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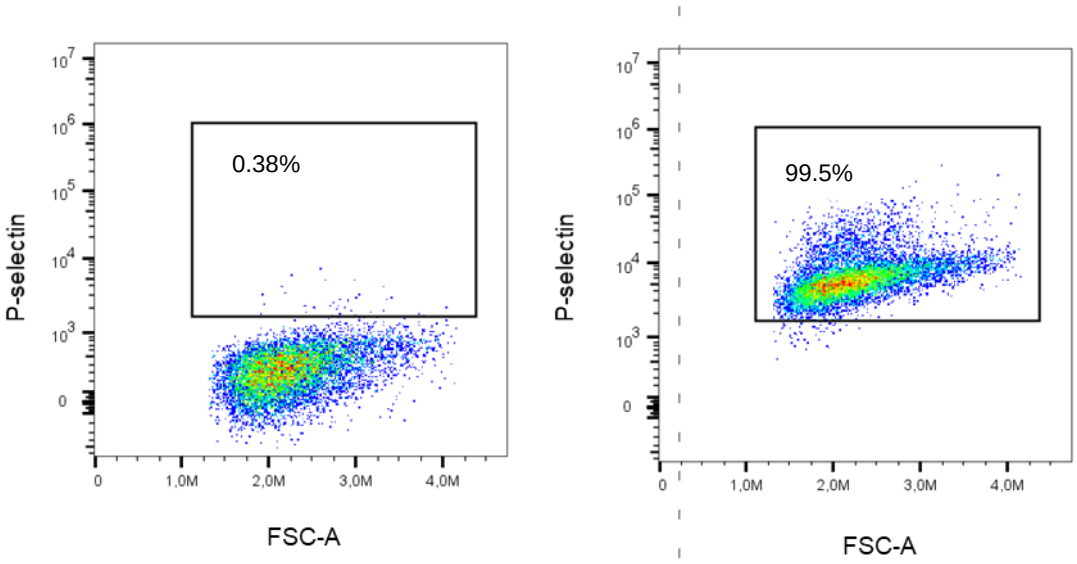
Supplemental information

Targeting transgenic proteins to alpha granules for platelet-directed gene therapy

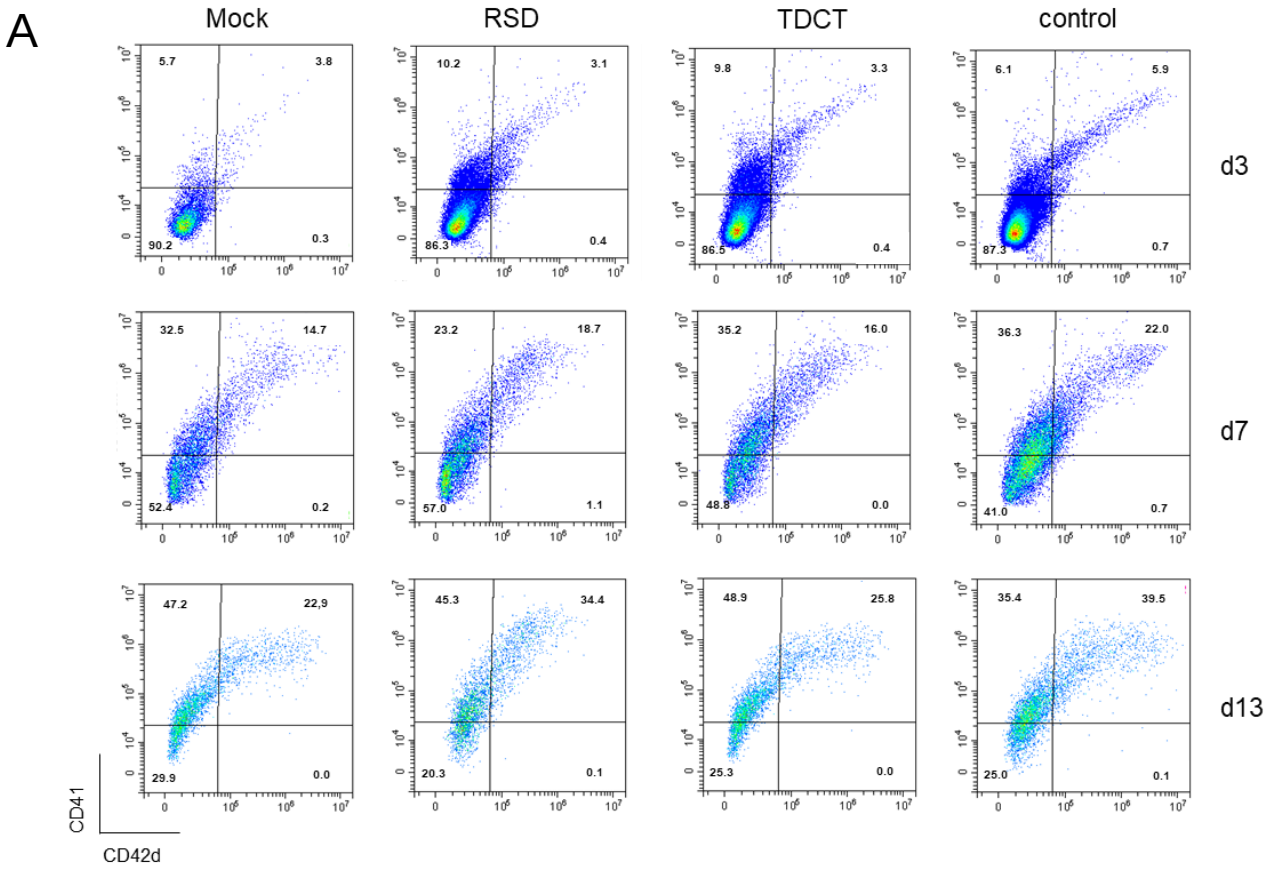
Vanessa M.A. Woods, Lisette J. Latorre-Rey, Franziska Schenk, Marcel G.E. Rommel, Thomas Moritz, and Ute Modlich

A

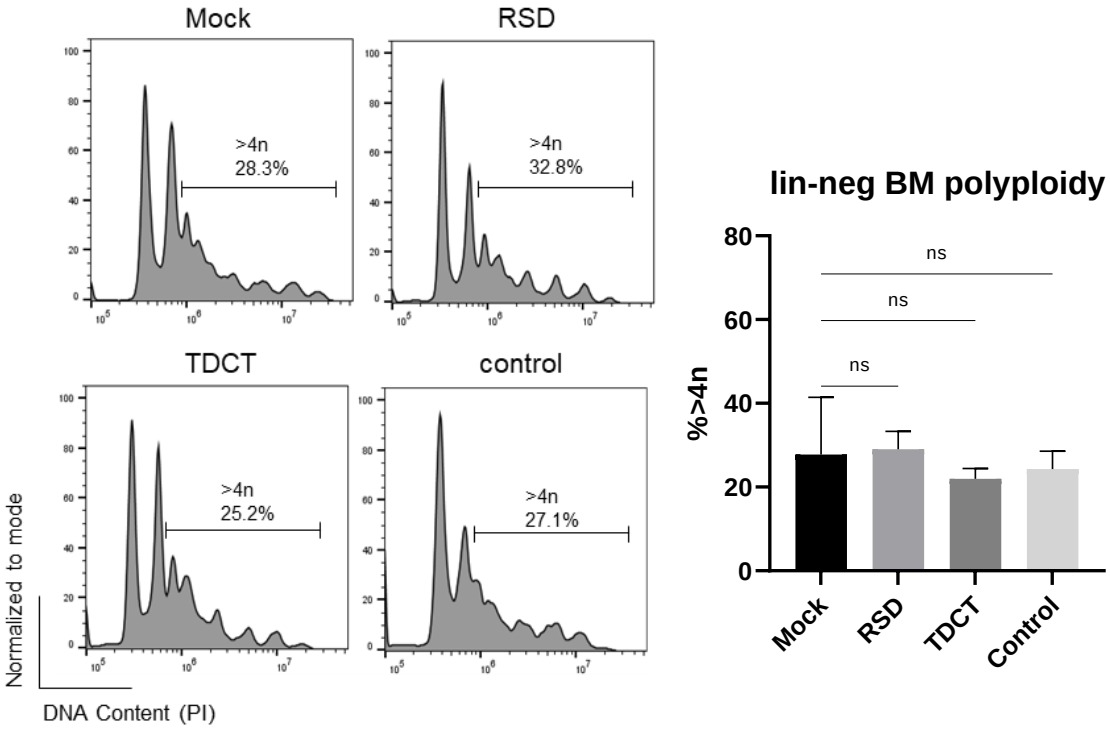


Supplemental Figure S1: P-selectin surface expression on MEG-01 cells

Representative flow cytometry plots showing P-selectin expression on MEG-01 cells (left: unstained and right: stained).

