to calculate significant differences in the percentage of proplatelet formation during shear conditions between cultures isolated from multiple tissues.

Bioinformatic analysis

scRNA-seq and CITE-seq datasets were analyzed using R (v4.1.3), the integrated development environment RStudio (v2022.7.2.576), and the package for single cell analysis Seurat (v4.2.0). scRNAseq count matrices from murine bone marrow and lung were obtained from the online repository GEO (accession: GSE152574) and processed using the authors' parameters as specified in their paper. Briefly, low-quality cells were filtered out based on mitochondrial content (>12%) or the number of detected genes (<800). Then, Unique Molecular Identifiers (UMIs) were normalized and scaled using Seurat's *sctransform*. Next, we performed Principal Component Analysis (PCA) and used the top 20 principal components to compute the unsupervised Uniform Manifold Approximation and Projection (UMAP) and the clusters (Louvain method, resolution = 0.75). The pro-platelet formation signature (PPF) was calculated using the function *AddModuleScore* from the Seurat package and the list of genes from Aokhan. Seurat objects for human lung and bone marrow single cell atlases were obtained from the following repositories: Lung, BoneMarrow. Data were imported to R and visualized using Seurat and ggplot2 (v3.4.0). MKs were identified using the metadata provided on the objects.

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4. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods.* 2012;9(7):671-675.