

Dual role of IL-21 in megakaryopoiesis and platelet homeostasis

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Received: February 15, 2016.

Accepted: January 4, 2017.

Pre-published: January 5, 2017.

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MATERIALS AND METHODS

Antibodies. A PE-conjugated mouse anti-human CD34 antibody and its isotype control were purchased from Miltenyi Biotec and a PE-Cyanine7-conjugated anti-human CD34 antibody from eBioscience. Mouse anti-human CD41/CD61 (ALMA.17), CD42b (ALMA.12) and CD42d (V.1) and rat anti-mouse and human CD42c (RAM.1) antibodies were produced, purified and fluorochrome-conjugated in our laboratory [24-27]. Isotype controls, mouse IgG1 (MOPC-21) and rat IgG1 (R3-34), were from Sigma-Aldrich and BD Bioscience respectively. An APC-conjugated mouse anti-human IL-21R α antibody (17A12) and its isotype control were from Biolegend. An unconjugated mouse anti-human IL-21R α antibody (152504) was from R&D Systems. Alexa Fluor® (Alexa) 647-conjugated mouse anti-phospho-Stat3 (4/P-STAT3) and Alexa488-conjugated mouse anti-phospho-Stat5 (Clone 47) antibodies were from BD Bioscience. A biotinylated goat anti-mouse IgG antibody was from Beckman Coulter. The humanized and neutralizing anti-IL-21R monoclonal antibody ATR-107 was kindly provided by Pfizer Inc.

Human CD34⁺ cell isolation. Adult human peripheral blood cells were obtained with informed consent from healthy blood donors of the Etablissement Français du Sang-Alsace. The cells were recovered by elution from leukocyte depletion filters as previously described [28]. After 15 min incubation with RosetteSep® Human Granulocyte Depletion Cocktail (StemCell Technologies), mononuclear cells were isolated by Histopaque®-1077 (Sigma-Aldrich) density gradient separation for 30 min at 400g. CD34⁺ cells were separated from mononuclear cells using a CD34 MicroBead Kit and an AutoMACS™ device (Miltenyi Biotec). The purity of the CD34⁺ cells was greater than 85% as measured by flow cytometry. The cells were counted and their viability was determined using an automatic cell counter and propidium iodide exclusion (ADAM, Digital-Bio, Korea).

In vitro differentiation of MKs from human CD34⁺ cells. Adult human peripheral blood cells were obtained with informed consent from healthy blood donors of the Etablissement Français du Sang-Alsace. The cells were recovered by elution from leukocyte depletion filters as

previously described [28]. CD34⁺ cells were separated from mononuclear cells using a CD34 MicroBead Kit and an AutoMACS™ device (Miltenyi Biotec). Batches of 40,000 viable CD34⁺ cells/mL were cultured for 7 days in StemSpan™ Serum-Free Expansion Medium, supplemented with 20 µg/mL human LDL and recombinant human (rh) cytokines (CC220 cocktail, containing TPO, IL-6, IL-9 and SCF), or when indicated rhTPO alone (all from StemCell Technologies). On day 7, aliquots of 50,000 viable cells/mL were seeded and differentiated for 6 days in fresh serum-free medium, supplemented with human LDL and rhTPO (30 ng/mL). rhIL-21 (PeproTech) was added at the indicated times and concentrations (see Results section). For experiments with JAK/STAT inhibitors, on day 7 the cells were washed and preincubated for 1 h in the culture medium containing the STAT3 inhibitor Stattic (300 nM) or JAK3 inhibitor Tofacitinib (1500 nM), or 0.25% DMSO vehicle (all from Sigma-Aldrich). Afterwards, rhTPO (30 ng/mL) or rhTPO + rhIL-21 (100 ng/mL) was added and the cells were cultured for a further 5 days.

For ATR-107 blocking experiments, CD34⁺ cells were cultured in the presence of TPO (30 ng/mL) +/- IL-21 (100 ng/mL) and 100 µg/ml human IgGs (for intravenous administration, Privigen). ATR-107 (100 ng/ml) was added on day 1 and 3 of the culture. Preliminary experiments showed that the addition of the IgG did not impact the differentiation of the cells into MKs (index of proliferation and % of CD41⁺ cells), as assayed on day 7 of the culture (data not shown).

All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere.

CFU-MK assays. For human cells, CFU-MK assays were performed using a MegaCult™-C Complete Kit with cytokines (StemCell Technologies) according to the manufacturer's instructions. Briefly, 2,500 CD34⁺ cells were cultured in 750 µL of collagen-based medium containing TPO, IL-3 and IL-6, supplemented or not with rhIL-21 (100 ng/mL), in a humidified 5% CO₂ atmosphere for 10 days. After methanol/acetone fixation, megakaryocytes were visualized with an anti-human CD41a antibody using alkaline phosphatase staining. Nuclei

were counterstained with Evans blue. The stained colonies were counted under a Leica DMLB inverted microscope and 3 independent experiments were performed in quadruplicate.

For mouse cells, bone marrow cells were flushed from the femora, tibia and iliac crests with 2 mL of Iscove's 2% FBS medium, dissociated with a 23 gauge needle and filtered through a 40 µm cell strainer. CFU-MKs were grown on quadruplicate chamber slides in collagen-based MegaCult-C medium supplemented with rhTPO, rhIL-6 and rmlL-3 (StemCell Technologies) according to the manufacturer's recommendations. After 7 days, the cells were fixed in ice cold acetone and MK colonies were stained for acetylcholinesterase activity and counted under a Leica DMLB inverted microscope.

