Supplementary Methods

Mice

To generate the conditional *pitpβ*$^{-/-}$ mouse, we targeted exons 4-6 of Pitpβ, introducing LoxP sites flanking this region. These mice were crossed with mice expressing CRE recombinase driven by the platelet factor 4 (PF4) promoter (obtained from Radek Skoda of the University of Basel, Switzerland) to generate *pitpβ*$^{fl/fl}$ *pf4cre*$^{+}$ mice which do not express Pitpβ specifically in their platelets and megakaryocytes. These mice were further crossed with *pitpα*$^{fl/fl}$ *pf4cre*$^{+}$ [1] to generate double-knockouts (*pitpα*$^{-/-}$*/β*$^{-/-}$*)*. Genomic DNA isolated from tail biopsies was used for the genotyping of the conditional alleles of the *pitpα* gene by PCR, using forward primer 5′-GAACAAGAAACTATCCAGCAGACAGACT -3′ and reverse primer 5′-CTTCCTCTGCCTTGTAATCCTGAG -3′. Primer pair 5′- GAGGACTGCTGTGTCTGCTGC -3′ and 5′- GTTTAGCTATGTAAGGGTTACTGTGCA -3′ was used for genotyping of *pitpβ* conditional allele.

Determination of protein concentration by ELISA

For TGF-β1 ELISA, a 96-well microplate was coated with TGF-β1 capture antibody overnight at 4°C. The plate was washed (0.05% Tween 20 in PBS) and blocked for one hour with blocking buffer (5% Tween 20 in PBS). During this period, CM or BM flush fluid were treated to activate TGF-β1 by incubation with 1 N HCl for 10 minutes at room temperature (RT) and neutralized with 1.2 N NaOH/ 0.5 M HEPES. The plate was blocked and incubated with biotinylated TGF-β1 detection antibody for 2 hours at RT. The plate was incubated with streptavidin-HRP for 20 minutes in the dark at RT. After each step, the plate was washed. The plate was developed with a substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) for 20 minutes, and the color change reaction stopped with 2 N H_2SO_4. Absorbance was read at 450 nm and compared to a standard curve of rTGF-β1.

Western blot

To detect Pitpα and Pitpβ levels in lineage negative, CD61 negative bone marrow (BM) cells, BM cells flushed from the femurs and tibias of *pitpα* WT, *pitpα*$^{-/-}$, *pitpα/β* WT and *pitpα*$^{-/-}$/*β*$^{-/-}$ mice were lineage depleted using a Direct Lineage Cell Depletion Kit (mouse; Miltenyi Biotec) and CD61 depleted using a CD61 MicroBeads Mouse and Rat Kit (Miltenyi Biotec) according to manufacturer's instructions. These cells plus splenocytes from a wildtype C57BL/6 mouse were then processed to make lysates and used for Westerns as described in primary Methods section. The primary antibodies used in this study include rabbit-anti-mouse TGF-β(1,2,3) (catalogue # 3711, Cell Signaling, Danvers, MA; 1:1000), rabbit-anti-mouse beta-actin (clone 13E5, Cell Signaling; 1:1000), rabbit-anti-mouse TSP-1 (catalogue # 14778, Cell Signaling; 1:1000), and rabbit-anti-human vWF (catalogue # A0082, Dako, Glostrup, Denmark; 1:1000), mouse anti-human PITPα (5F12) (sc-13569, Santa Cruz Biotechnology, Santa Cruz, CA; 1:400); rabbit anti-human PITPβ (ab83795, Abcam, Cambridge, MA; 1:1000). The secondary antibodies are HRP conjugated anti-rabbit IgG (catalogue # 7074, Cell Signaling; 1:2500) and HRP conjugated anti-mouse IgG (catalogue # 7076, Cell Signaling; 1:2500).

Transmission electron microscopy

After subsequent buffer washes, the samples were post-fixed in 2.0% osmium tetroxide for 1 hour at room temperature, and rinsed in distilled H_2O prior to en bloc staining with 2% uranyl acetate. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in EMbed-812 (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate.

Immunophenotyping of BM HSC and HPC by flow cytometry

All antibodies were purchased from either BD Biosciences (San Diego, CA) or BioLegend (San Diego, CA). For BM HSC/HPC analysis the following panel of antibodies were used: FITC-anti-mouse lineage (lin) cocktail (anti-CD3/Gr-1/CD11b/B220/Ter-119; BioLegend), PE-CF594-anti-mouse Sca1 (clone D7), APC-H7-anti-mouse c-kit (clone 2B8), BV421-anti-mouse CD34 (clone RAM34), APC-anti-mouse Flt3 (clone A2F10.1), and PerCp-Cy5.5-anti-mouse FcγR (clone 2.4G2) all purchased from BD Biosciences with their appropriate isotype controls. For transplantation analysis, APC-anti-mouse CD45.2 (clone 104), FITC-anti-mouse CD45.1 (clone A20), PE-Cy7-anti-mouse CD11b (clone M1/70), PerCP-Cy5.5-anti-mouse CD45R/B220 (clone RA3-6B2) and BV421-anti-mouse CD3e (clone 145-2C11) were purchased from BD Bioscience. Single color compensation and isotype controls were included in each experiment. Data analysis was performed using FlowJo 7.6.3 software (TreeStar, WA). Gates were determined using fluorescence-minus-one controls. The percent of each population and that of live cells were used to calculate absolute numbers of each cell type per femur.