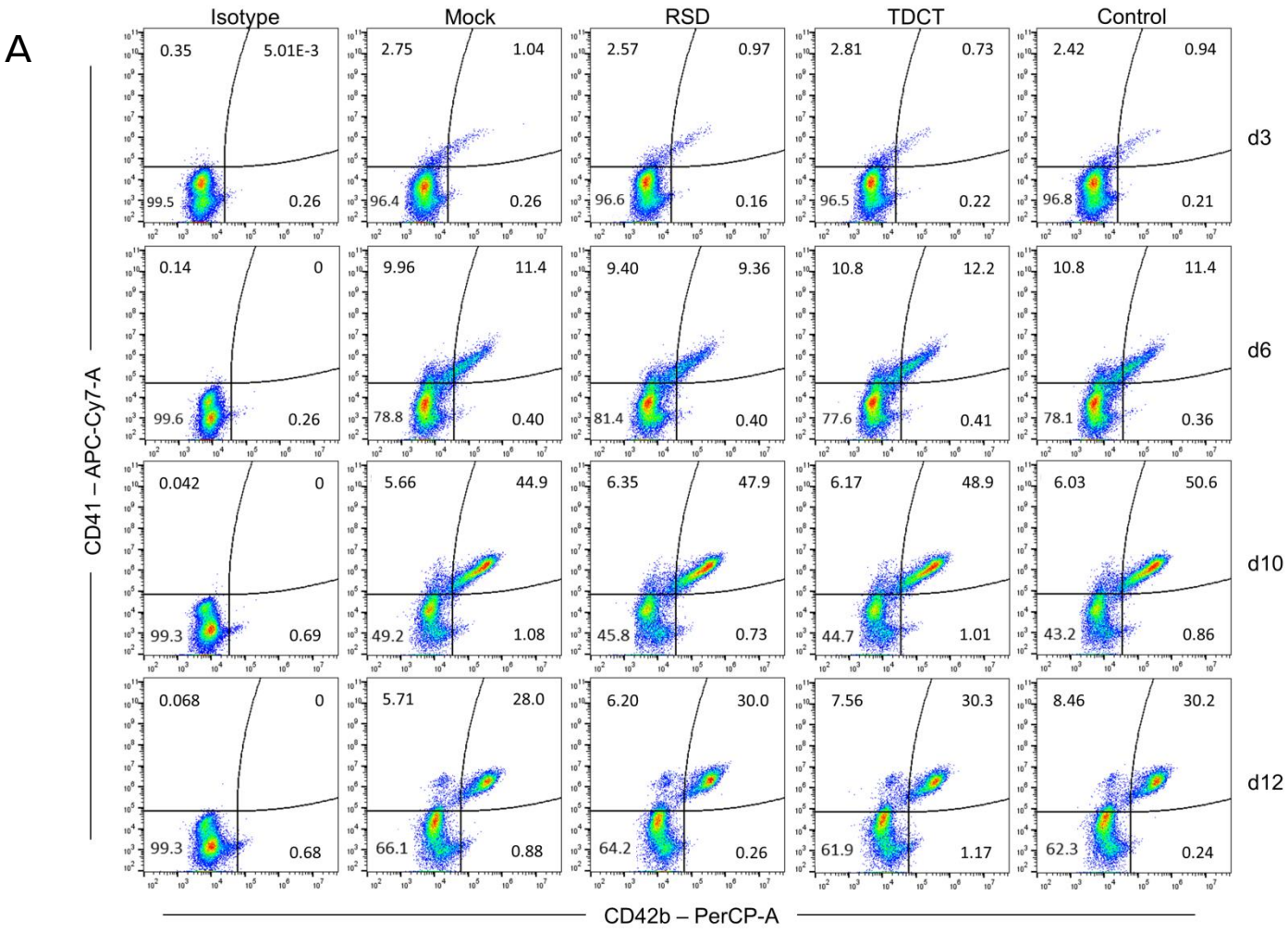

A
Mock
RSD
TDCT
control
d3
d7
d13
CD41
CD42d
B
% CD41+ MK
% CD41+ CD42d+ MK
time [d]
RSD
TDCT
Control
Mock
ns
C
%GFP on CD41+ cells
%GFP CD41- cells
time [d]
RSD
TDCT
Control
ns
*

D



Supplementary Figure S2: Characterization of *in vitro* differentiated murine MK.

Murine lineage marker negative BM cells from C57BL6 mice were transduced with alpha granule targeting vectors (RSD, TDCT) or the non-targeting control or not transduced (Mock), followed by differentiation towards MK using murine THPO (50 ng/ml). **(A)** Representative dot plots showing $CD41^+/CD42d^+$ surface marker expression of lineage marker negative derived MK at different time-points for transduced and non-transduced (Mock) cells. **(B)** Summary of percentages $CD41^+$ and $CD41^+/CD42d^+$ MK harvested at the indicated time-points (n = 6; two indipendent transductions). **(C)** Percentage of GFP expressing cells in MK ($CD41^+$) and non-megakaryocytes/non-differentiated cells ($CD41^-$) differentiated from lineage marker BM cells (n=6). **(D)** Representative flow cytometry histograms and quantification of DNA content of mature MK (>4n) stained with propidium iodide (PI) staining solution (n=4-5, two indipendent transduction). All data are shown as mean ± SD. Multiple comparisons in (B) to the Mock sample were performed by Two-Way ANOVA with Sidaks comparison test and (D) One-way ANOVA with Dunett's comparison test ns = not significant.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Isotype
Mock
RSD
TDCT
Control
d3
d6
d10
d12
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5.01E-3
99.5
0.26
2.75
1.04
96.4
0.26
2.57
0.97
96.6
0.16
2.81
0.73
96.5
0.22
2.42
0.94
96.8
0.21
0.14
0
99.6
0.26
9.96
11.4
78.8
0.40
9.40
9.36
81.4
0.40
10.8
12.2
77.6
0.41
10.8
11.4
78.1
0.36
0.042
0
99.3
0.69
5.66
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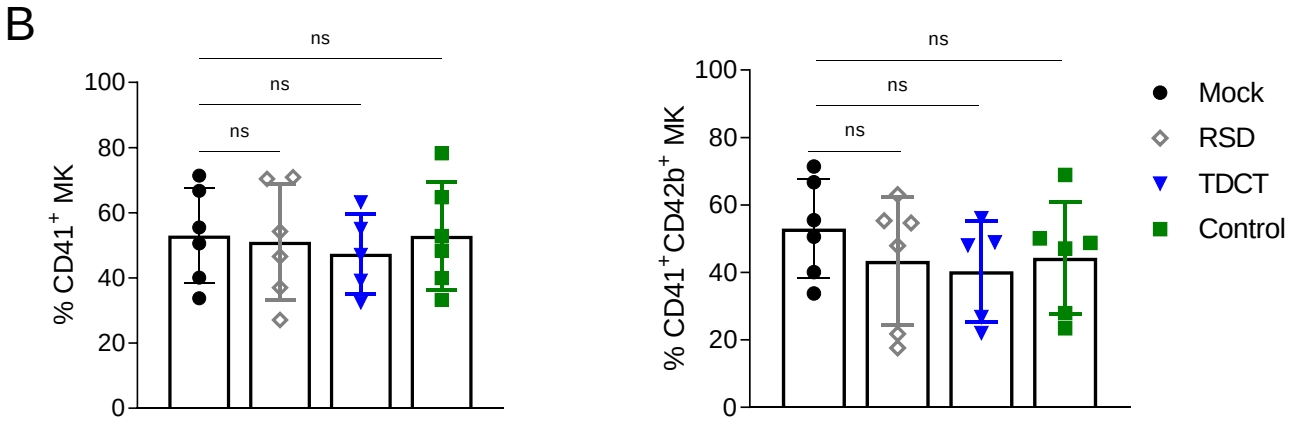
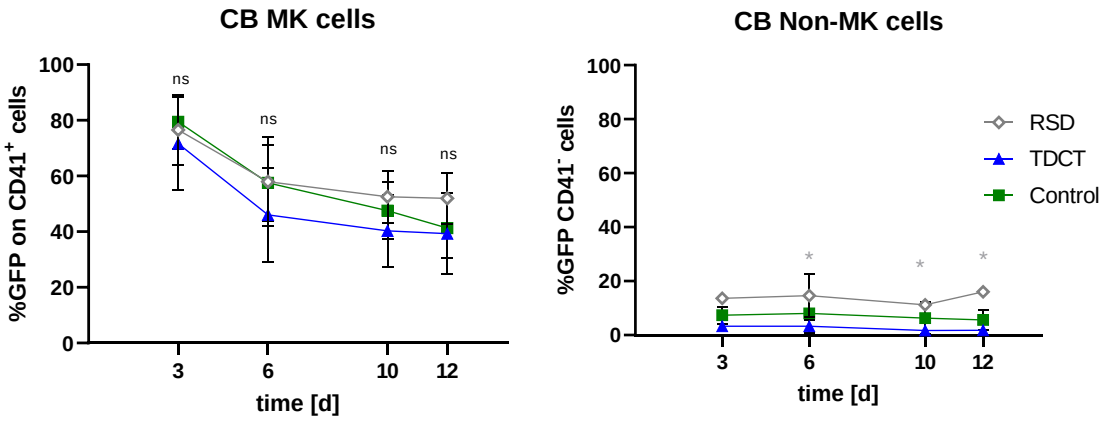
49.2
1.08
6.35
47.9
45.8
0.73
6.17
48.9
44.7
1.01
6.03
50.6
43.2
0.86
0.068
0
99.3
0.68
5.71
28.0
66.1
0.88
6.20
30.0
64.2
0.26
7.56
30.3
61.9
1.17
8.46
30.2
62.3
0.24
CD41 – APC-Cy7-A
CD42b – PerCP-A


B
ns
ns
ns
100
80
60
40
20
0
% CD41+ MK
% CD41+CD42b+ MK
Mock
RSD
TDCT
Control

C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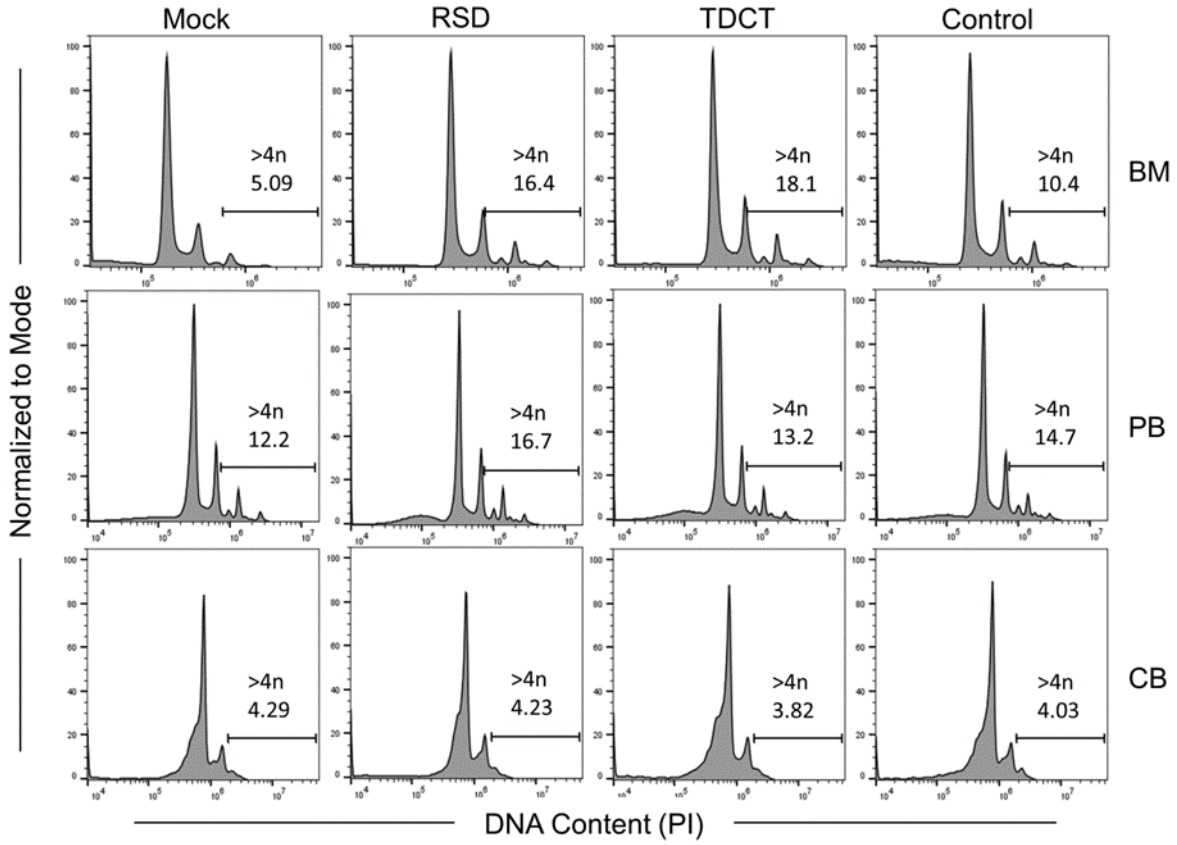
%GFP on CD41+ cells
100
80
60
40
20
0
ns
ns
ns
ns
3
6
10
12
time [d]
CB Non-MK cells
%GFP CD41- cells
RSD
TDCT
Control
*
*
*
time [d]