RT-PCR analyses. CD41/CD61⁺ cells were isolated on days 4, 7 and 10 of culture using the antibody ALMA.17 and magnetic beads (EasySep® "Do-It-Yourself" Selection Kit, StemCell Technologies). The purity of the CD41/CD61⁺ cells was greater than 90%, as measured by flow cytometry. Total RNA was extracted from freshly isolated CD34⁺ progenitors and cultured CD41/CD61⁺ cells using an RNeasy® Mini kit (QIAGEN). Aliquots containing 250 ng were reverse transcribed with SuperScript III Reverse Transcriptase (InVitrogen) in a final reaction volume of 30 µL. Samples (1 µL) of cDNA were amplified using iProof High Fidelity DNA polymerase (BIORAD) in a reaction volume of 20 µL, containing 0.2 mM dNTPs and 0.5 µM of each primer. The sense primers were 5'-GCTTTCTCAGACCCTCATCTGTC-3', 5'-CCTCTTATTCCTGCAGCTGCC-3' and 5'-GACTCAACACGGGGAAACT-3' and the antisense primers were 5'-CGTCCTTCAGCTCTTCATACTGG-3', 5'-TTGCATCTGTGTGGCCTGTCT-3' and 5'-ATTCCTCGTTGAAGAGCA-3' for *IL21R*, *IL2RG* and *18S* respectively. The PCR products (5 µL) were separated on 2% (wt/vol) agarose gels and stained with 1/10,000 (v/v) GelRed Nucleic Acid Stain (BIOTIUM). Images of the stained gels were acquired using an ImageQuant 350 system and analyzed using ImageQuantTL software (GE Healthcare). The numbers of cycles were empirically determined so that the PCR products were generated during the linear phase of amplification; in practice 14, 31 and 28 cycles were used to amplify

18S rRNA and *IL21R* and *IL2RG* cDNAs, respectively. The identities of the RT-PCR products were confirmed by DNA sequencing.

Immunofluorescence microscopy. Human bone marrow specimens having a normal megakaryocytic lineage were obtained with informed consent from individuals undergoing bone marrow aspiration from the iliac crest. The samples were fixed in 2% paraformaldehyde for 4 h, cryoprotected in 10% and then 20% sucrose solutions overnight at 4°C, embedded in OCT medium and cryosectioned at 8 µm. Cultured megakaryocytes at day 13 of culture were fixed in 2% paraformaldehyde for 15 min and cytopun onto poly L-lysine-coated slides. The samples were blocked for 1 h in PBS containing 100 mM glycine, 10% normal goat serum (Vector Laboratories) and 5% human FcR blocking reagent (Miltenyi) and then with an avidin/biotin blocking reagent according to the manufacturer's instructions (Vector Laboratories). After incubation for 1 h at room temperature (RT) with an unconjugated anti-human IL-21R α antibody or its isotype control, the sections were washed and incubated with biotinylated goat anti-mouse IgG for 30 min. After further washing and fixation with 1% paraformaldehyde for 10 min, endogenous peroxidases were inhibited by incubation for 30 min in methanol containing 3% hydrogen peroxide (Sigma-Aldrich). Peroxidase-labeled streptavidin (Immunotech) was added for 15 min and the staining was revealed by incubation with FITC Tyramide for 3 min (TSA PLUS Fluorescence Kit, Perkin Elmer). The sections were washed, blocked in PBS containing 2% normal rat serum (StemCell Technologies) and counterstained with an Alexa 555-conjugated anti-CD42c antibody in the same buffer at RT for 1 h. The slides were mounted using ProLong® Gold Antifade Reagent containing DAPI (Life Technologies) and examined under a confocal microscope (TCS SP5, Leica Microsystems).

Mice were sacrificed and the murine femora, spleens and livers were harvested and fixed overnight in 4% paraformaldehyde. Femora were decalcified in 10% EDTA in water (wt/vol, pH 7.4) at 4°C. The organs were cryoprotected overnight at 4°C in 10% and then 20% sucrose solutions and embedded in OCT medium and 8 µm thick cryosections were prepared. The sections were fixed in ice cold acetone for 2 min, blocked for 1 h in PBS containing 100 mM

glycine, 10% normal goat serum (Vector Laboratories) and 5% mouse FcR blocking reagent (Miltenyi), labeled for 1 h with an F4/80 mAb in PBS 0.05% Tween containing 5% normal goat serum and revealed with Alexa 647-conjugated goat anti-rat IgG for 30 min. The washed sections were blocked for 30 min in PBS containing 2% normal rat serum (StemCell Technologies) and counterstained overnight with an Alexa 555-conjugated anti-CD42c antibody. The slides were mounted in ProLong® Gold Antifade Reagent containing DAPI (Life Technologies) and examined under a confocal microscope. Three 120 µm spaced sections from each organ were labeled and three immunofluorescence micrographs were taken from each section. Mosaic scans of transversal sections of the entire bone marrow were obtained using Leica Application Suite AF software. Quantitative analysis of the micrographs using ImageJ software enabled measurement of the CD42c and F4/80 positive areas in the spleen and liver and the density of MKs in the bone marrow.