HPC assays

For experiments examining effects of MK CM from *pitpα*$^{-/-}$, *pitpα*$^{-/-}$/*β*$^{-/-}$ or *pitp* WT mice on colony formation of wild type C57Bl/6 BM HPC, 100 µL CM or control media was added to each plate with indicated growth factors. To assess effects of TGF-β1 or IL-4, control media with rTGF-β1 or rIL-4 or CM were incubated with 1 µg/mL anti-mouse TGF-β(1,2,3) neutralizing antibody (clone 1D11; R&D Systems, Minneapolis), anti-mouse IL-4 neutralizing antibody (clone 30340; R&D Systems, Minneapolis) or Rat IgG_1 isotype control (clone 43414; R&D Systems, Minneapolis) for 1 hour at 4°C prior to assaying the CM. 10 ng/mL rTGF-β1 or rmIL-4 (R&D Systems, Minneapolis) served as a positive control. Doses of neutralizing antibodies were used according to manufacturer's dose curves. Percent HPC in the S-phase of the cell cycle was estimated by high-specific-activity tritiated thymidine kill by pretreating BM cells with tritiated thymidine for 40 minutes at RT.

Hematopoietic stem and progenitor cell engrafting studies

Donor BM cells were collected from *pitp* WT and *pitpα*$^{-/-}$/*β*$^{-/-}$ mice (CD45.1^{-} CD45.2^{+}). These cells were mixed at various doses (25 000, 50 000, or 100 000) with 1x10^{5} BM cells from Boy/J competitors (CD45.1^{+} CD45.2^{-}) then intravenously injected into F1 mice (CD45.1^{+} CD45.2^{+}) that had received 10Gy total body irradiation 24 hours before transplantation. Following 1, 2, 3 and 4 months peripheral blood (PB) was collected and analyzed for the percent donor cell engraftment by flow cytometry. At 4 months, the mice were euthanized and the BM was examined for the percent donor cells by flow cytometry. The number of mice with greater than 10% donor-derived BM cells was determined for each dose of cells used. The CRU frequency was calculated using L-Calc software (Stem Cell Technologies, Vancouver, BC, Canada) and plotted using ELDA software (http://bioinf.wehi.edu.au/software/elda/).

Determination of cytokine/growth factor concentrations by BioPlex

The *pitpα*$^{-/-}$, *pitpα*$^{-/-}$/*β*$^{-/-}$ and WT MK CM was produced as previously described in Methods then analyzed for the presence of cytokines and chemokines using the MILLIPLEX® MAP Kit Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA) according to manufacturer's specifications. The cytokines/chemokines chosen for analysis include: IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-9, IL-10, IL-17, IL-20, IL-21,IL-22, MIP2, MDC, TIMP1, KC, MIP3β, TARC, RANTES, TNFα, IL-4, IL-5, IL-12p40, IL-12p70, IL-13, IL-15, IL-23, IL-25, IL-27, IL-28B, M-CSF, G-CSF, MIG, MIP1β, MCP1, MCP5, MIP1α, MIP3α, GM-CSF, VEGF, eotaxin, and IFNγ.

Complete blood counts

Peripheral blood was collected through retro-orbital puncture. Complete blood counts and mean platelet volumes were analyzed using a Drew Hemavet Hemacytometer (HV1700).

Counting MK and ploidy

To measure the MK population, BM was collected from femurs of 8 week old *pitpα*$^{-/-}$/*β*$^{-/-}$ and *pitp* WT mice. Femurs were flushed out with a 26 gauge needle with CATCH buffer (3.5% BSA, 0.38% trisodium citrate, 2 mM theophylline, 1 mM adenosine in DPBS) and the marrow was aspirated several times to segregate the tissue into individual cells. The cell suspension is further filtered through a 70 μm cell strainer (Fisherbrand) to remove remaining cell clumps. These cells were spun down at 70g for 10 min and incubated with RBC lysis solution (Qiagen) at room temperature to remove red blood cells. The cells were spun again and washed before stained with anti-CD41 (integrin αIIb, clone MWReg31, BD Biosciences) antibodies conjugated with Alexa 488 (BD Biosciences) and CD41^{+} cells were counted on the Facs Calibur flow cytometer (BD Biosciences) then gated for size to discriminate platelets which were also CD41^{+}. To determine the DNA ploidy of the MK, BM were also counterstained with a DNA dye propidium iodide (Sigma Aldrich) to quantify the relative DNA content of each cell. FlowJo analysis software (version 7.6.5) was used to analyze all flow cytometry data.