D


Mock
RSD
TDCT
Control
BM
PB
CB
>4n 5.09
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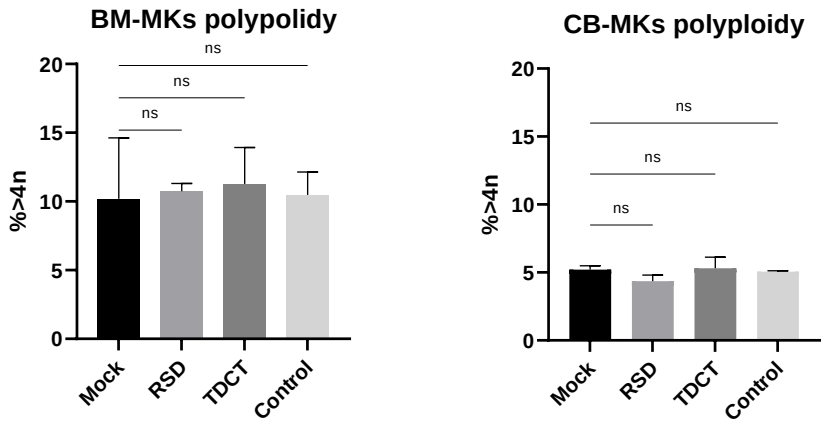
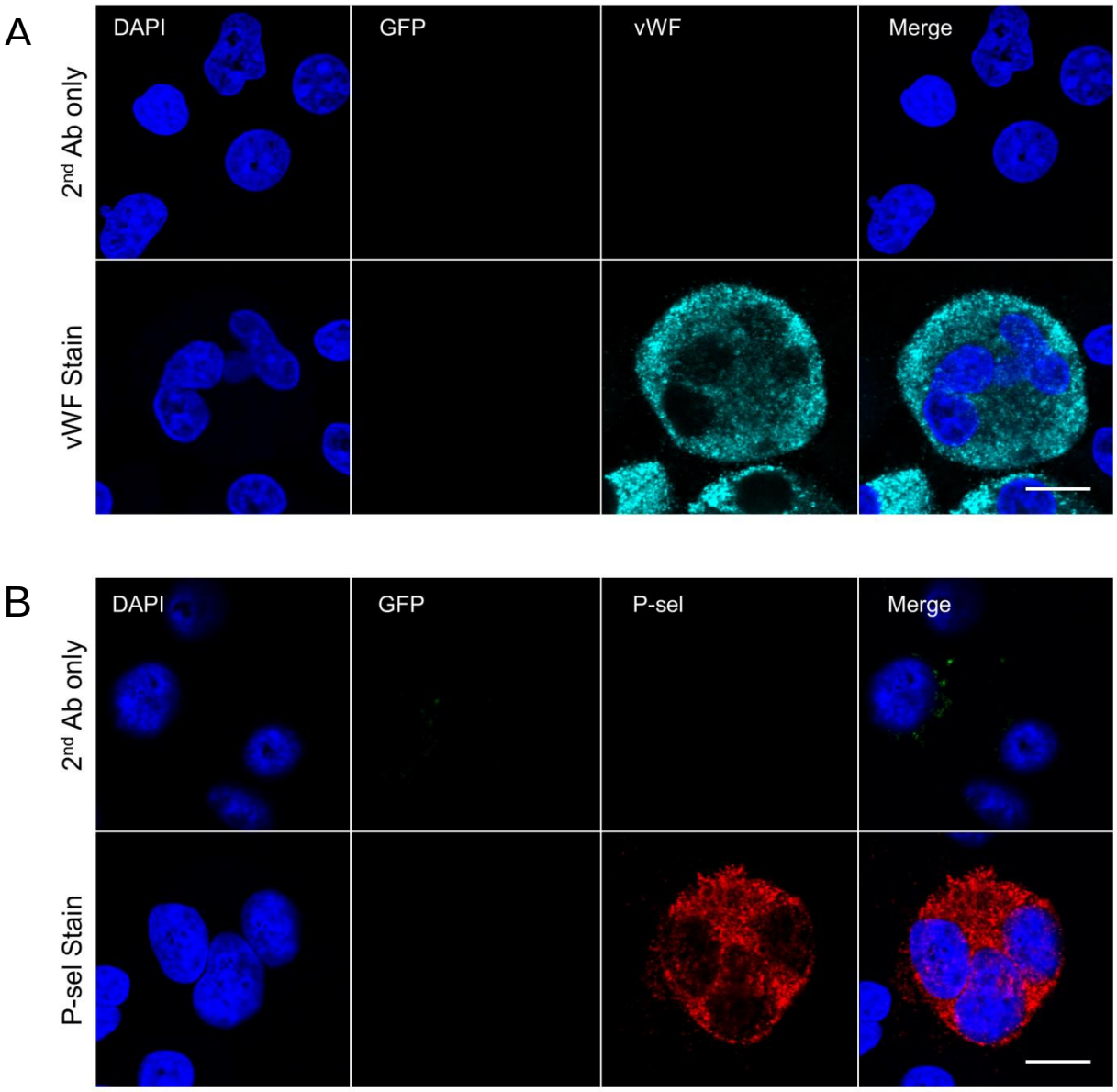
>4n 16.7
>4n 13.2
>4n 14.7
>4n 4.29
>4n 4.23
>4n 3.82
>4n 4.03
Normalized to Mode
DNA Content (PI)

E


BM-MKs polypoidy
%>4n
20
15
10
5
0
ns
ns
ns
Mock
RSD
TDCT
Control
CB-MKs polyploidy
%>4n
20
15
10
5
0
ns
ns
ns
Mock
RSD
TDCT
Control

Supplementary Figure S3: Characterization of *in vitro* differentiated human MK.

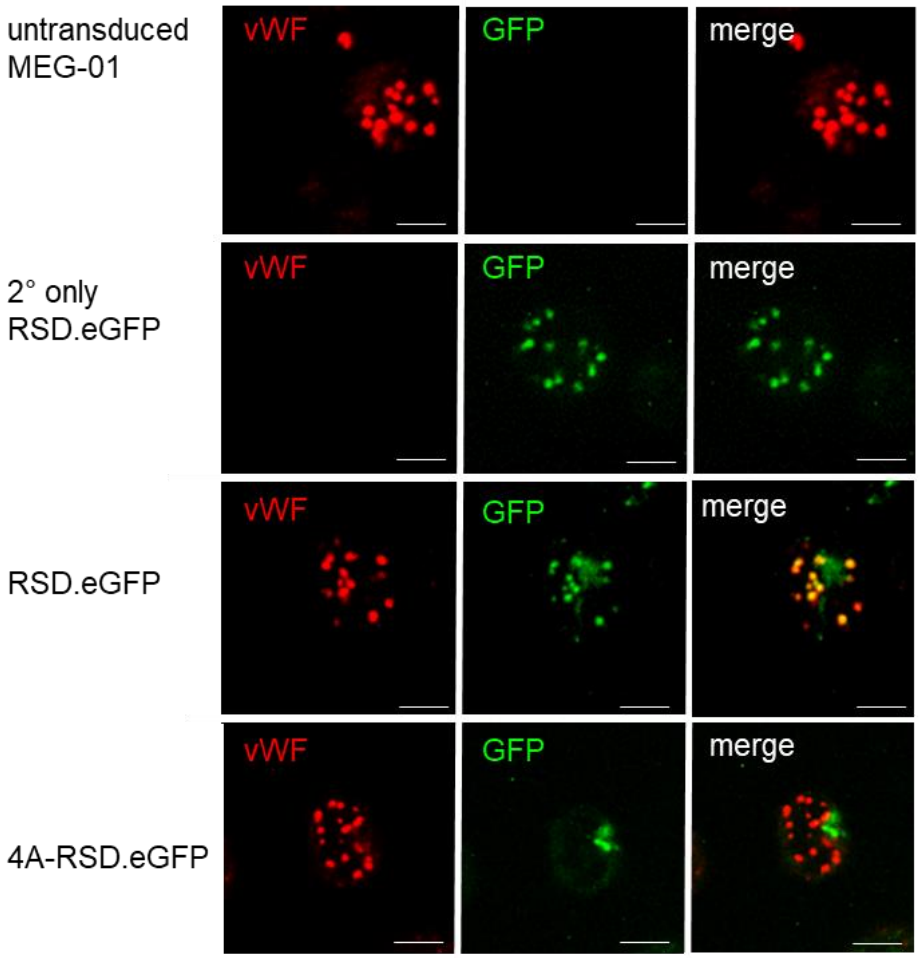
Human $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) were isolated from different cell sources, transduced with alpha granule targeting vectors (RSD, TDCT) or the non-targeting control, followed by differentiation towards MK using human cytokines (THPO, SCF and IL-6) for 12 – 14 days. **(A)** Representative dot plots showing $CD41^+$/$CD42b^+$ surface marker expression of cord blood (CB)-derived megakaryocytes at different time-points for transduced cells and non-transduced mock cells. **(B)** Summary of percentages $CD41^+$ and $CD41^+$/$CD42b^+$ MK harvested at the latest differentiation time-point (n=5-6 individual cultures). **(C)** Percentage of GFP expressing cells in megakaryocytes ($CD41^+$) and non-megakaryocytes/non-differentiated cells ($CD41^-$) differentiated from CB. **(D)** Representative flow cytometry histograms showing DNA content of mature MK differentiated from bone marrow (BM), apheresis (PB) or CB HSPCs and stained with propidium iodide (PI) staining solution. **(E)** Quantification of polyploidy (>4n) of MK differentiated from bone marrow (n = 3, two indipendent transductions) and cord blood (n = 4 – 5, two to three indipendent transudctions). Data represent mean ± SD. Multiple comparisons to the Mock sample (B and E) were performed by One-way ANOVA with Dunett's comparison test ns = not significant. Multiple comparision between RSD, TDCT and control (C) were performed by Two-way ANOVA with Sidak's comparison test ns = not significant, *$p \leq 0.05$.