Flow cytometry. Cultured cells were labeled with an Alexa 488-conjugated anti-CD41/CD61 mAb and an APC-conjugated anti-IL-21R α mAb. The washed cells were resuspended in PBS containing 7-AAD and analyzed by flow cytometry. Washed human platelets were prepared from acid-citrate-dextrose anticoagulated blood obtained from healthy volunteers as previously described [29]. The platelets, in the resting state, activated with 1 U/mL thrombin for 10 min at 37°C, or fixed with 2% paraformaldehyde and permeabilized with 0.1% triton, were labeled with an APC-conjugated anti-IL-21R α mAb and PE-conjugated anti-P-selectin or anti-CD63 antibodies for 15 min at RT.

Signal transduction. After 7 or 12 days of culture, cells were washed, starved of cytokines for 5 h in a serum-free medium and then stimulated with 30 ng/mL TPO and/or 30 ng/mL IL-21 for 15 min at 37°C. Afterwards, the cells were fixed in 1.5% formaldehyde for 10 min, washed twice, permeabilized in ice-cold methanol and labeled with anti-CD41/CD61-ECD (PE-Texas Red), anti-phospho-STAT3-Alexa 647 and anti-phospho-STAT5-Alexa 488 antibodies for 30 min at RT. The washed cells were analyzed by flow cytometry. To measure their ploidy, anti-

CD41/CD61-Alexa 488 and anti-phospho-STAT3-Alexa 647 labeled cells were stained in 50 µg/mL propidium iodide solution in the presence of 100 µg/mL RNase A for 30 min at 37°C.

To inhibit IL-21R mediated signal transduction, ATR107 antibody (100 ng/ml) was added during the last 30 min of cytokine starvation. Cells were washed, stimulated with IL-21, then STAT3 phosphorylation was assessed using the same protocol.

Analysis of the phenotype of MKs. On days 7 and 12 of culture, cells were incubated for 2 h at 37°C with Hoechst 33342 and then stained with anti-CD34-PE-Cy7, -CD41-PE, -CD42c-Alexa 488 and -CD42d-Alexa 647 mAbs. The washed cells were resuspended in PBS containing 7-AAD and analyzed by flow cytometry.

Quantification of platelet release. On day 13 of culture, 1 µmol/L prostaglandin E1 (Sigma-Aldrich) and 0.02 U/mL apyrase, purified from potatoes as previously described [29], were added to the culture medium and the cells were subjected to repetitive pipetting as previously described [30]. The cells were then labeled with anti-CD41/CD61-Alexa 647 and anti-CD42c-Alexa 488 antibodies for 15 min at RT before direct analysis by flow cytometry. Platelets released during culture were identified as CD41/CD61 and CD42c double positive events having the same scattering properties as human blood platelets.

Analysis of murine bone marrow MK progenitors. Bone marrow cell suspensions were stained with biotinylated rat antibodies against mouse Ly76, Ly6G, CD11b, CD45R (B220), CD3e, CD5, CD4, CD8a, Ly6A and IL7R α chain (all from eBioscience), counterstained with streptavidin-APC-Cy7 and then directly labeled with rat anti-mouse CD9-FITC, CD16/32-PE, CD150-PE-Cy7, CD105-PerCP 5.5 and cKit-APC. The cells were resuspended in PBS containing 0.4 ng/mL DAPI (Invitrogen) and analyzed by flow cytometry.

Samples were analyzed on a Gallios cytometer or a BD LSRFortessa cytometer and the acquired data were analyzed with respectively Kaluza software (Beckman Coulter) or BD FACSDiva software (BD Biosciences).

Quantification of proplatelet-bearing human MKs and of platelet release. On day 13 of culture, cells were examined under an inverted microscope (Axio Vert A1, Zeiss) coupled to a camera (AxioCam ERc 5c, Zeiss). Images of ten different wide fields in the culture wells (24 well plates) were acquired and round and proplatelet-bearing MKs were counted. On day 13 of culture, 1 $\mu\text{mol/L}$ prostaglandin E1 (Sigma-Aldrich) and 0.02 U/mL apyrase were added to the culture medium and the cells were subjected to repetitive pipetting as previously described [30].

In vivo experiments in mice. Wild type C57BL/6J mice were obtained from Charles River Laboratories, while the HR35 mouse strain (C57BL/6J background) ubiquitously expressing EGFP under the control of the beta-actin promoter was kindly provided by P Chambon (Strasbourg).