Megakaryocyte culture for proplatelet and platelet survival infusion assays

Fetal livers (E13.5) were harvested from *pitpα*$^{-/-}$/*β*$^{-/-}$ and *pitp* WT mice and aspirated several times through a 21 gauge needle to create a single cell suspension. It was filtered with a 70 μm cell strainer (Fisherbrand) to remove tissue debris and cell clumps. Fetal liver cells were cultured for eight days with 100 ng/ml recombinant mouse thrombopoietin (rmTPO; R&D Systems, Minneapolis, MN) in culture media (IMDM (Invitrogen), 10% FBS (Sigma-Aldrich), 1% Pen/Streptomycin (Invitrogen)). Eight to ten day old, cultured MK were spun down in a 1.5%/ 3% BSA step gradient.[2] The cells were resuspended to 2.5 million cells per 250 μL. After, anesthetization with 1.8% isoflurane in O_2 (Piramal Healthcare), MK were injected into the retro-orbital sinus of transgenic mice (mouse (m) αII$^{+/+}$/human (h) αII$^{-/-}$) that lack mouse αII but express human αII on their platelets, which allowed discrimination of between donor (mouse (m)

αIIb$^{+/+}$/human (h) αIIb$^{-/-}$) and recipient (mαIIb$^{-/-}$/hαIIb$^{+/+}$) platelets described previously.[3] After injection, blood was collected retro-orbitally for at various time points. Cells were double stained with monoclonal FITC-conjugated mouse anti–human CD41 Ab (h-αIIb, clone HIP8, eBioscience) and monoclonal phycoerythrin-conjugated rat anti–mouse CD41 Ab (m-αIIb, clone MWReg30, BD Biosciences) for 30 minutes and analyzed by flow cytometry to determine the relative platelet population over time as described.[1] To determine percent of MK with proplatelets, seven day old cultured MK (derived from fetal liver cells as described above) were plated onto glass bottom dishes (MatTek Corporation) and imaged after 24 hour incubation (20X). After 24 hours, cells which had at least one proplatelet were counted as a percentage of all the cells in a field.

Histology and Immunohistochemistry

All slides were prepared by the Comparative Pathology Core at University of Pennsylvania School of Veterinary Medicine. Femurs were fixed overnight in 10% formalin (Fisherbrand), decalcified for three days in Cal-Ex decalcifier solution (Fisherbrand), and embedded in paraffin before sectioned onto slides. For immunohistochemical staining, the slides were de-paraffinized with xylene, dehydrated in ethanol, and then treated with 20 µg/ml proteinase K in TE buffer, pH 8.0 containing 0.5% triton X-100 for 15 min at 37 °C. The slides were then treated with 3% H_2O_2 for 15 min to block endogenous peroxidase, and then treated with 2.5% normal serum for 60 min to block non-specific antibody binding. The tissue sections were stained with goat anti-human Integrin αIIb (C-20) (sc6602, Santa Cruz Biotechnology; 1:75) and peroxidase secondary antibody as instructed by the ImmPRESS reagent kit (Vector Laboratories). The signals were visualized by developing with DAB as indicated by the ImmPact DAB peroxidase substrate kit (Vector Laboratories; 2.5 minutes DAB exposure).

References

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2. Fuentes R, Wang Y, Hirsch J, et al. Infusion of mature megakaryocytes into mice yields functional platelets. *J Clin Invest.* 2010;120(11):3917-3922.

3. Thornton MA, Zhang C, Kowalska MA, Poncz M. Identification of distal regulatory regions in the human alpha IIb gene locus necessary for consistent, high-level megakaryocyte expression. *Blood.* 2002;100(10):3588-3596.

Supplemental Figure Legends

Supplemental Figure 1. (A) Western blot of *pitp* WT *and* KO MK lysates probed with anti-pitp antibodies to confirm deletion. (B) Western blot of *pitp* WT and KO lineage negative and CD61 negative BM cells examining Pitpα and Pitpβ levels. Splenocytes were used for a positive control where necessary.

Supplemental Figure 2. Bone marrow (BM) was collected from *pitp*$\alpha^{-/-}$ (n=4), *pitp*$\alpha^{-/-}/\beta^{-/-}$ (n=3), or littermate WT control mice (n=5) and analyzed by flow cytometry. The number of phenotypically-defined GMP (A), CMP (B), and MPP (C) per femur was determined by flow cytometry. Data are the mean ± SEM.