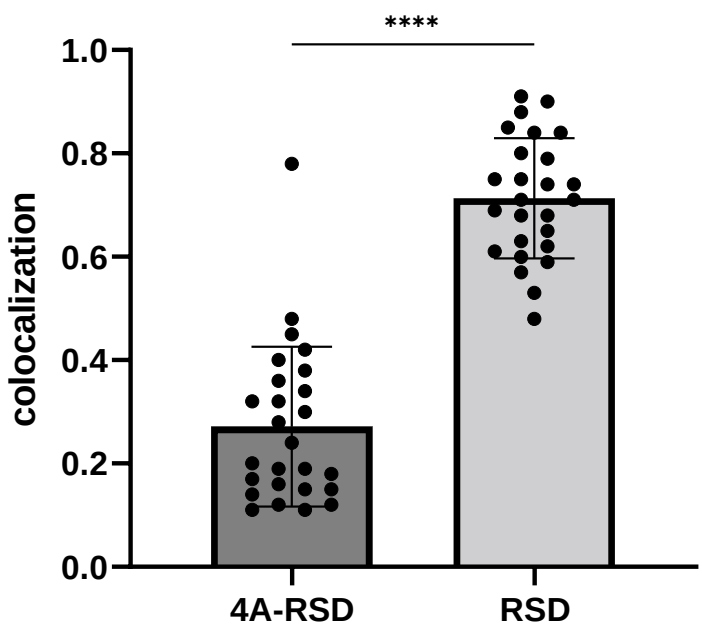
Supplementary Figure S4: Human MK immunofluorescence controls

Non-transduced human CD34$^+$ cord blood HSPCs were differentiated towards MKs by stimulation with human THPO (50 ng/ml), SCF (25 ng/ml) and IL-6 (7.5 ng/ml). Mature cells harvested at day 12 were immuno-stained for alpha granule proteins using anti-von Willebrand factor (vWF, turquoise) and P-selectin (P-sel, red) antibodies. **(A)** vWF secondary antibody control - goat anti-rabbit IgG Alexa Fluor 647 (1:2000) (top panel) and vWF stain – anti-vWF, rabbit polyclonal (1:100) (bottom panel). **(B)** P-sel secondary antibody control – goat anti-mouse IgG Cy3 (1:2000, top panel) and P-sel stain – anti-human/mouse PE-conjugated CD62P (1:100, bottom panel). Nuclear counterstain was done with 4,6-diamidino-2-phenylindole *(*DAPI, blue,1:5000). Background autofluorescence in the GFP channel was subtracted based on the secondary antibody controls. Images were acquired with 63X oil immersion objective using a Leica DMi8 Inverted-3 confocal microscope, scale bar = 10µm.

A

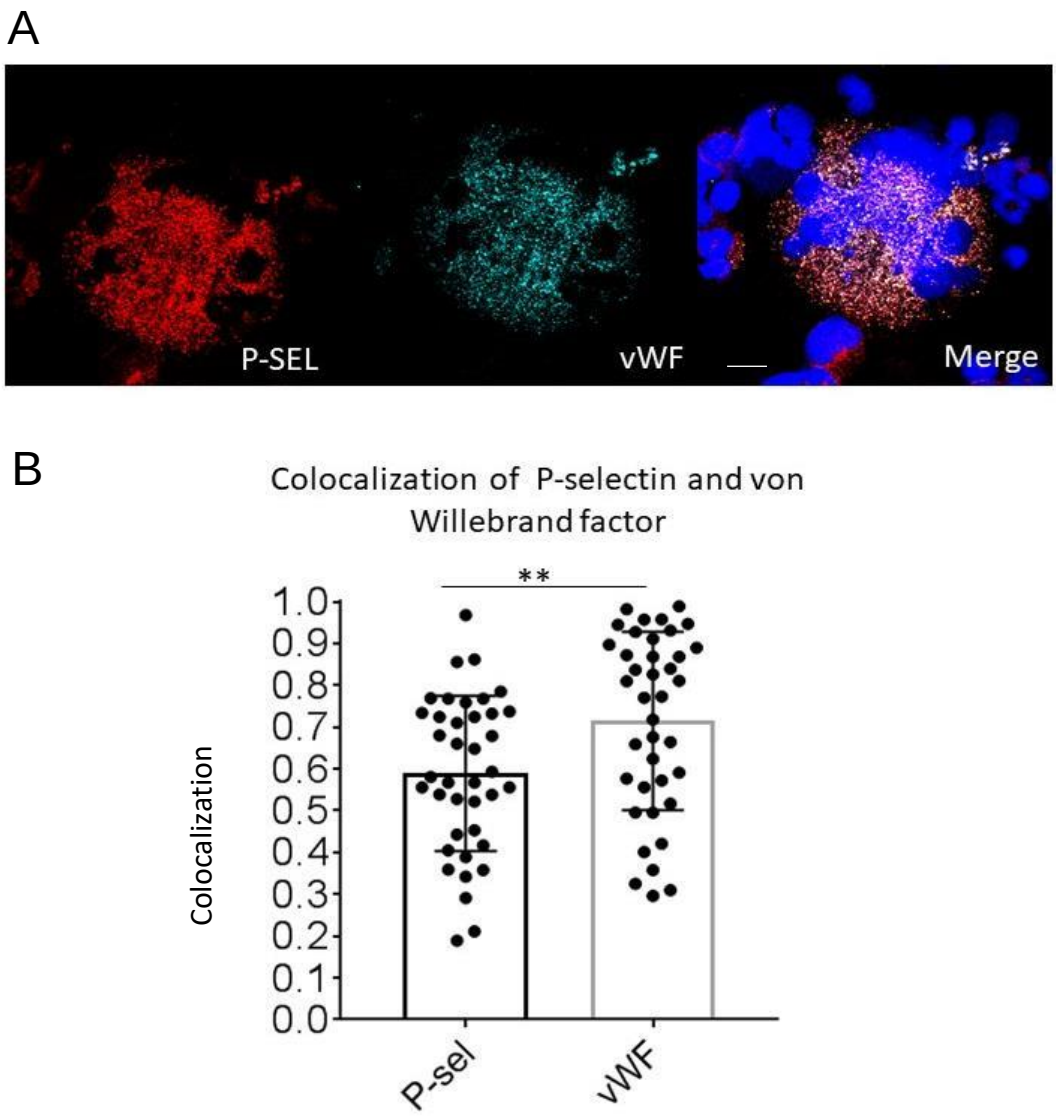

untransduced MEG-01
vWF
GFP
merge
2° only RSD.eGFP
vWF
GFP
merge
RSD.eGFP
vWF
GFP
merge
4A-RSD.eGFP
vWF
GFP
merge

B


****
1.0
0.8
0.6
0.4
0.2
0.0
colocalization
4A-RSD
RSD

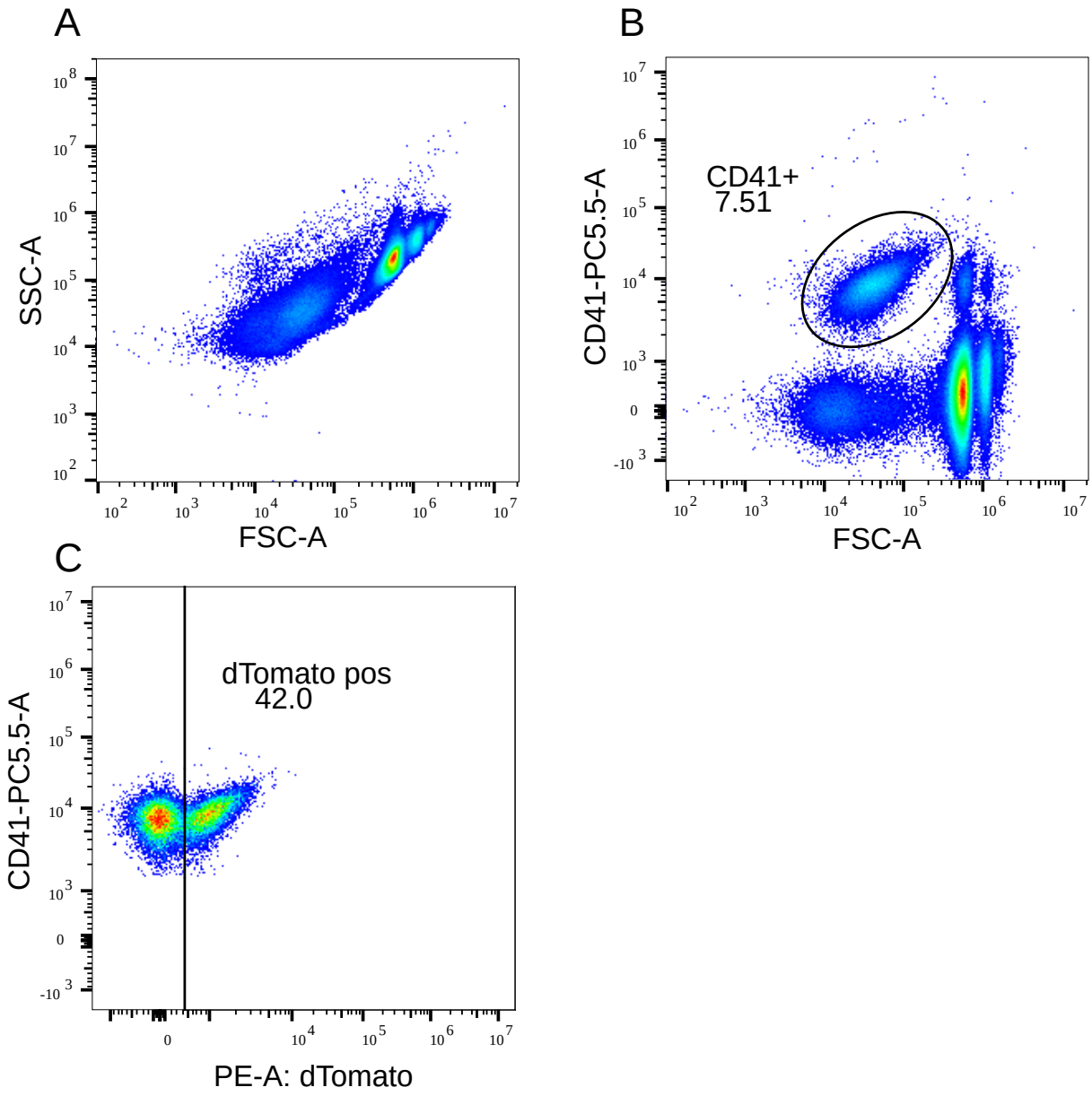
Supplementary Figure S5: Colocalization of GFP expressed from the RSD and 4A-RSD vectors with αG marker vWF in MEG-01 cells

(A) MEG-01 cells were transduced with RSD.eGFP (RSD) or 4A-RSD.eGFP (4A-RSD, 4 amino acids in the main RANTES sorting domain (TRKN) substituted by alanine (AAAA)) vectors with the MOI of 1 and confocal immunofluorescence images were taken. MEG-01 cells were stained with primary rabbit antibody against the αG protein vWF (1:100) visualized with secondary goat anti-rabbit antibody Alexa Flour 647 (1:1000, red). RSD transduced MEG-01 cells were used for the secondary antibody only control. The merged images of RSD show that GFP (green) colocalized with vWF (red). GFP expressed from the RSD vector showed a dotted appearance, while GFP expressed from the 4A-RSD vector was present at isolated areas in the transduced MEG-01 cell not colocalizing with vWF. Confocal images of stained MEG-01 cells were acquired in a 96-well plate with the Operetta high imaging system and a 40X objective (scale bar = 10 µm). **(B)** Quantification of colocalization of GFP expressed from RSD vector (n = 26 individual cells, two transductions) and 4A-RSD vector (n = 26, individual cells, two transductions) with vWF. Data represents mean ± SD. Statistical analysis were performed by Mann Whitney Test, ****p<0.0001.