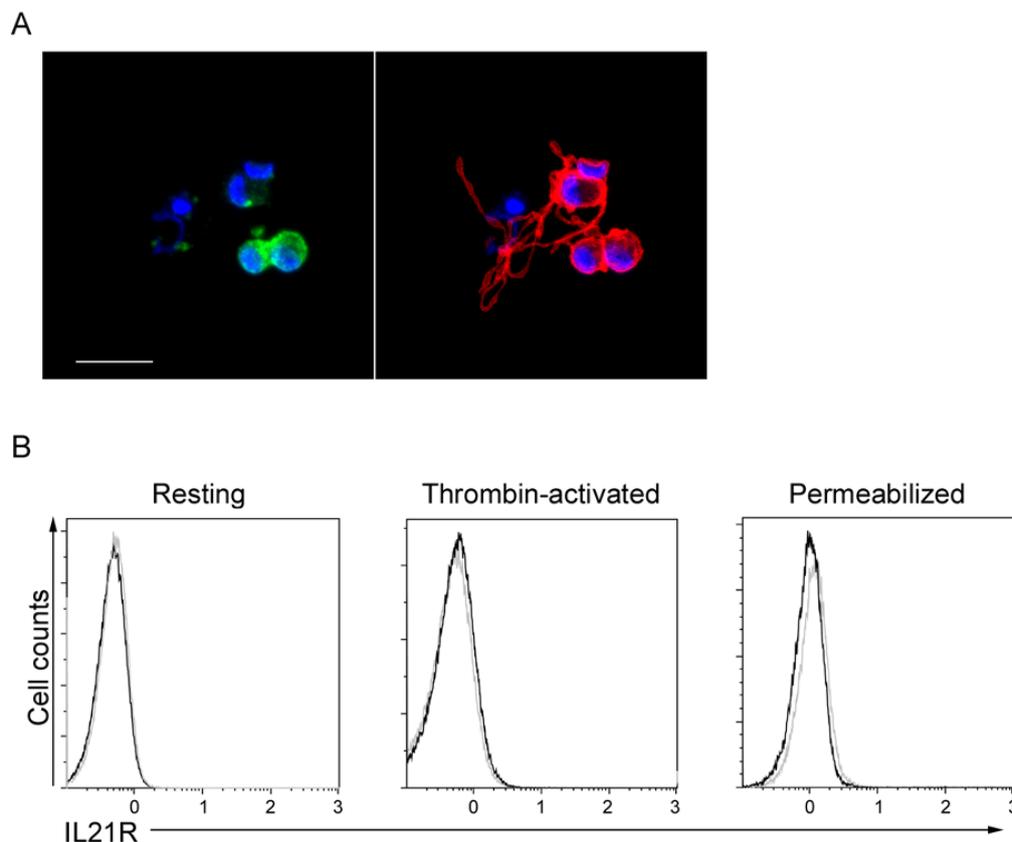
For *in vivo* expression, murine IL-21 cDNA was cloned into the pLIVE expression vector under the albumin promoter (Mirus Bio LLC). For hydrodynamic transfection, 2 mL of TransIT®-EE Delivery Solution (Mirus Bio LLC) containing empty or recombinant plasmids (10 $\mu\text{g/mL}$) were intravenously injected into 18 to 20 g male C57BL/6J mice (Liu et al., 1999). At the indicated times, plasma samples were prepared from EDTA-anticoagulated blood and stored at -80°C . The plasma concentration of IL-21 was measured by enzyme-linked immunoadsorbent assay (Mouse IL-21 ELISA Ready-SET-Go!®, eBioscience).

Mouse platelets. Blood samples were collected into EDTA anticoagulant (6 mM) from the tail of isoflurane-anesthetized mice. Platelet counts were determined using a Scil Vet abc Plus+ analyzer (HORIBA Medical). The blood samples were labeled with anti-IL-21R α -PE and anti-CD42c-Alexa 488 in the presence of an Fc blocking reagent. The percentage of reticulated platelets was determined by adding 2 μL of whole blood to 50 μL of PBS containing 6 mM EDTA, 1 $\mu\text{g/mL}$ Thiazole Orange (Sigma-Aldrich) and 0.5 $\mu\text{g/mL}$ anti-CD42c-Alexa 647. After incubation for 15 min, the cells were fixed in 500 μL of 1% paraformaldehyde and immediately analyzed by flow cytometry (a representative analysis is shown on Supplementary Figure 7).

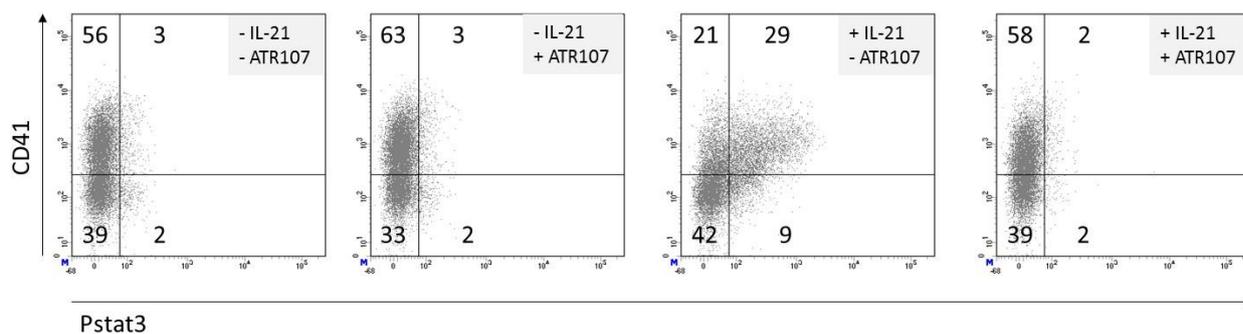
To measure platelet survival, washed EGFP⁺ platelets (1.2×10^6 platelets/ μL) were prepared from HR35 mice as previously described (Cazenave et al., 2004) and retro-orbitally injected into isoflurane-anesthetized mice, 5 days after hydrodynamic transfection (100 μL per mouse). The ratio of EGFP⁺ transfused to EGFP⁻ endogenous platelets was determined by flow cytometric analysis of EDTA-anticoagulated blood samples drawn at the indicated times.

Statistics. All values are reported as the mean \pm SEM. Statistical analyses were performed with GraphPad software (Prism Version 5.0) using Student's t test, or 1-way or 2-way ANOVA and a Bonferroni post-test. The level of statistical significance was set to $P < 0.05$.