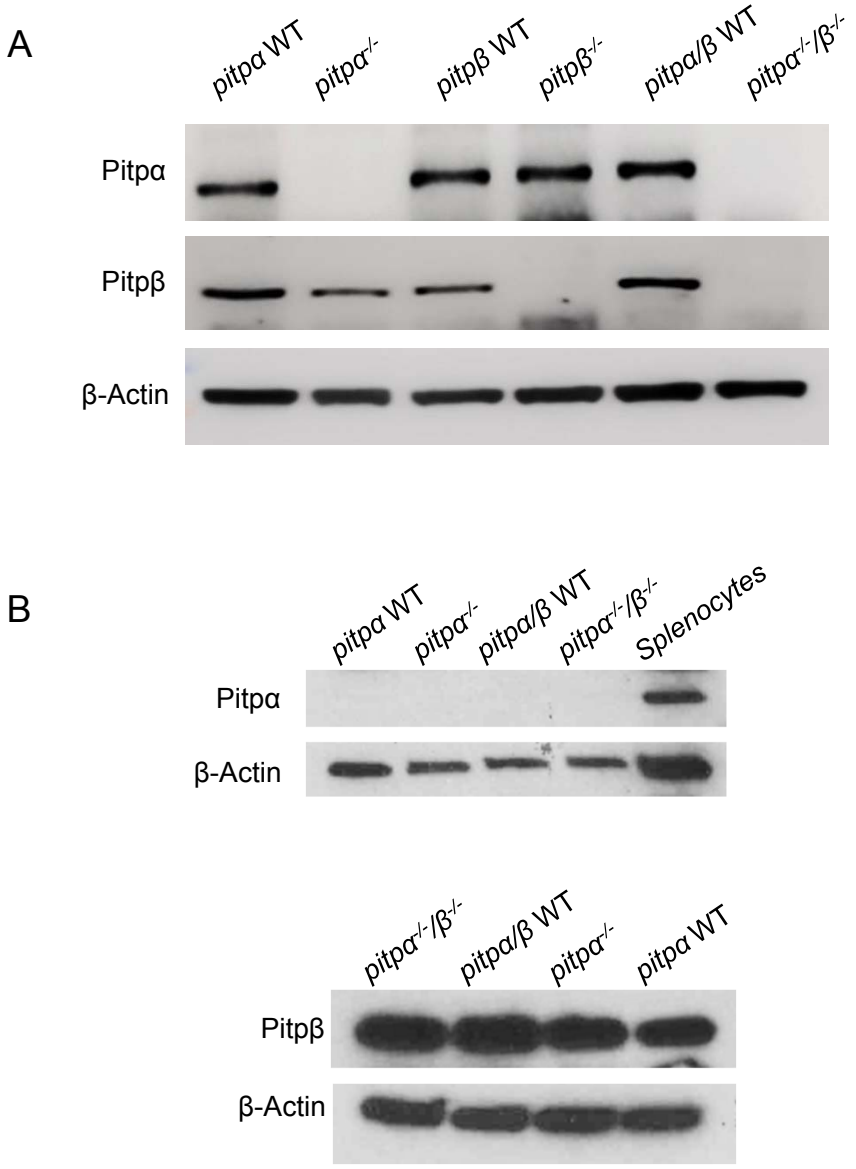
Supplemental Figure 3. (A) Platelet counts were performed using a Drew Hemavet Hemacytometer. Data are the mean ± SEM (n=6-12 mice per group). * $p < 0.05$ when compared to WT as determined by Student's T tests. (B) MKs in BM flushes were counted by flow cytometry after labeling with anti-CD41 antibodies. Data are the mean ± SEM (n=3 mice per group). (C) The overall BM nucleated cellularity number per femurs was compared in the indicated animals. Data are the mean ± SEM (n=3-5 mice per group). (D) H&E and immunohistochemistry staining for CD41 on femurs. Scale bars are 500μm. (E) $CD41^+$ MK counts from the sections were tallied from three microscope fields (10X). Data are the mean ± SEM of 3 mice per group. (F&G) Fetal liver tissue (E13.5) was cultured for seven days to assess changes in DNA ploidy (F) and proplatelet development (G). (F) DNA ploidy was measured by flow cytometry after staining for MK (CD41) and DNA content (propidium iodide). Data are the mean ± SEM (n=3). (G) Seven day old cultured MK were transferred to glass bottom dishes and proplatelet development was captured on video. Cells with at least one proplatelet were counted and expressed as a percentage of total number of cells within each field. Data are the mean ± SEM (n=3). (H) Lifespan of pitp$\alpha^{-/-}/\beta^{-/-}$ platelets in circulation was

assessed by injecting cultured fetal liver derived megakaryocytes into mice expressing human CD41 on its platelets. This allowed discrimination between host and donor platelets by identifying the species of the CD41 marker. The platelet lifespan was monitored over several hours to measure the rate of platelet release and clearance. Data are the mean ± SEM (n=3).