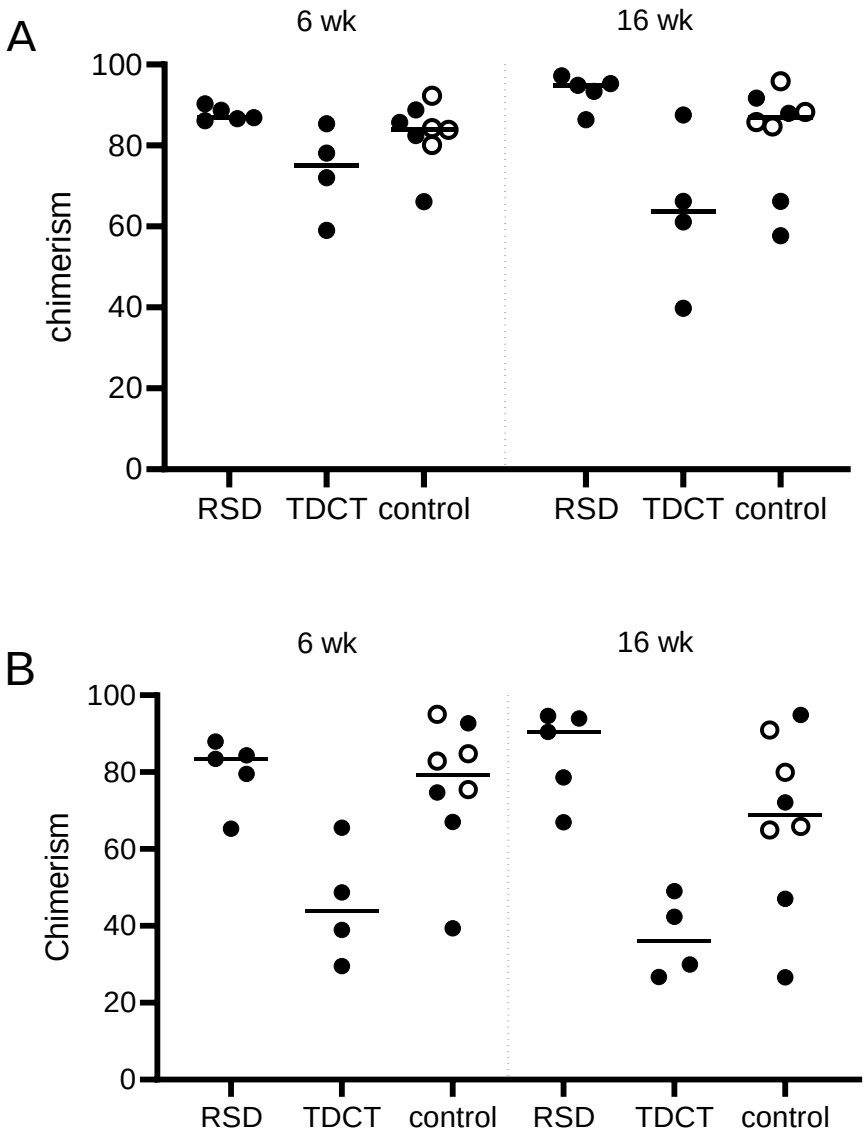
Supplementary Figure S6: Alpha granule protein localization in murine MK.

Lineage marker negative cells were isolated from whole bone marrow of wild type C57BL/6J mice, and differentiated towards megakaryocytes by the stimulation of THPO (50 ng/ml). Mature non-transduced cells were harvested and immuno-stained for alpha granule proteins using anti-P-selectin and anti-vWF antibodies. **(A)** Representative confocal images of MK showing P-sel (red) and vWF (turquoise). Merge image shows overlapping P-sel and vWF signals (white) and nuclear stain DAPI (blue). Images were acquired with 100x oil immersion objective using the Zeiss LSM510 Axiovert500M confocal microscope, scale bar = 10 µm. **(B)** Summary of colocalization of P-sel and vWF, where each dot represents one cell on the bar graph (n=37-39, indipendent cells). Statistical analysis was performed by Mann Whitney Test, **p=0.0056. Data represents mean ± SD.



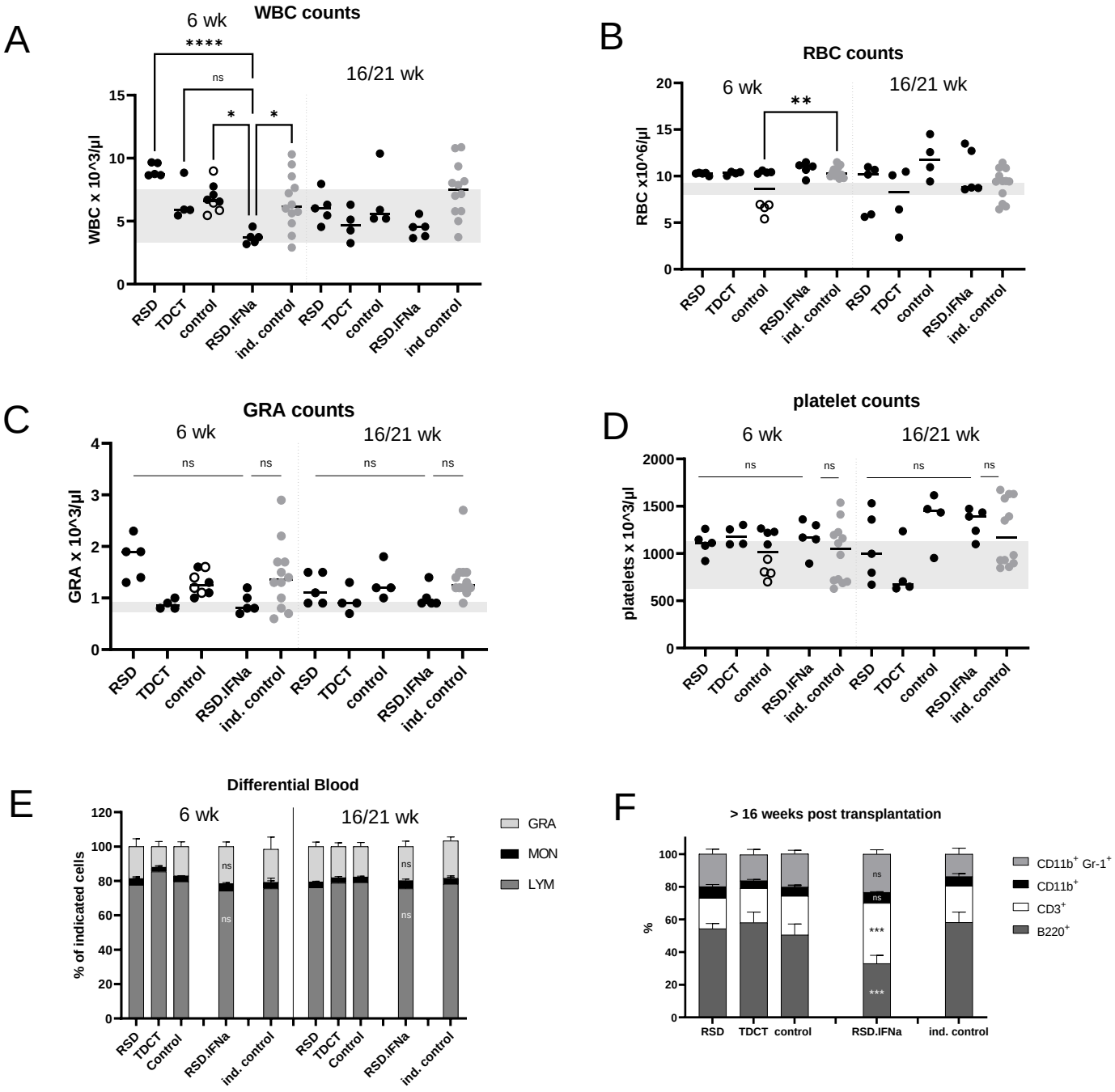
Supplementary Figure S7: Gating strategy of murine blood platelets

Whole blood from mice was analyzed by flow cytometry. **(A)** Platelets were identified by size and **(B)** by CD41 cell surface staining. **(C)** In the CD41+ platelet population, donor platelets were identified by dTomato expression.



Supplementary Figure S8: Chimerism after bone marrow transplantation.

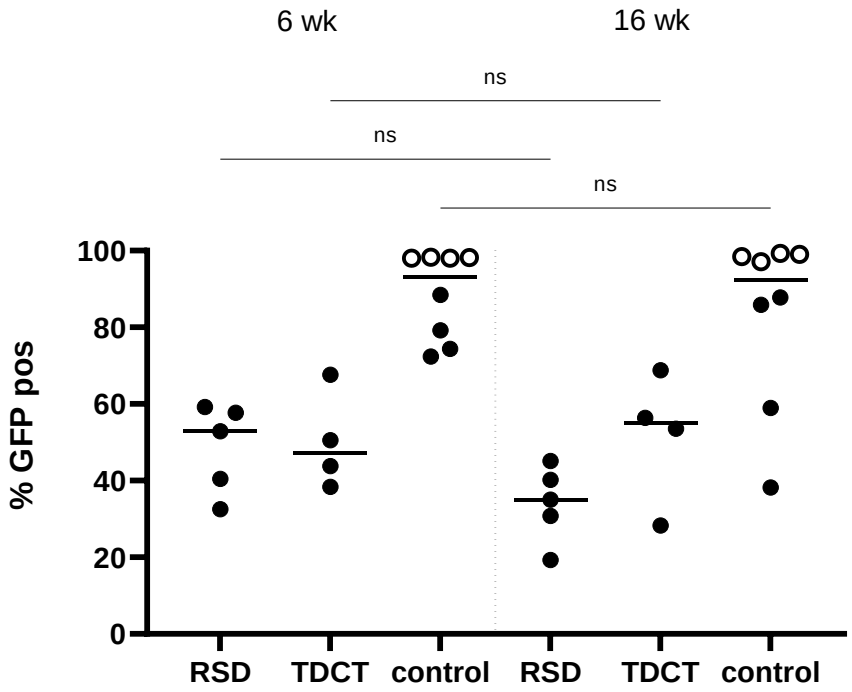
Detection of chimerism of mTmG mice donor cells on **(A)** peripheral blood leukocytes and **(B)** platelets, 6 weeks (left) and 16 weeks (right) after BMT. Line drawn at mean, n=4-5 mice per treatment group. (Open circles: high VCN mice, closed circles: low VCN mice of the control group).