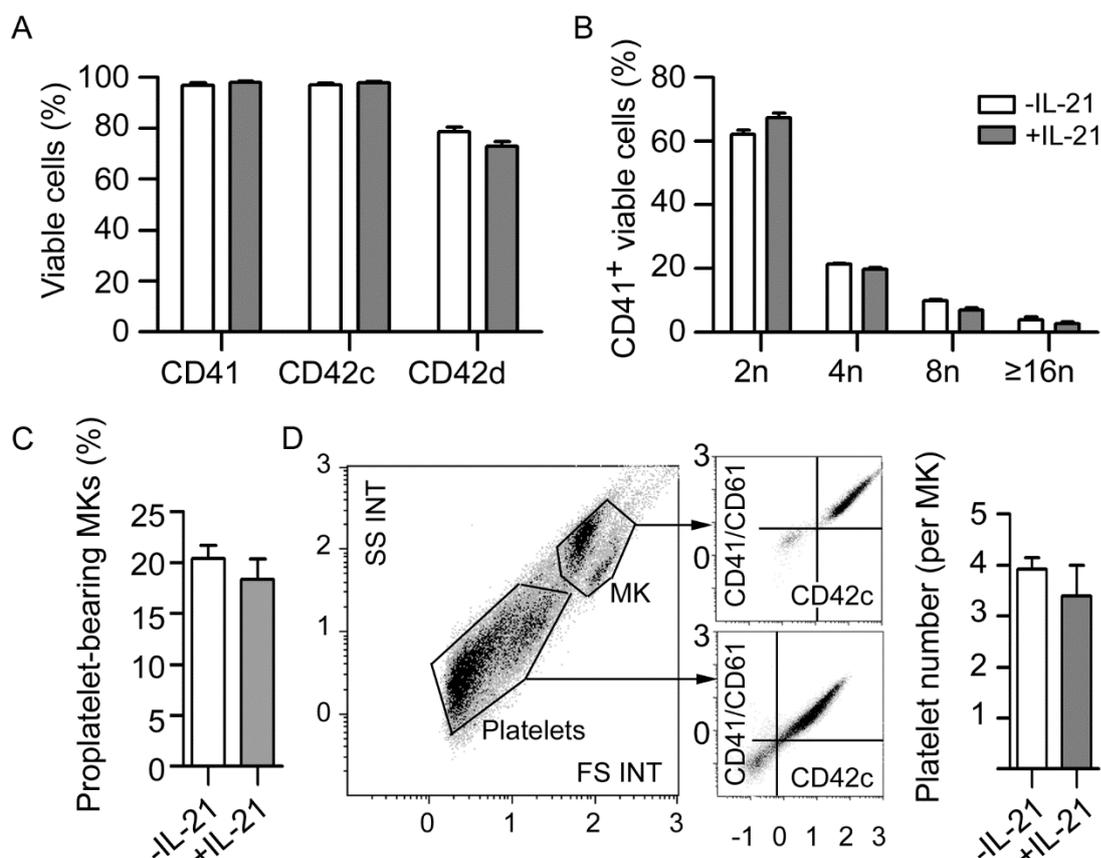
Study ethics. The human studies were performed according to the Helsinki Declaration. Experiments were performed using residue of samples from individuals who had given their written informed consent. Mice were housed and murine experiments were performed according to the French regulations and, was approved by the Regional Ethic Committee for Animal Experimentation of Strasbourg, C.R.E.M.E.A.S.(CEEA 35).



Supplementary Figure 1. Expression of IL-21R on *in vitro* differentiated megakaryocytes or blood platelets. A, CD34⁺ progenitors were differentiated into megakaryocytes under standard conditions (Figure 1A, Scheme 1, without IL-21). On day 13 on the culture, cells were cytopun, fixed and then immunostained as described for bone marrow megakaryocytes (left, anti-IL-21R, right anti-CD42c). Scale bar, 20 μ m. B, Freshly isolated human blood platelets were immunolabeled with anti-IL-21R α -APC and anti-P-selectin-PE antibodies, in the resting state or after thrombin stimulation, and with an anti-CD63-PE antibody after fixation and permeabilization. The flow cytometry histograms are representative of three independent experiments.

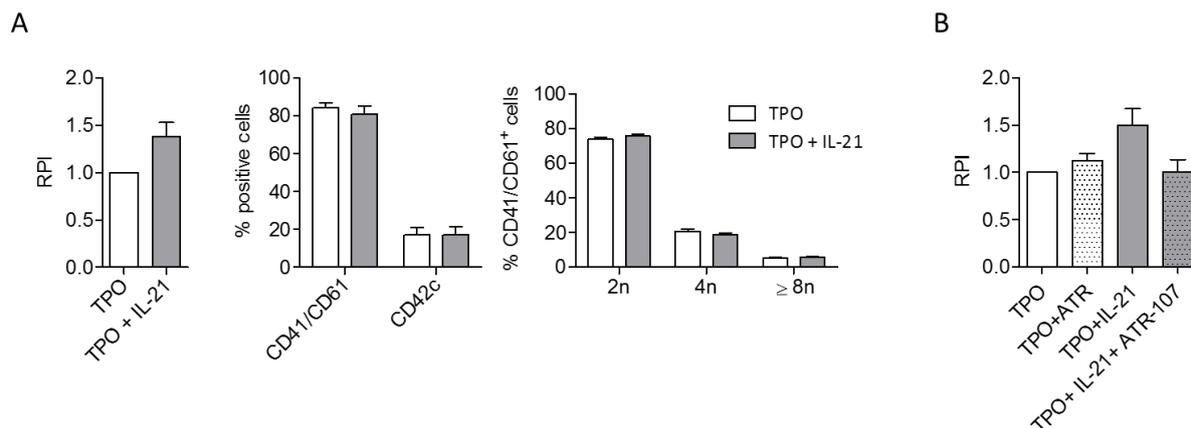


Supplementary Figure 2. IL-21-induced phosphorylation of STAT3 in megakaryocytic cells is mediated by IL-21R. CD34⁺ cells were differentiated for 7 cells days in the MK Expansion Supplement cocktail. Cells were washed and starved from cytokines for 4 hours. During the last 30 min of cytokine starvation, ATR-107 was added to the culture medium (100ng/ml). Then, pSTAT3 assay was performed as described in main section, Fig 2, in the absence, or the presence of IL-21. The percentage of events in each quadrants are shown. Two independent experiments resulted in very similar results.



