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Supplementary Materials for

Noninvasive low-level laser therapy for thrombocytopenia

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Supplementary Materials

Materials and Methods

Flow cytometry analysis and cell sorting

Mouse BM cells were flushed from femur, tibia, vertebrae, and pelvis bones, and filtered through a 100-μm strainer, followed by removal of red blood cells with ACK lysing buffer (Life technologies). The resultant cells were suspended in phosphate-buffered saline (PBS) plus 5% fetal bovine serum (FBS, Life technologies) and incubated with APC-conjugated anti-mouse CD41 antibody (BioLegend) for 20 min on ice. MKs were sorted on a FACSAria (BD Bioscience) on the basis of CD41 expression and FSC. The sorted $CD41^+$ FSC^{high} MKs were differentiated in MK medium comprised of 100 ng/ml mouse TPO (PeproTech) in Serum-Free Expansion Medium (STEMCELL Technologies) for 1 or 3 days to generate proplatelets and platelets, respectively. Alternatively, mouse BM cells were incubated first with FITC-anti-CD41 antibody at 4°C for 30 min and then with 10 μM Hoechst 33342 (Molecular Probes) at 37°C for another hr. MKs were sorted into a 2N/4N and ≥8N fractions as previously described (*29*). Platelets were collected from day-3 cultures and quantified by FACS on the basis of CD41 expression and FSC. Mitochondria of MKs were stained with 200 nM MitoTracker Deep Red FM (Molecular Probes) at 37°C for 30 min. The relative mitochondrial content was determined by flow cytometric analysis of the mean fluorescence intensity at 660 nm. Mitochondrial membrane potential ($\Delta\psi_m$) of MKs was estimated by flow cytometric analysis of red J-aggregate fluorescence at 590 nm after incubation of the cells with 10 μg/ml tetraethylbenzimidazolylcarbocyanine iodide (JC1, Molecular Probes) at 37°C for 30 min.

Luminescent assays of ATP and caspase-3/7

MKs were sorted and seeded in 96-well plates at $5x10^4$ cells per well in MK medium, to which 100 μl CellTiter-Glo reagents (Promega) were added 1 h after LLL or sham light treatment. A luminescent signal of each sample was measured after incubation for 10 min at room temperature on a microplate reader (Molecular Devices) per manufacturer's instruction, which was proportional to the amount of ATP. Alternatively, 100 μl Caspase-Glo 3/7 reagents (Promega) were added to each well at 10 hr post-IR to examine the caspase cleavage of a luminogenic substrate. The luminescence of each sample was measured similarly as above after gently mixing

in a plate shaker for 30 seconds and incubation at room temperature for 1 hr. All samples were tested in triplicate.

Proplatelet formation assays and *in vivo* tracking of platelet formation from MKs

$CD41^+$ FSC^{high} MKs were sorted, treated with or without LLL, and placed in MK medium supplemented with 3.75 g/l methylcellulose (Sigma). The cells were differentiated in a chamber with 5% CO^2 at 37 °C. Phase contrast live cell images were recorded up to 24 h by a time-lapse microscope (Zeiss Axio Observer Z1) using a 40x objective. The longest or major diameter of PPF-MK was measured by AxioVision software (Zeiss). PPF-MKs with a diameter <100 μm were defined as "small", and a diameter ≥100 μm as "large". To estimate a ratio of PPF-MK, 500 $CD41^+$ FSC^{high} MKs were plated in each well, and the percentage of PPF-MKs was manually calculated from at least 6 samples per group.

Infusion of mouse MKs into recipients was performed as described previously (*11*). In brief, $CD41^+$ FSC^{high} MKs were sorted from mouse BMs and treated with 3 J/cm^2 of LLL or sham light, followed by staining for 15 minutes on ice with 5 μM CFSE (Invitrogen) prepared in 2.5% FBS in PBS. After washing twice, the stained MKs were administered into recipients at $1x10^5$ MKs per mouse via tail vein. $CD41^+$ $CFSE^+$ platelets in the recipients were analyzed by flow cytometry in blood samples collected every day following the infusion.

Transmission electron microscopy

To assess ultrastructures of mature MKs, mouse BM cells were collected, treated with LLL or sham light, and then differentiated in MK medium for 24 hr, after which the cells were pelleted and fixed in Karnovsky's fixative at 4°C for overnight. The fixed cells were washed with 0.1 M sodium cacodylate buffer, postfixed in 2% OsO_4 in sodium cacodylate buffer, dehydrated, and embedded in Epon t812 (Tousimis). Ultrathin sections were stained and examined on a Philips CM-10 transmission electron microscope (Eindhoven). Images were recorded with an undermount XR41M 4 Mpixel cooled camera. To count mitochondria by Image J software, a total of 30 polyploid MKs were selected randomly from each group by an investigator blinded to the treatment. The number of mitochondria of each MK, and distances of individual mitochondria to the nearest nucleus were calculated manually in the randomly selected cells.