Supplementary Figure S9: Peripheral blood cell counts

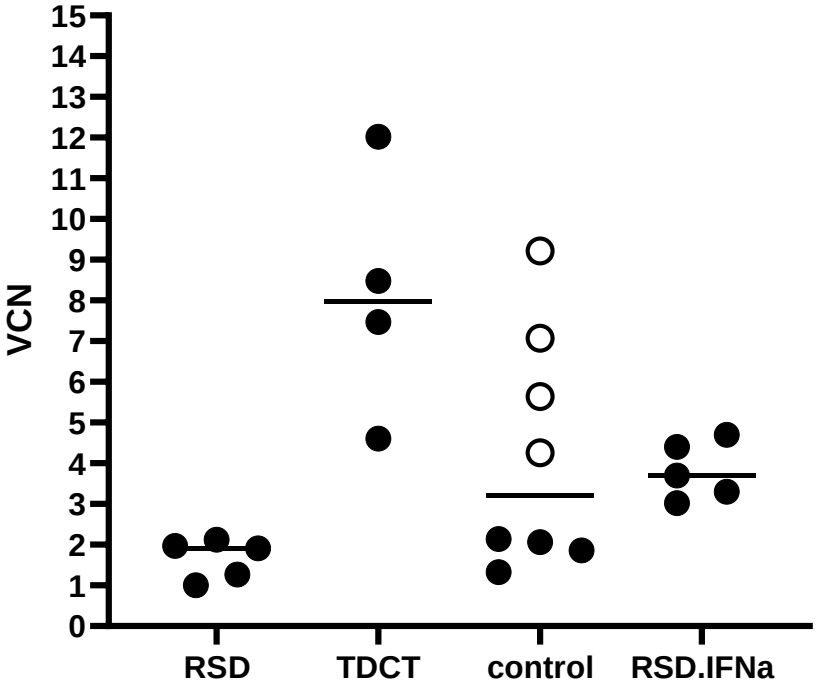
Six weeks and sixteen or twenty-one weeks after transplantation of lentiviral transduced BM cells into C57Bl/6J mice, peripheral blood was taken from the retro-orbital venous plexus and blood cell counts determined. **(A)** White blood cell (WBC) counts, **(B)** red blood cell (RBC) counts, **(C)** granulocyte (GRA) counts, **(D)** platelet counts, **(E)** differential blood cell contribution in the peripheral blood determined by automated blood analyzer, **(F)** contribution of $CD11b^+Gr1^+$ granulocytes, $CD11b^+$ myeloid cells, $CD3^+$ T-cells and $B220^+$ B-cells to the peripheral blood determined by flow cytometry. Each dot represents one mouse from the experimental groups. (Open circles: high VCN mice, closed circles: low VCN mice of the control group). Blood from the RSD.IFNa mice was taken 6 and 21 weeks after transplantation. The shaded area represents the physiological range in C57Bl/6J age matched mice published by the Jackson Laboratories. The independent (ind.) control (grey icons) is comprised of

unpublished data from mice transplanted with lentiviral vectors expressing GFP from three independent transplantations and are shown here as additional control. Multipe comparison between the different vector constructs (A - F) were performed by Two-way ANOVA with Sidak's comaprison test ns = not significant, *p≤0.05, **p<0.01, ***p<0.001 and ****p<0.0001.



Supplementary Figure S10: GFP expression on donor platelets

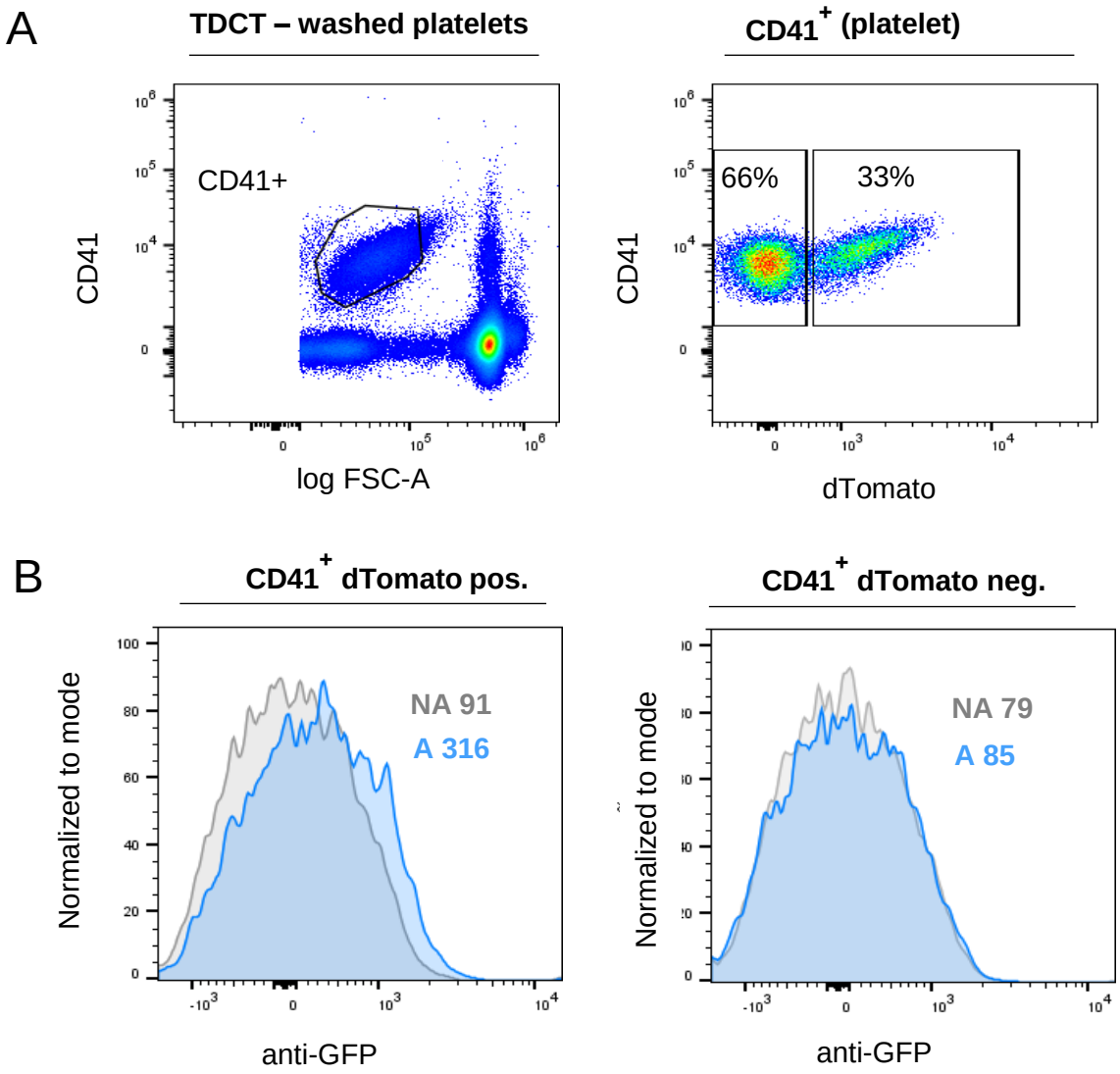
The percentage of GFP was measured by flow cytometry in platelets in the peripheral blood 6 and 16 weeks after transplantation. The GFP percentage presented was pre-gated on donor platelets, based on the expression of dTomato (see Figure S4). (Open circles: high VCN mice, closed circles: low VCN mice of the control group). Multiple comparison between 6 and 16 weeks were performed by One-way ANOVA with Dunett's comparison ns = non-signficiant.



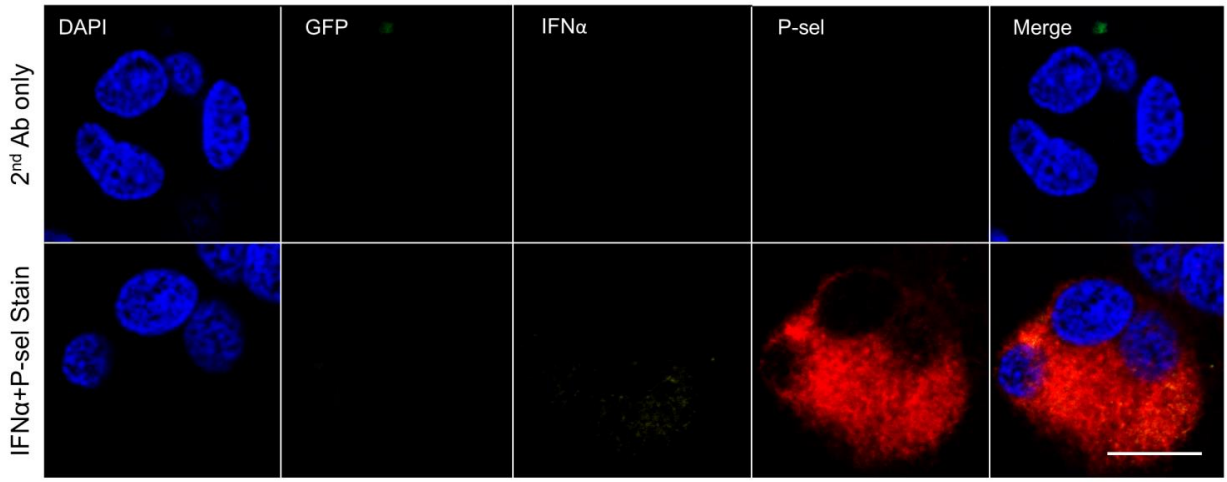
Supplementary Figure S11: Mean vector copy (VCN) number in transplanted mice

The vector copy number was determined by quantitative PCR on genomic DNA isolated from peripheral blood leukocytes of transplanted mice.