Supplementary Figure 3. IL-21 preserves MK differentiation and platelet production.

CD34⁺ cells were cultured as described in Figure 1 in the presence (filled bars) or absence (empty bars) of IL-21 during the second phase of culture. **(A and B)** On day 12 of culture, the cells were labeled with anti-CD41-PE, -CD42c-Alexa488 and -CD42d-Alexa 647 antibodies, Hoechst 33342 for a ploidy analysis, 7AAD for a viability assay and analyzed by flow cytometry. **(C)** On day 13 of culture, images of the cells in the culture wells were acquired under an inverted microscope and the percentages of proplatelet-bearing MKs were counted. **(D)** On day 13 of culture, the cells were labeled with anti-CD41/CD61-Alexa 647 and anti-CD42c-Alexa 488 antibodies and analyzed by flow cytometry. Culture-derived platelet-like particles were identified through their forward and side scattering properties (identical to blood platelets) and CD41/CD61 and CD42c expression. Results are the mean \pm SEM in three independent experiments.



Supplementary Figure 4. IL-21 increases the number of megakaryocytic cells

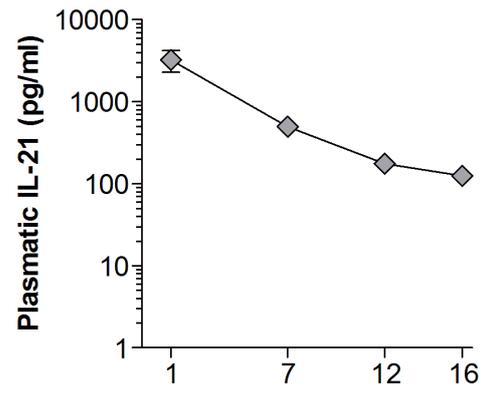
differentiated from mouse Lin⁻ progenitors, without incidence on their phenotype.

Bone marrow Lin⁻ cells (2×10^6 cells/ml) were cultured for 3 days in DMEM culture medium supplemented with 10% fetal calf serum (hematopoietic stem cell tested, Stemcell Technologies), 50 ng/ml of TPO, 100 U/ml hirudin, without or with IL21 (100 ng/ml). (A) At day 3, cells were counted and the relative proliferation indices were calculated, their phenotype and the ploidy of CD41⁺ cells were determined by flow cytometry. To analyze the ploidy, cells were fixed in cold ethanol, treated with RNase, stained with propidium iodide, as described in the method section for human MKs. Values are reported as the mean \pm SEM (n=4 or 5). (B) Bone marrow Lin⁻ cells (2×10^6 cells/ml) were cultured for 3 days in the same conditions, with or without IL-21 and/or ATR-107 (1 μ g/ml) and analyzed as in A (n=4). ATR-107 was used at a higher concentration to compensate for the 8 fold higher value of its KD with respect to mouse IL-21R, as compared to human IL-21R.

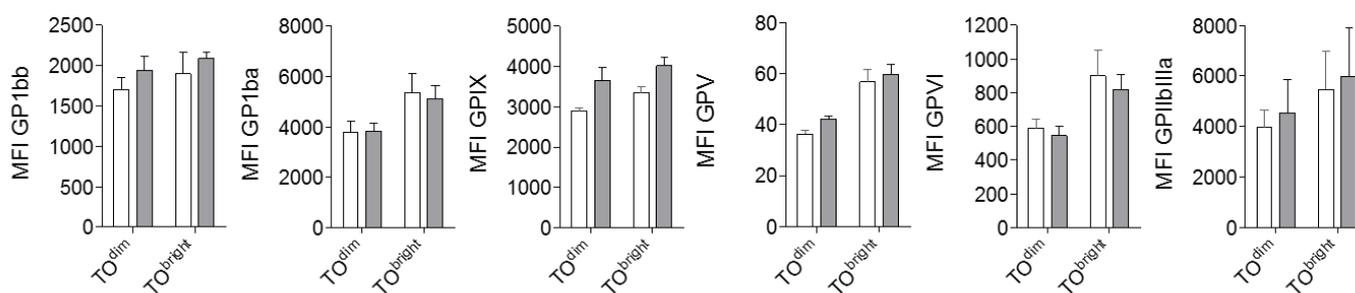
Supplementary Figure 5. Plasma concentration of IL-21 expressed after hydrodynamic gene transfer. IL-21 expressing vector (pLIVE-IL21) or control empty vector (pLIVE) were

hydrodynamically transferred in mice. At indicated days after transfer, IL-21 plasma levels was measured. The plasma concentrations of IL-21 in pLIVE transfected animals were below the limit of