Mitochondrial/Nuclear DNA ratios by quantitative real-time PCR

Sorted $CD41^+$ MKs were subjected to LLL or sham light and cultured in MK medium for 24 hr. Total genomic DNA was isolated using a QIAamp DNA mini kit (Qiagen) according to manufacturer's instruction. A relative copy number of mitochondrial DNA (mtDNA) was determined by quantitative real-time PCR and normalized to nuclear DNA. The primers were 5′-CCTATCACCCTTGCCATCAT-3' (forward) and 5′-GGGTTGTATT-GATGAGATTAGT-3′ (reverse) for mtDNA; and 5′-CCCTACAGTGCTGTGGGTTT-3′ (forward) and 5′-GAGACATGCAAGGAGTGCAA -3′ (reverse) for nuclear *β-actin*. Real-time PCR was performed on a Roche Lightcycler 480 with a SYBR Green I Master kit (Roche Diagnostics). The PCR program was pre-incubation at 95°C, 5 min, followed by 45 cycles of 95°C, 10s, 60°C, 10s, and 72°C, 10s. All assays were carried out in triplicate using 10 ng DNA per 20 µl reaction. The relative mtDNA copy number was calculated using comparative Ct method $2^{-\Delta\Delta Ct}$ ($\Delta Ct = Ct_{mtDNA}\text{-}Ct_{\beta\text{-}actin}$).

Real-time RT- PCR

Total RNA was isolated from sorted $CD41^+$ MKs for quantification of the expression of *Pgc1α* and mitochondrial component genes at indicated times after LLL treatment. The RNA (1 µg) was reverse-transcribed with a high capacity RNA-to-cDNA kit (Applied Biosystems). Real-time PCR was performed on a Roche Lightcycler 480 with a SYBR Green I Master kit (Roche Diagnostics). A relative amount mRNA of each target gene was normalized to endogenous *Gapdh* and calculated using comparative Ct method (ΔΔCt method). The primer sequences used in this study are listed in table S1.

Immunofluorescence analysis of caspase activation

Sorted $CD41^+$ FSC^{high} MKs were subjected to 3-Gy IR and cultured on a Poly-D-Lysine coated coverslip (neuVitro) in MK medium, followed by treatment with LLL or sham light at 6 hr post-IR. MKs were fixed 18 h later with 4% paraformaldehyde and permeabilized with 0.1% (w/v) saponin for 15 min. The permeabilized MKs were incubated for overnight at 4°C with primary antibody against caspase 3 (Cell Signaling Technology), followed with Alexa Fluor-conjugated secondary antibody (BioLegend) for 30 min at 37°C. The slides were mounted with DAPI-contained mounting medium (Invitrogen) and examined using Olympus FV1000 confocal

microscope (Olympus). Acquired images from at least 6 different samples in each group were then analyzed using FV10-ASW 4.0 Viewer software (Olympus).

Tracking femur MKs

At 24 h after whole body LLL illumination, FITC-anti-CD41 and PE-anti-CD105 antibodies (BioLegend) each at 12 μg per mouse were intravenously administered. The mice were sacrificed 15 min later and the femurs were removed and examined by confocal microscopy. At least 50 MKs were tracked in 6 views randomly selected from each femur and the percentages of PPF-MKs were calculated from 6 samples per group in a sample-blind manner.

Platelet aggregation assay

About 50 μl whole blood was collected from indicated mice by retro-orbital puncture, and centrifuged 15 min at 250 g to collect platelet-rich plasma. The platelet-rich plasma from each mouse was divided into two parts equally: one was labeled with PE-anti-CD9 and another with APC-anti-CD31 antibody. The two parts were then mixed and stimulated with 100 ng/ml phorbol myristate acetate (Sigma) at 37°C while shaking at 600 rpm. At indicated time points, samples were collected and fixed by paraformaldehyde and measured by flow cytometry. The percentage of double-colored events was quantified by flow cytometry and analyzed by FlowJo software as aggregated platelets.

Mouse blood cell count

For complete blood cell counts, 50 μl of mouse blood was collected into EDTA-coated microtainer tubes (BD Bioscience) and analyzed on a HemaTrue veterinary hematology analyzer (Heska Corporation).

Mouse bleeding times

Mouse bleeding time was assessed by transecting the mouse tail at 3 mm from the tip and immersed into a tube containing 37°C pre-warmed PBS, and time to cessation was measured.