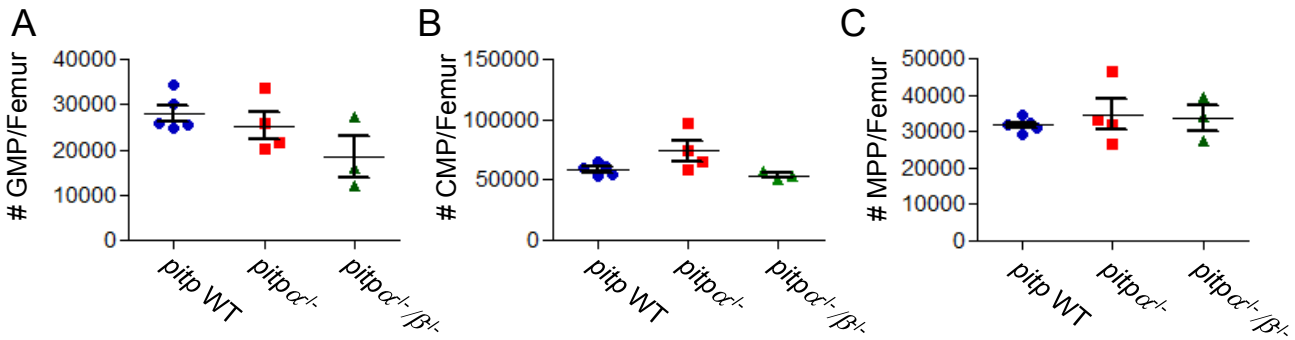
Supplemental Figure 4. (A) ELISAs were performed examining PF4 levels in control media and MK CM made from *pitp*$\alpha^{-/-}$ and *pitp*$\alpha^{-/-}/\beta^{-/-}$ and littermate WT control MK. Data are the mean ± standard deviation. * $p < 0.05$ and ** $p < 0.005$ when compared to WT control MK CM as determined by Student's T test. (B) ELISAs were performed examining thrombopoietin (TPO) concentrations in the serum of *pitp*$\alpha^{-/-}$ and *pitp*$\alpha^{-/-}/\beta^{-/-}$ and littermate WT control mice (n=5 mice per group). Data are the mean ± SEM. (C) *Pitp*$\alpha^{-/-}/\beta^{-/-}$ and *pitp*α/β WT mice were given intraperitoneal injections once per day for two days with neutralizing anti-TGF-β antibody or isotype control. Twenty-four hours following final injection, BM was collected and analyzed for HPC numbers per femur. The number of phenotypically-defined MPP (Ci), CMP (Cii) and GMP (Ciii) was determined by flow cytometry. All data are ± SEM of 3 mice per group. * $p < 0.014$ when compared to WT isotype control as determined by Student's T test.

Supplemental Figure 5. Ultrastructure of *pitp*α/β WT MK (A, C, & E) and *pitp*$\alpha^{-/-}/\beta^{-/-}$ MK (B, D, & E). These vacuolated spaces in *pitp*$\alpha^{-/-}/\beta^{-/-}$ MK (red arrows, B) are distinct from the open canalicular systems seen in *pitp*α/β WT MK (white arrows, C) and other areas of *pitp*$\alpha^{-/-}/\beta^{-/-}$ MK (white arrows, D). Compared to *pitp*α/β WT MK (E), *pitp*$\alpha^{-/-}/\beta^{-/-}$ MK also display numerous autophagic compartments (red arrowheads, F). Magnification is 40000X for A and B. Magnification is 15000X for C-F. All scale bars represent 500nm.

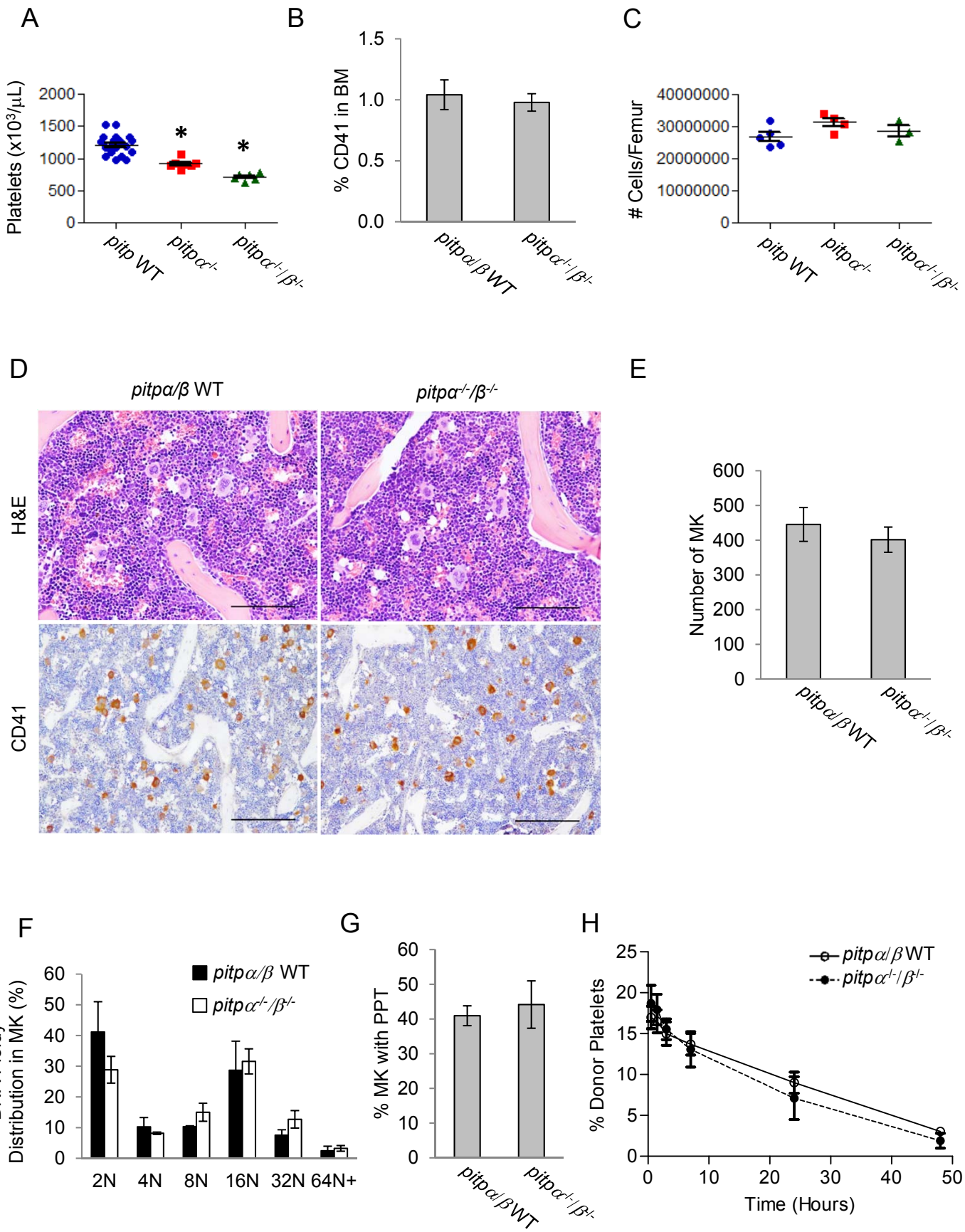
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