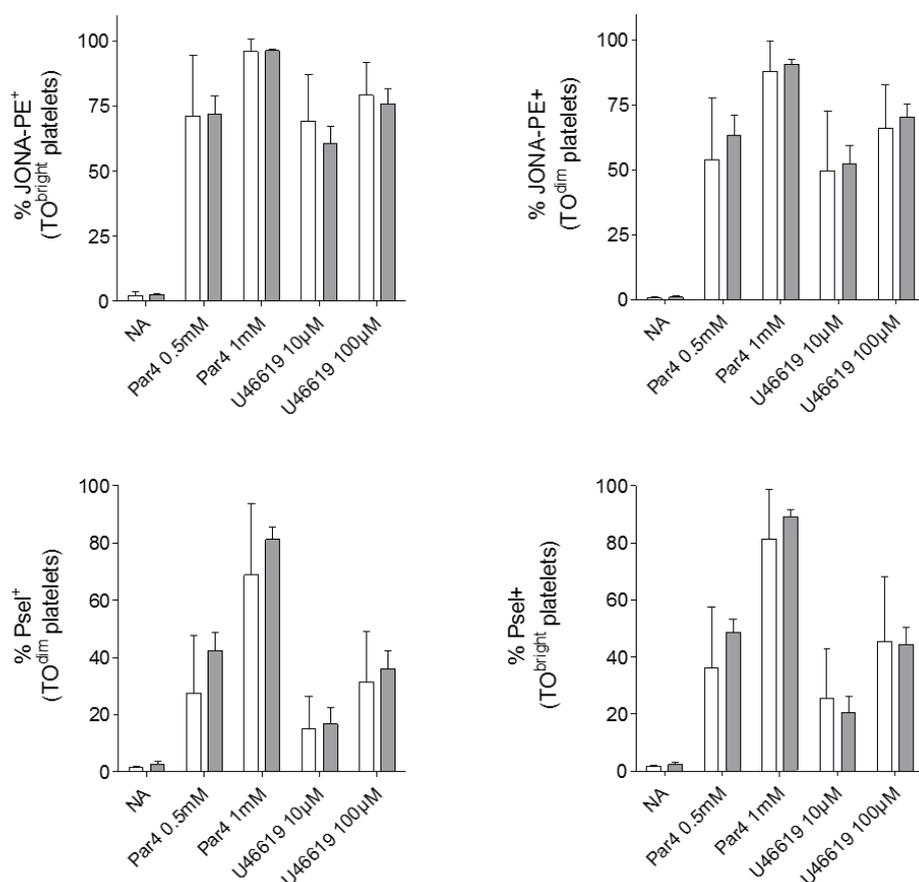
the detection, i.e. <64 pg/ml, thus were not depicted. Mean \pm SEM values are reported, n=7.