(Open circles: high VCN mice, closed circles: low VCN mice of the control group)

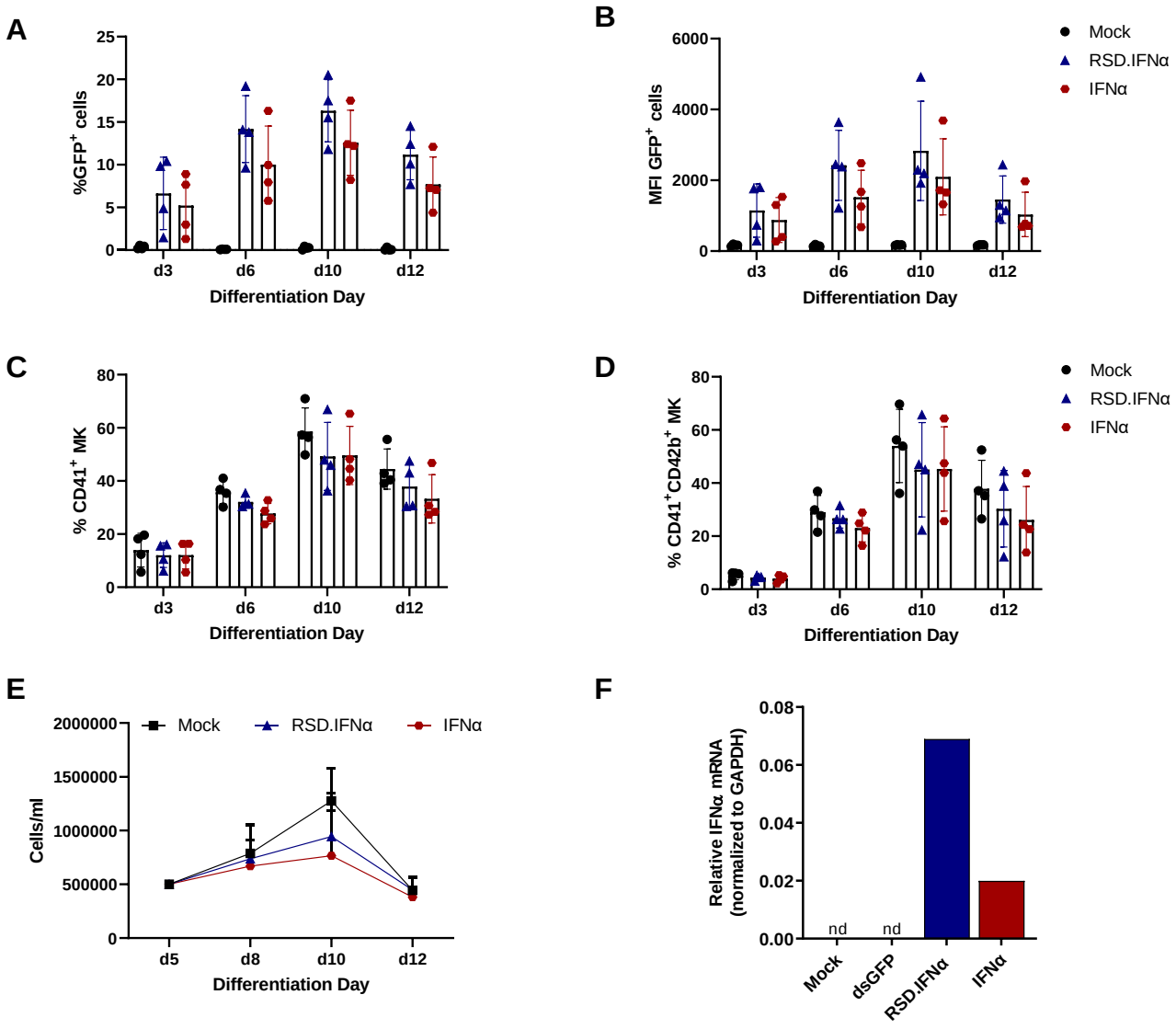


Supplemental Figure S12: GFP presentation on the surface after activation of platelets from mice transplanted with BM cells transduced with the TDCT vector (A) Flow cytometeric analysis of washed platelets (CD41+) from mice transplanted mTmG lineage marker neg BM donor cells (dTomato+) transduced with the TDCT vector. **(B)** Representative flow cytometric histograms of non-activated (NA) and activated (A) platelets from donor derived (dTomato pos.) or recipient (dTomato neg.) cells. Platelets were activated with 0.1 U thrombin for 12 min and stained with anti-GFP antibodies (clone: 1A12-6-18, BD Bioscience).



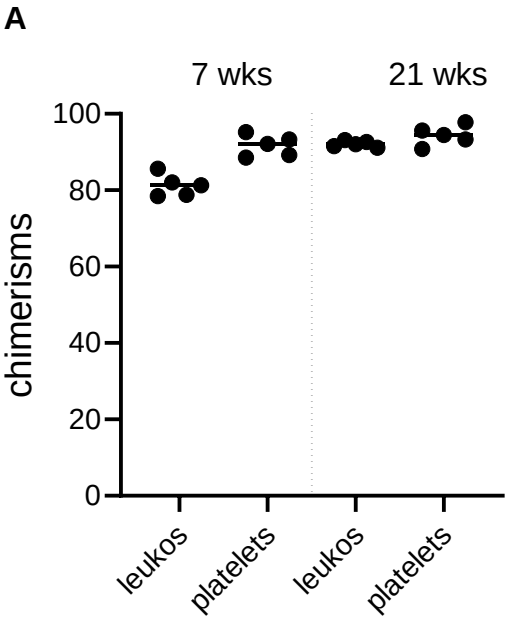
Supplementary Figure S13: Human MK immunofluorescence controls for IFNα gene transfer vectors.

Non-transduced human $CD34^+$ cord blood HSPCs were differentiated towards megakaryocytes by stimulation with THPO (50ng/ml), SCF (25ng/ml) and IL-6 (7.5ng/ml). Mature cells harvested at day 12 were immuno-stained for P-selectin (P-sel, red) and IFNα (yellow). Top panel: IFNα secondary antibody control - goat anti-rabbit IgG Alexa Fluor 647 (1:2000) and P-sel secondary antibody control – anti goat anti-mouse IgG Cy3 (1:2000). Bottom panel: IFNα stain with human IFNα (1:50) plus P-sel stain with anti-human/mouse PE-conjugated CD62P (1:100). Nuclear counterstain was done with DAPI (blue) (1:5000). Background autofluorescence in the GFP channel was subtracted based on the secondary antibody controls. Images were acquired with 63x oil immersion objective using a Leica DMi8 Inverted-3 confocal microscope, scale bar = 10µm.



Supplementary Figure S14: Differentiation characterization of cord blood derived MK expressing IFNα gene transfer vectors

CD34+ HSPCs were isolated from cord blood and transduced with RSD.IFNα and IFNα vector supernatants at MOI 40. Mock and vector-transduced cells were differentiated towards MK for 12 days. Flow cytometry determination of **(A)** Percentage of GFP+ cells, **(B)** MFI of GFP+ cells, **(C)** percentage of CD41+ and **(D)** CD41+/CD42b+ cells. **(E)** Cell proliferation from d5 – d12. A-E: n = 4 from 3 donors, data represent mean ± SD. **(F)** Relative IFNα mRNA expression of Mock, dsGFP, RSD.IFNα and IFNα MK harvested on d12, n=1 from one donor.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100
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60
40
20
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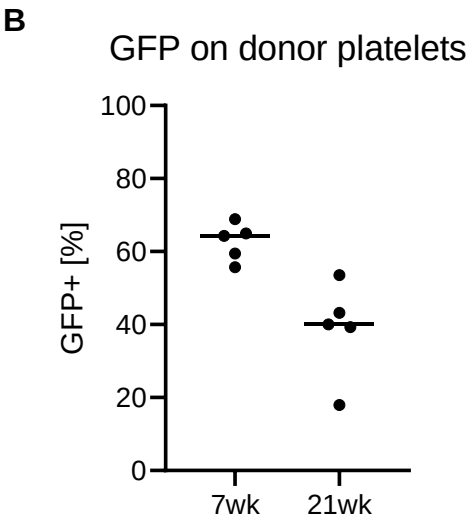
leukos
platelets
leukos
platelets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GFP+ [%]
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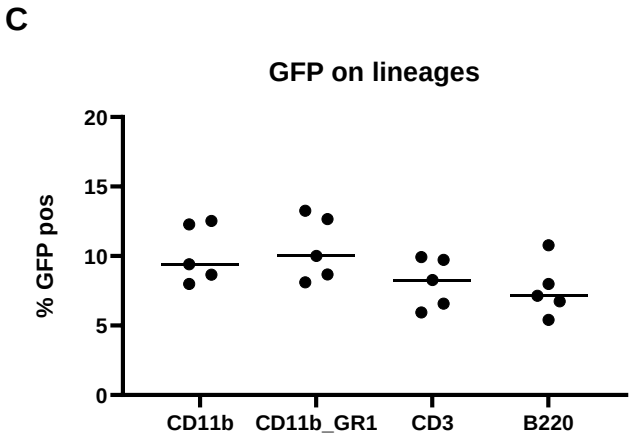
80
60
40
20
0
7wk
21wk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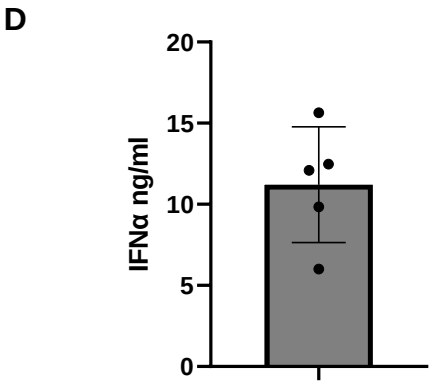
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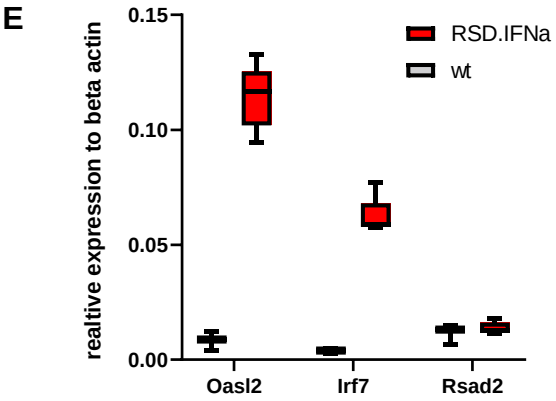
15
10
5
0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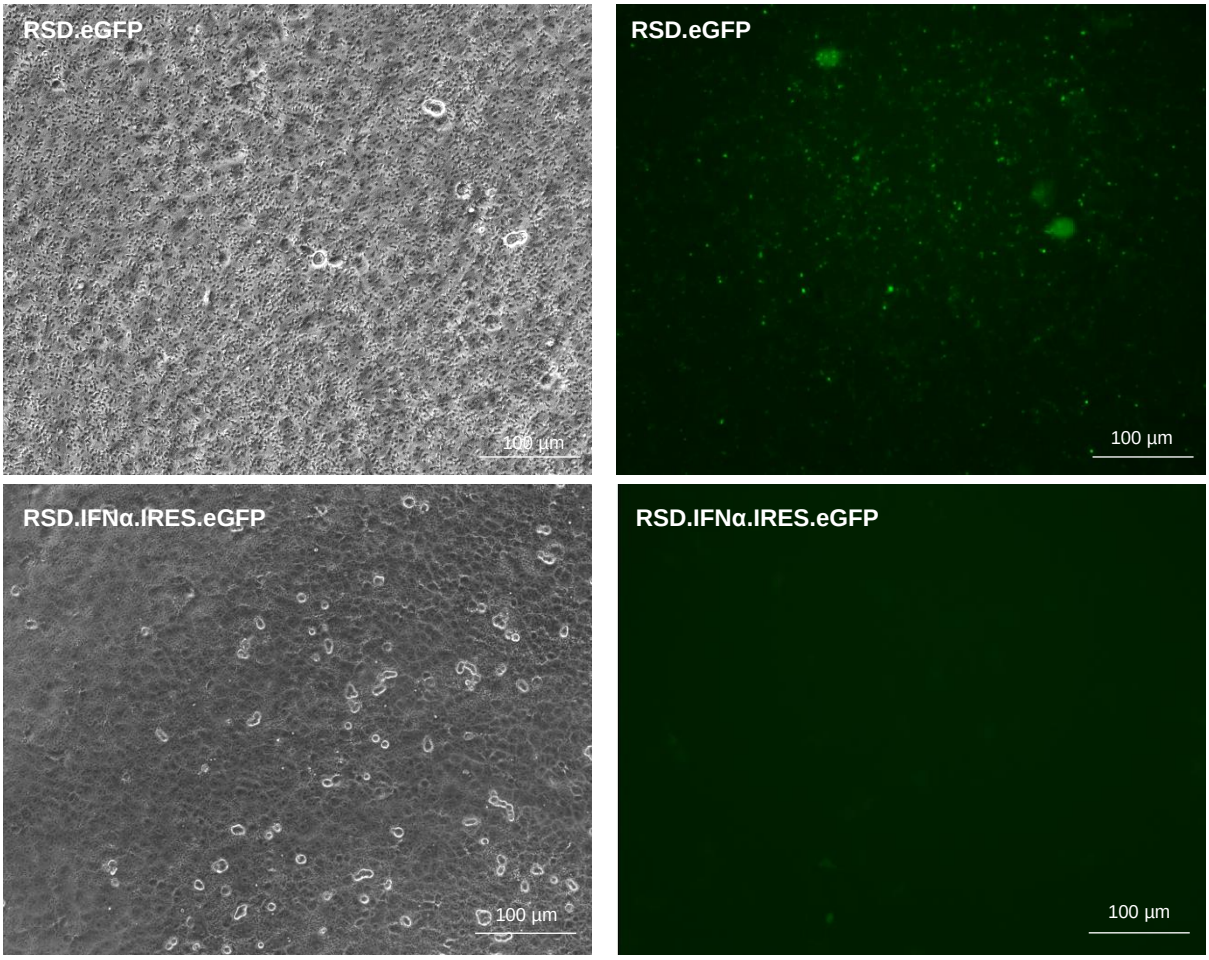
0.10
0.05
0.00
RSD.IFNa
wt
Oasl2
Irf7
Rsad2