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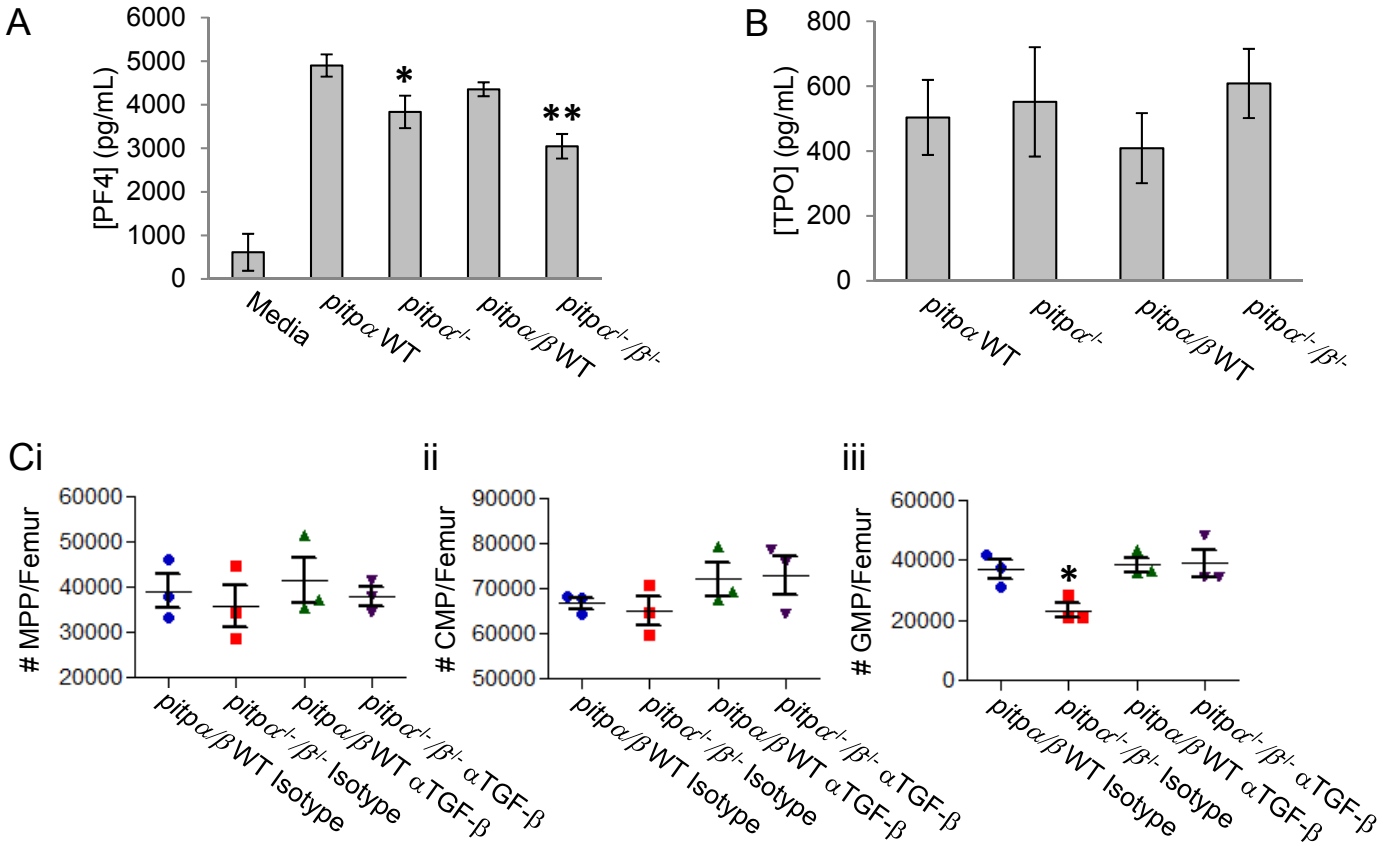
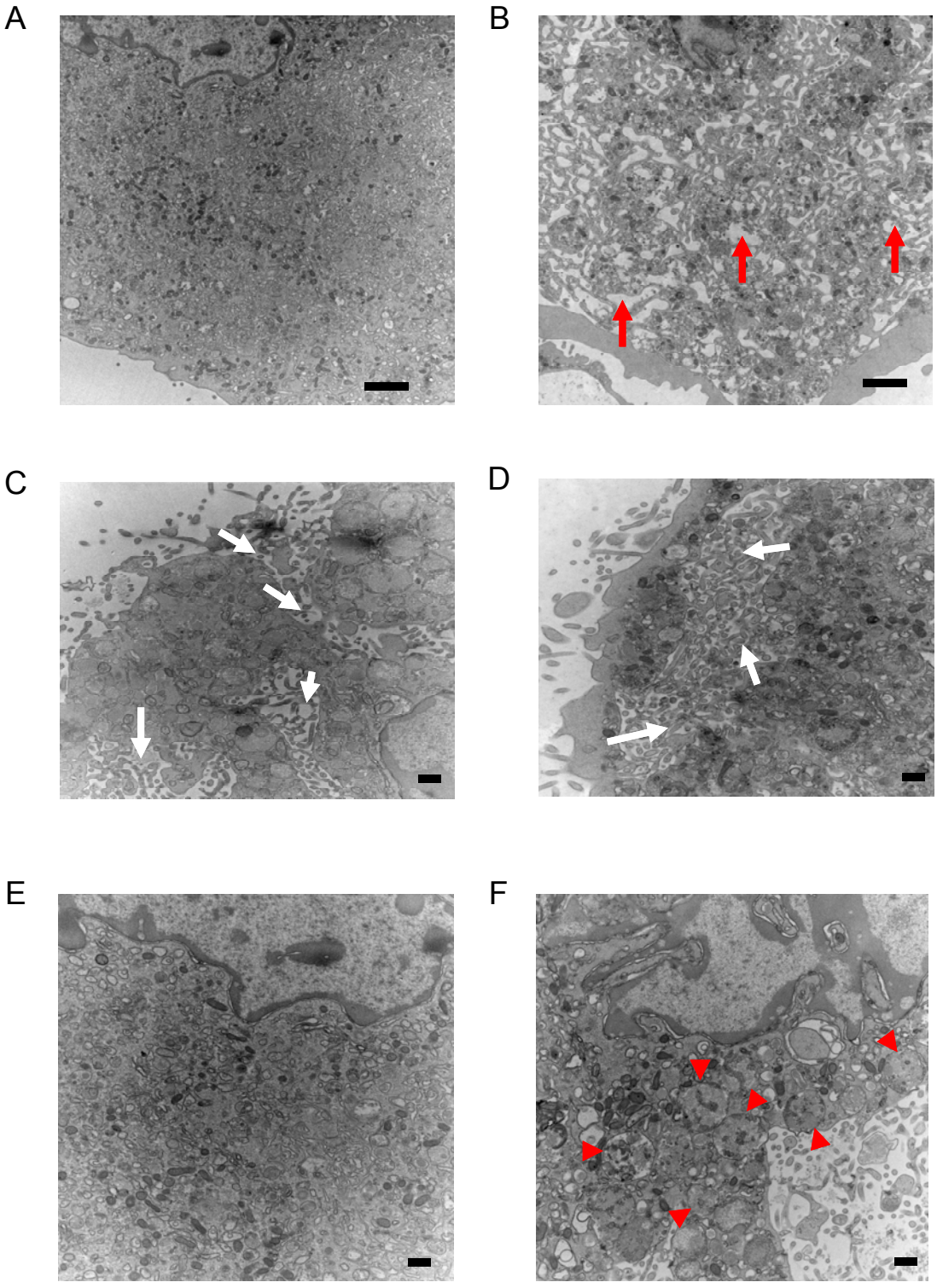