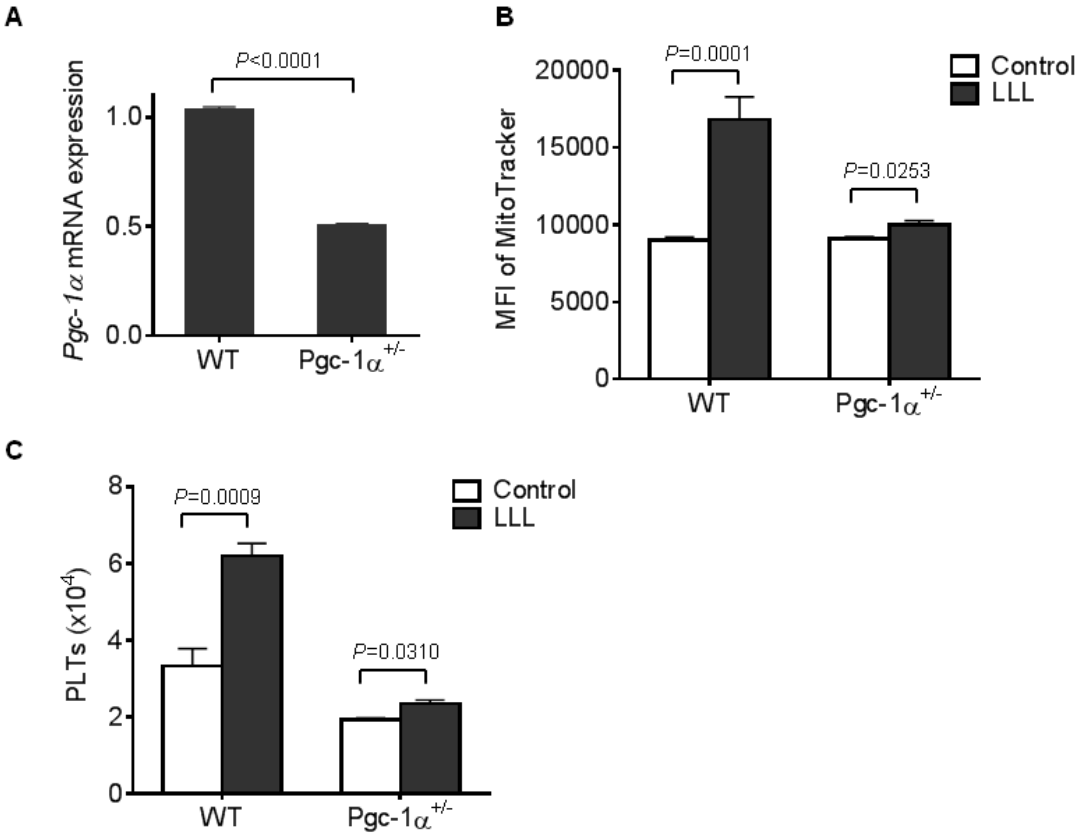

Figure S1. PGC-1α plays a role in LLL-mediated platelet biogenesis. LLL has limited effects on mature MKs from *Pgc1a*$^{+/-}$ mice. (**A**) CD41^{+} FSC^{high} MKs were sorted from wild-type or *Pgc1a*$^{+/-}$ mice, and the relative *Pgc1a* expression was assessed by real-time RT-PCR. (**B**) Sorted MKs from wild-type or *Pgc1a*$^{+/-}$ mice were subjected to sham-light or LLL, and then cultured in MK medium. At 24 h post-LLL, MKs were stained with MitoTracker for mitochondrial mass analysis. (**C**) Alternatively, MKs were differentiated into platelets in 3 days. In all panels, data are means ± SEM (n = 6) and P values were determined by two-tailed Student's t test.