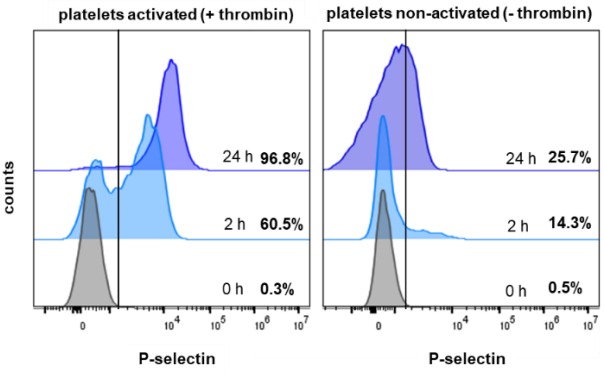
Supplementary Figure S15: Engraftment of RSD.IFNa transduced BM cells

(A) Chimerism of C57Bl/6 mice transplanted with RSD.IFNα-transduced Lin- BM cells of mTmG donor mice (n = 5). **(B)** GFP expression on donor platelets in transplanted mice**. (C)** GFP expression in myeloid cells (CD11b), granulocytes (CD11b_Gr1), T-cells (CD3) and B-cells (B220) in the blood of mice transplanted with RSD.IFNα-transduced Lin- BM cells. **(D)** IFNα concentration in the serum of RSD.IFNa transplanted mice (n=5) **(E)** Relative mRNA expression of three IFNa target genes in the bone marrow of mice of the RSD.IFNa group (n = 5 in three technical replicates) compared to wt mice (n = 3 in three technical replicates).

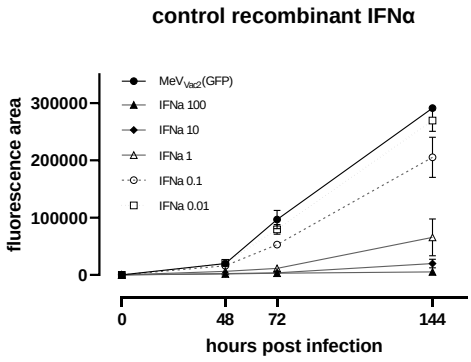
A



B



C

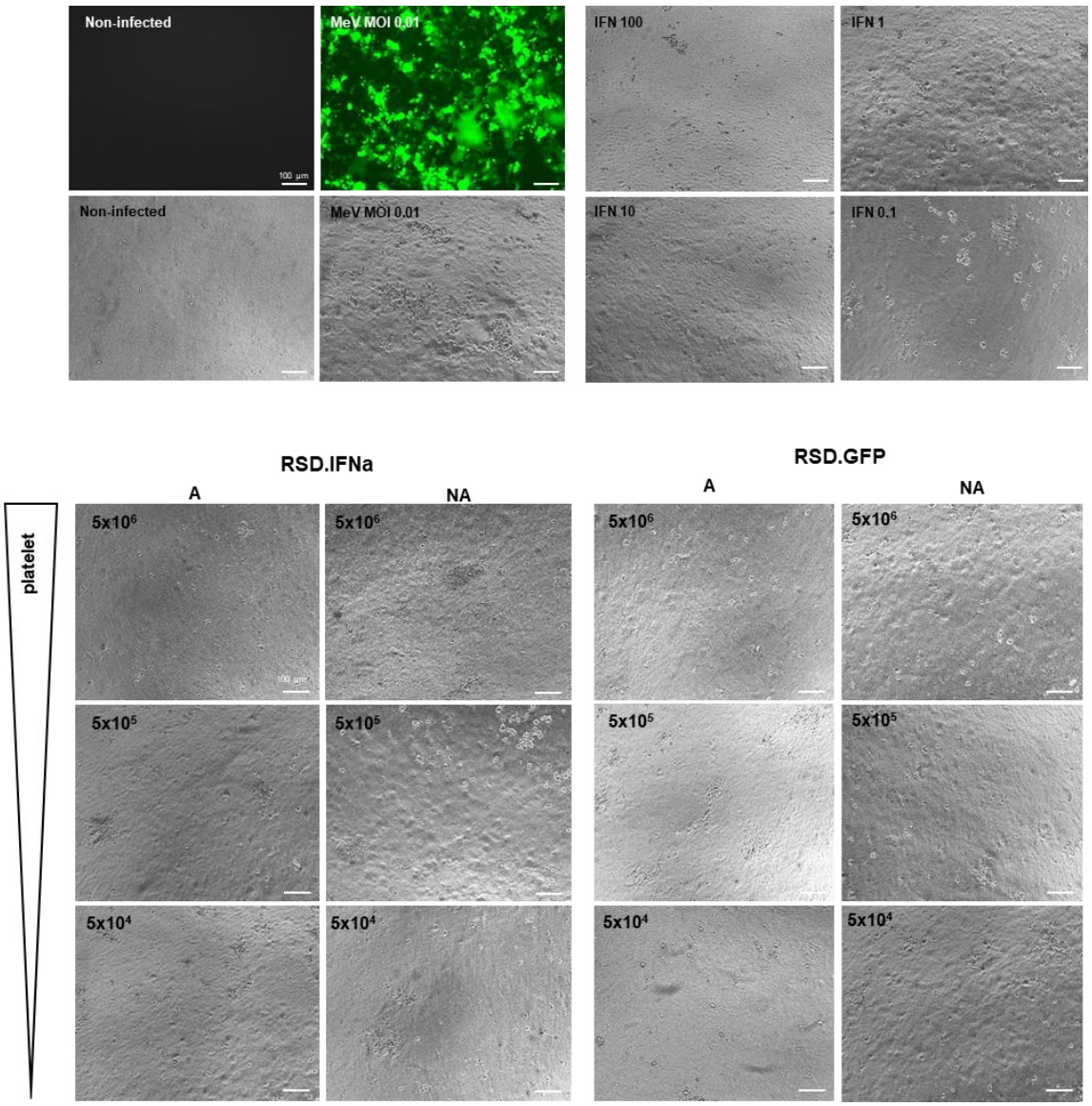


Supplementary Figure S16: Platelets in co-culture with Vero-E6 cells.

(A) Confluent Vero-E6 cells with platelets in the medium (dark grey, left images); platelets were isolated from mice transplanted with BMC transduced with the RSD.eGFP (top) and RSD.IFNα.IRES.eGFP (bottom) vector. GFP-fluorescent platelets (top right image) isolated from mice that express from the RSD.eGFP vector. Magnification 200x, scale bar = 100 µm. GFP-fluoresces of platelets ioslated from mice transplanted with RSD.IFNα.IRES.eGFP could not be deteced via the fluorescences microscope. **(B)** P-selectin surface expression of thrombin activated platelets (activation was done in the well) and non-activated platelets at 0, 2 and 24 hours co-cultivation with Vero-E6 cells. **(C)** Time course of virus replication (MOI

0.01) treated with decreasing doses of IFNα (100-0.01 U) measured by the fluorescent area at 48 h, 72 h and 144 h after infection (mean +/- SD, experiments was done twice, three images per well were analyzed at magnification 40x).

A



Supplementary Figure S17: Measles virus replication assay controls.

(A) Upper left: No GFP background fluorescence signal from Vero-E6 cells. Rest: Measles virus replication starting with an MOI of 0.01 did not alter the cell viability of the Vero-E6 cells. Vero-E6 cells were at all time points confluent during the measles virus replication assay and were not altered by the IFNa treatment or co-cultivation with activated or non-activated RSD.IFNα or RSD.eGFP platelets. Magnification 200x, scale bar = 100 µm.

Protein	Species	Sequence	Reference
RANTES sorting domain	*Mus musculus*	VVFVTRRNRQ	(uniprot p30882)
	Homo sapines	VVFVTRKNRQ	(uniprot p13501)
	Sequence identity: 90% Sequence similarity: 100%		
P-selectin transmembrane domain	*Mus musculus*	RKRLRKKDDGKCPLNPHSHLGTYGVFTNAAYDPTP	(uniprot p30882)
	Homo sapines	RKRFRQKDDGKCPLNPHSHLGTYGVFTNAAFDPSP	(uniprot p13501)
	Sequence identity: 89% Sequence similarity: 97%		
P-selectin cytoplasmic tail	*Mus musculus*	LTYLGGAVASTTGLAVGGTLLALL	(uniprot p30882)
	Homo sapines	LTYFGGAVASTIGLIMGGTLLALL	(uniprot p13501)
	Sequence identity: 83% Sequence similarity: 88%		

Supplementary Figure S18: Aligment of the murine and human aa sequence of the sorting domains

Aa sequence aligment of the RANTES sorting domain, P-selectin transmemebrane domain and cytoplasmic tail from mouse and human. Yellow labeled aa represent an aa exchange to an aa with similar proteries and blue labeled aa an aa exchange to an aa with different propeties.