Supplemental Figure 5



Supplementary Table 1: CRU frequency transplanted				
Mouse	Cell number transplanted	# of mice with >10% CD45.2+ cell chimerism in BM/total # of mice	CRU frequency	95% confidence interval
pitp WT	25,000	2/6	1/24,744	1/44,929 to 1/13,628
	50,000	7/7		
	100,000	10/10		
pitp $\alpha^{-/-}/\beta^{-/-}$	25,000	0/6	1/177,984	1/370,209 to 1/85,569
	50,000	1/9		
	100,000	6/10		

Supplementary Table 2: CBC of *pitp WT and KO* mice			
	pitp WT	*pitpα KO*	*pitpα/β KO*
WBC (1×10^3/μl)	8.016 ± 0.338	7.508 ± 0.465	5.860 ± 0.129**
RBC (1×10^3/μl)	10.62 ± 0.168	10.83 ± 0.134	10.61 ± 0.504
Hg (g/dl)	16.04 ± 0.164	15.91 ± 0.225	16.08 ± 0.699
Ht (%)	50.81 ± 0.408	51.10 ± 0.670	50.32 ± 2.262
MCV (fl)	48.00 ± 0.707	47.25 ± 0.329	47.60 ± 0.245
MCH (pg)	15.13 ± 0.169	14.99 ± 0.325	15.22 ± 0.102
MCHC (g/dl)	31.59 ± 0.298	31.14 ± 0.209	31.98 ± 0.159
RDW (%)	15.68 ± 0.293	14.48 ± 0.147**	14.64 ± 0.160*
MPV (fl)	5.800 ± 0.110	5.727 ± 0.149	6.425 ± 0.329**
Protein (g/dl)	6.957 ± 0.100	7.222 ± 0.133	8.200 ± 0.458**
PCV (%)	49.36 ± 0.746	48.13 ± 0.999	47.33 ± 1.764
Neu (1×10^3/μl)	1.315 ± 0.208	1.193 ± 0.113	1.046 ± 0.197
Lym (1×10^3/μl)	6.541 ± 0.355	5.985 ± 0.435	4.638 ± 0.371**
Mono (1×10^3/μl)	0.178 ± 0.042	0.216 ± 0.052	0.358 ± 0.180
Eosino (1×10^3/μl)	0.170 ± 0.036	0.123 ± 0.030	0.160 ± 0.030
*, $p<0.05$ when compared to WT.			
**, $p<0.007$ when compared to WT.			