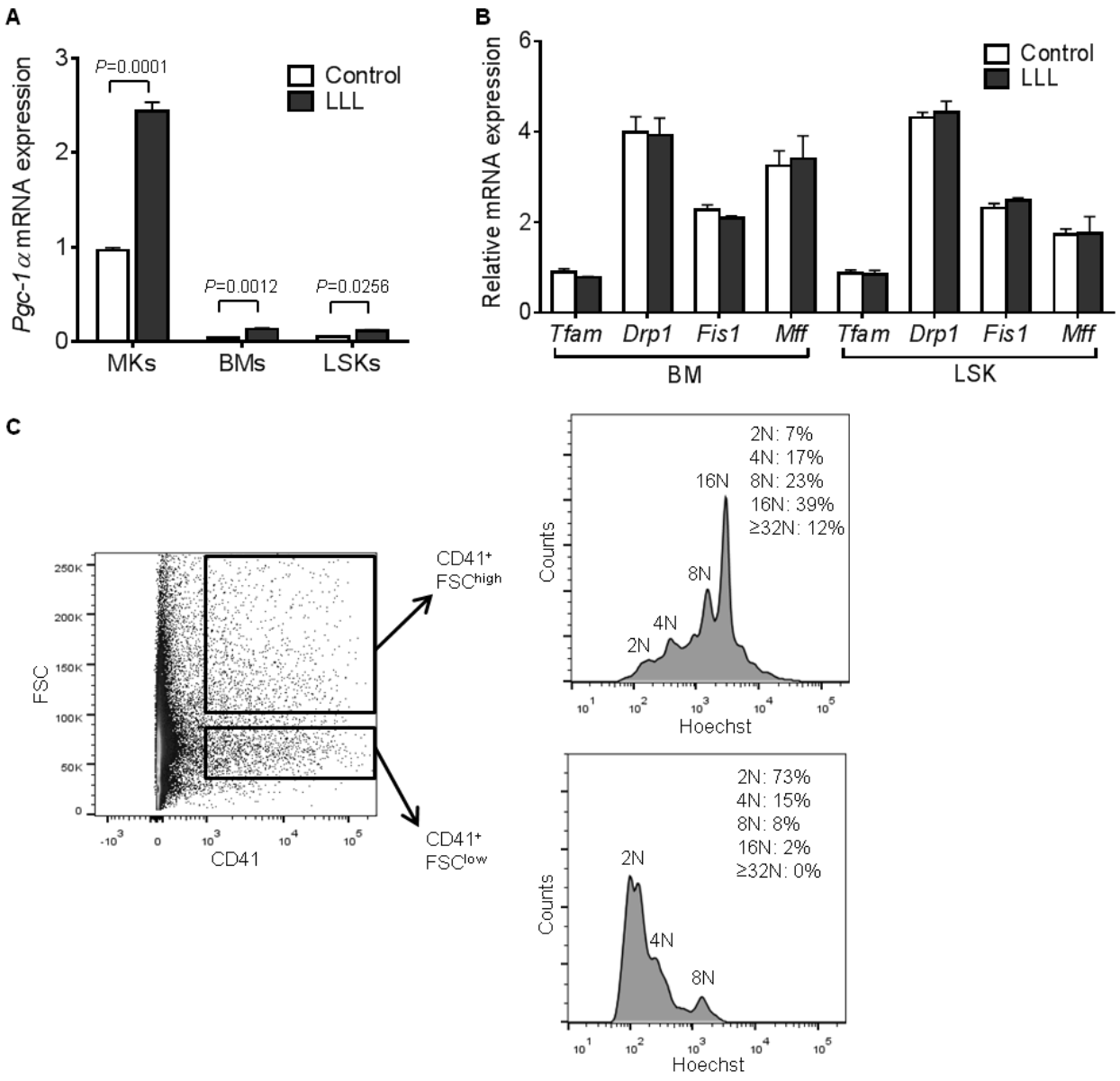

Figure S2. LLL increases *Pgc1a* transcription, but expression of downstream genes is unaltered in BM nucleated cells and LSKs. (**A**) *Pgc1a* expression in indicated cells was assessed at 4 h post-LLL by real-time RT-PCR and normalized to that of control MKs. Data are means ± SEM (n = 6). P values were determined by two-tailed Student's t test. (**B**) Transcripts of other downstream genes were determined similarly at 16 h post-LLL. The expression levels were normalized to the level of *Tfam* in control BM nucleated cells. Data are means ± SEM (n = 6). (**C**) Ploidy analysis of $CD41^{+}FCS^{low}$ and $CD41^{+}FCS^{high}$ MKs in the BM. Cells were stained with Hoechst 33342 and FITC-anti-CD41 antibody, followed by flow cytometric analysis of DNA content. Data represent three independent experiments.