A Platelet phenotype



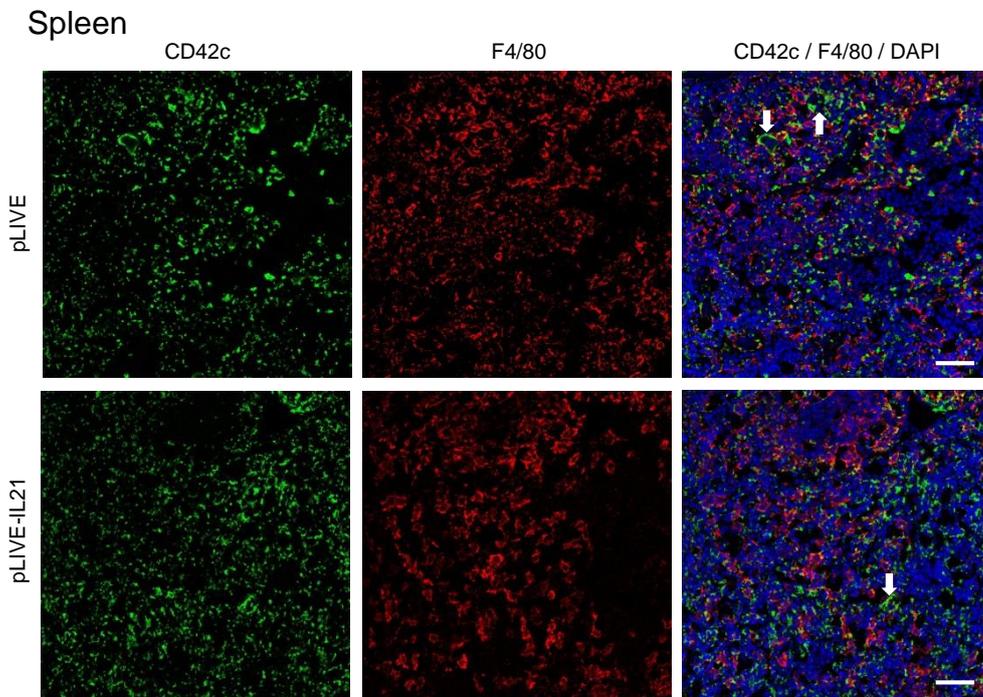
B Platelet responses



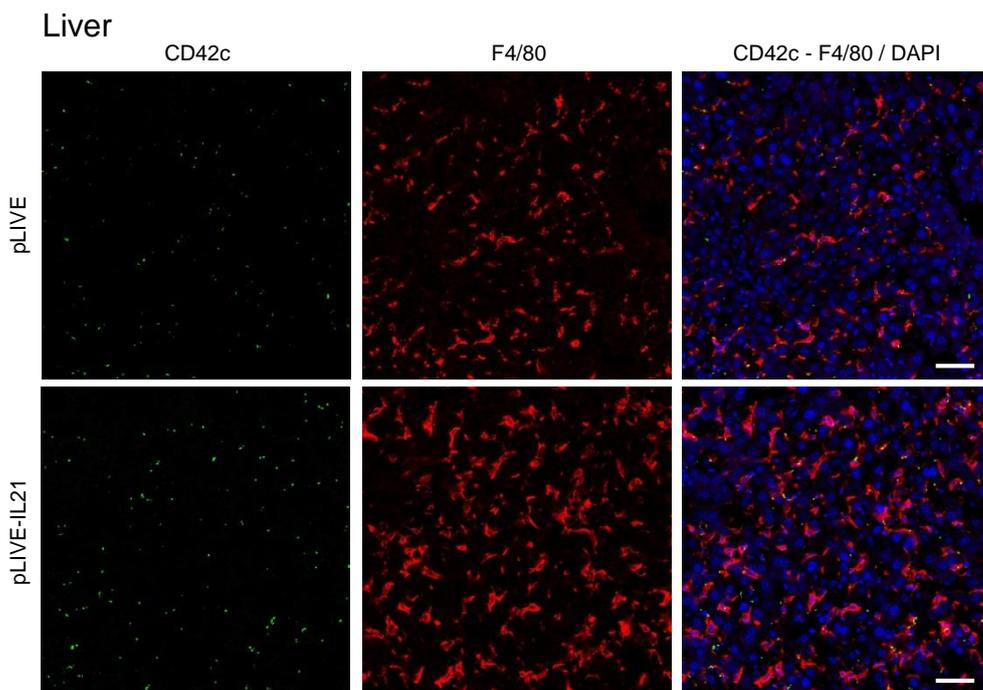
Supplementary Figure 6. Effect of IL-21 expression on the phenotype and on the reactivity of blood platelets. Six days after hydrodynamic transfection of pLIVE (white bars) or pLIVE-IL-21 (grey bars), blood was collected at the aorta in the presence of hirudin (100 U/ml). Standardized blood platelet suspensions (10^5 platelets/ μ L) were prepared by diluting blood in tyrode albumin containing hirudin (100 U/mL). (A) Platelet suspensions were mixed in 50 μ L plain Tyrode buffer containing hirudin and thiazole orange (1 μ g/mL). Platelets were labeled with the indicated mAbs for 15 min, then fixed by

adding 1 ml 1% paraformaldehyde and analyzed by flow cytometry within 45 min. (B) A mix containing thiazole orange, Alexa 647-conjugated anti-P-Selectin or JONA-PE antibody (anti-activated GpIIb/IIIa) was added to platelet suspension (10 μ L each). Then, 5 μ L 5 times concentrated agonist (AYPGKF peptide and U46619, PAR4 and thromboxane receptor agonist, respectively) in plain Tyrode buffer or the buffer alone were added. Platelets were incubated for 15 min at 37°C, then the reaction was stopped by adding 50 μ L PAF 1.5% containing RAM1 mAb (anti Gp1b β) conjugated to Alexa 555 (used in combination with anti-P-selectin labeling) or to Alexa647 (used in combination with JONA-PE mAb). The percentages of activated platelets were determined by flow cytometry within 45 min. Mean \pm SD values are reported, n=3.

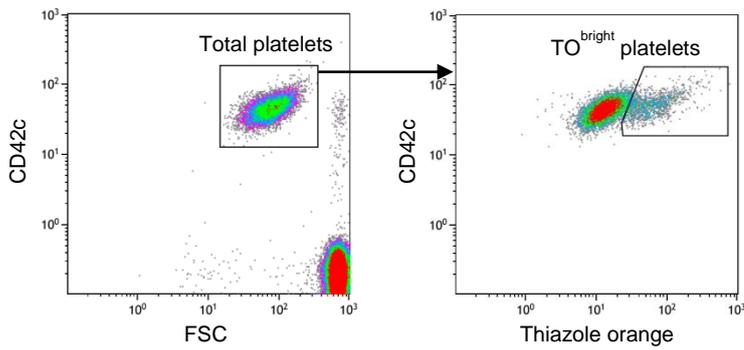
A



B



Supplementary Figure 7. Immunodetection of platelets and macrophages in the spleen and the liver. IL-21 expressing vector (pLIVE-IL21) or control empty vector (pLIVE) were hydrodynamically transferred in mice. Spleens **(A)** and livers **(B)** were removed 6 days after transfection, fixed, cryo-sectioned and immunolabelled with F4/80 mAb, revealed by Alexa 647-conjugated goat anti-rat antibody, and, after a blocking step with rat serum, counterstained with Alexa 555-conjugated anti-CD42c mAb and DAPI. Three sections per organ and a minimum of three micrographs per section were analyzed from three animals per group. Representative micrographs are shown. Megakaryocytic CD42c⁺ cells are indicated by arrows. Scale bar, 50 μ m.



Supplementary Figure 8. Analysis of young platelets by flow cytometry. Blood samples were incubated 15 min at room temperature with 1 μ M thiazole orange, and Alexa 647-conjugated anti-CD42c antibody, diluted in 20 volumes of 1% paraformaldehyde and analyzed by cell flow cytometry (GalliosTM cell analyzer, Beckman-Coulter) within 30 min. The ratio of TO^{hi} to total platelets was deduced, and then, the absolute number of TO^{hi}, based on the counts of all platelets. A representative dot plot analysis is shown.