Supplemental Table 3: Concentration (pg/mL) of cytokine in MK CM[a]

Cytokine	*pitp α* WT	*pitp α KO*	*pitp α/β* WT	*pitp α/β KO*
IL-1α	ND	ND	ND	ND
IL-1β	9.48 ± 3.04	9.74 ± 0.36	5.21 ± 1.12	12.9 ± 0.68*
IL-2	ND	ND	ND	ND
IL-3	ND	ND	ND	ND
IL-4	1.40 ± 0.40	15.2 ± 1.60**	0.65 ± 0.60	5.35 ± 0.01*
IL-5	3.80 ± 2.42	5.90 ± 1.31	2.89 ± 0.45	4.85 ± 0.36*
IL-6	22.0 ± 9.50	14.1 ± 1.67	7.78 ± 0.35	36.6 ± 3.91**
IL-7	ND	ND	ND	ND
IL-9	6.75 ± 6.70	17.3 ± 17.0	ND	5.04 ± 0.00**
IL-10	OOR	OOR	ND	OOR
IL-12p40	ND	ND	ND	ND
IL-12p70	ND	ND	ND	ND
IL-13	ND	ND	ND	ND
IL-15	ND	OOR	ND	OOR
IL-17	OOR	OOR	OOR	OOR
IL-20	ND	ND	ND	ND
IL-21	ND	ND	ND	ND
IL-22	0.17 ± 0.00	0.15 ± 0.02	0.06 ± 0.05	0.17 ± 0.00
IL-23	OOR	OOR	OOR	OOR
IL-25	ND	ND	ND	ND
IL-27	OOR	OOR	OOR	OOR
IL-28b	ND	ND	ND	ND
IL-33	ND	ND	ND	ND
M-CSF	OOR	4.02 ± 0.83*	OOR	5.04 ± 0.68*
G-CSF	11.0 ± 9.20	OOR	OOR	OOR
GM-CSF	ND	ND	ND	ND
VEGF	53.7 ± 21.1	67.3 ± 5.67	44.7 ± 6.61	95.4 ± 12.8*
IFNγ	ND	ND	ND	ND
LIF	0.75 ± 0.31	0.21 ± 0.00	0.11 ± 0.10	0.54 ± 0.03*
TNFα	12.6 ± 0.52	11.2 ± 1.55	7.10 ± 2.92	24.3 ± 1.37*
IP-10	98.8 ± 45.6	117 ± 11.1	49.8 ± 0.49	389 ± 59.9*
LIX/ENA-78	17.0 ± 16.9	54.4 ± 44.8	260 ± 120	176 ± 16.6
MIP-2	ND	1.32 ± 1.31	1.32 ± 1.31	21.3 ± 21.2
MDC	455 ± 289	499 ± 4.11	198 ± 26.1	584 ± 17.4**
KC	2.55 ± 2.54	ND	3.53 ± 3.52	5.03 ± 5.00
MIP-3β	ND	ND	ND	ND
TARC	ND	ND	ND	ND
RANTES	21.0 ± 13.5	20.2 ± 2.07	11.0 ± 3.43	43.2 ± 8.40*
MIG	62.0 ± 52.5	75.9 ± 2.14	19.92 ± 11.1	107 ± 75.7*
MIP-1β	2327 ± 1706	1245 ± 720	2246 ± 1922	1700 ± 1054
MCP-1	159 ± 135	59.8 ± 37.6	209 ± 204	89.6 ± 70.3
MCP-5	24.8 ± 12.3	14.5 ± 4.47	3.01 ± 0.98	54.5 ± 4.17**
MIP-1α	3212 ± 167	2196 ± 92.6*	3328 ± 120	3455 ± 860
MIP-3α	4.76 ± 0.25	5.01 ± 0.24	5.01 ± 0.24	5.17 ± 0.78
Eotaxin	ND	ND	ND	ND
TIMP-1	31.9 ± 31.8	ND	11.4 ± 11.3	ND

[a], Determined by BioPlex . Data is average ± SEM of 2 experiments.

*, Indicates a $p<0.05$ when compared to littermate MEG CM.

**, Indicates a $p<0.005$ when compared to littermate MEG CM.

ND, Not detected. OOR, Out of range of analysis.