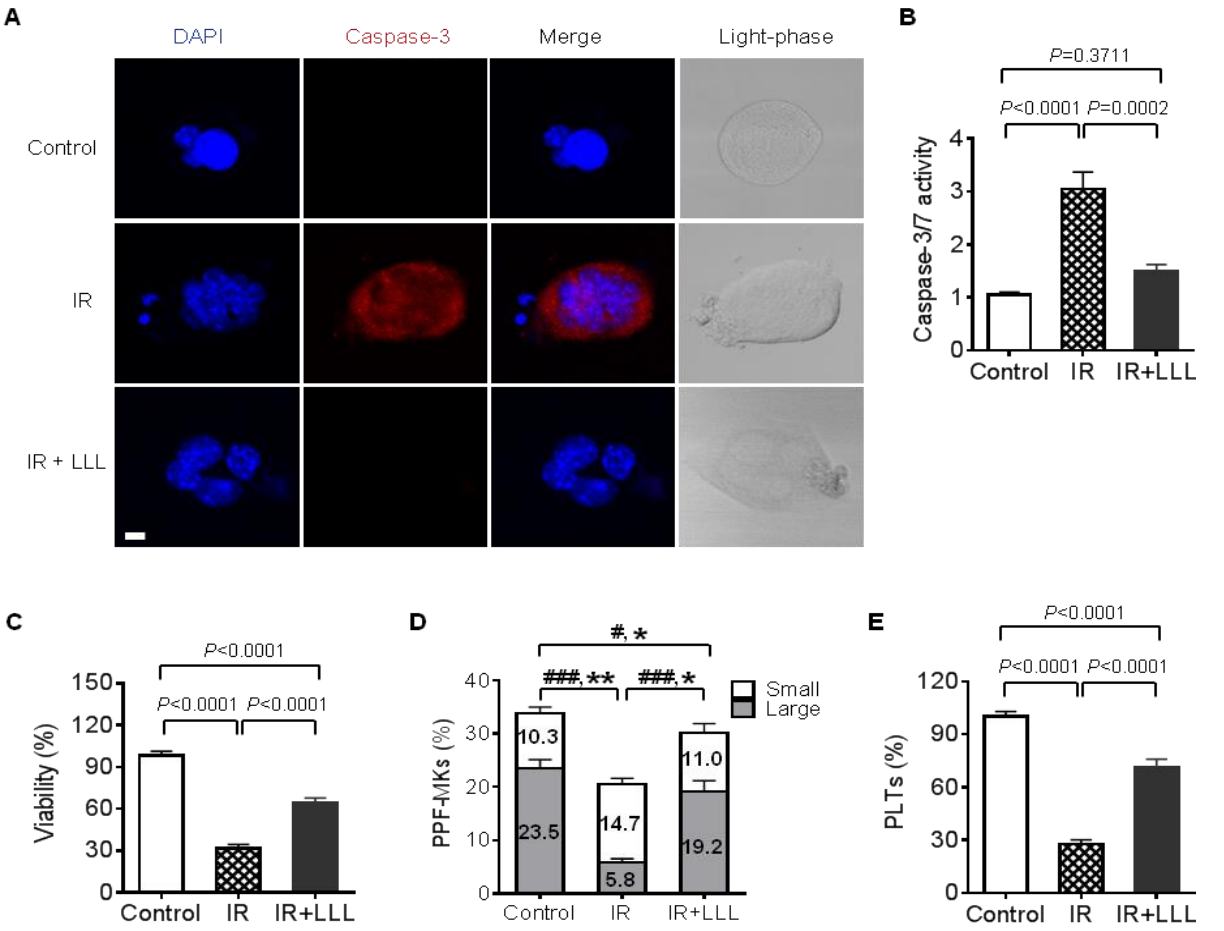

Figure S3. LLL protects mitochondrial function of γ-irradiated MKs. (**A**) Sorted MKs were subjected to 3-Gy IR and then exposed to LLL 6 h later. After overnight culture, MKs were immunostained with an antibody specific to caspase-3, followed by DAPI staining to label DNA. Representative images for caspase-3 and DAPI are from at least 6 different samples in each group. Scale bar, 10 μm. (**B**) Caspase-3/7 activity in MKs was measured at 10 hr post-IR using Caspase-Glo 3/7 reagents. Data are means ± SEM (n = 6). (**C**) Cell viability of MKs were measured 24 h post-IR using CellTiter-Glo reagents. Data are means ± SEM (n = 6). (**D**) Percentages of PPF-MKs were analyzed as Fig. 1F at 24 h post-IR from at least 6 samples per group. $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$, large PPF-MKs compared; $^{*}P < 0.05$ and $^{**}P < 0.01$, total PPF-MKs compared. (**E**) Platelet counts were determined in 3 days' MK culture by flow cytometry. Untreated control was set as 100%. Data are means ± SEM (n = 6). All P values were determined by one-way ANOVA.

SUPPLEMENTARY TABLE

Table S1. Primer pairs used for quantitative real-time RT-PCR.

Target	Forward	Reverse
Tfam	AAAGAAAGCACTGGTAAAGAGAAGAG	CCCTGAGCCGAATCATCCT
Drp1	TCACCCGGAGACCTCTCATT	TGCTTCAACTCCATTTTCTTCTCC
Fis1	ACGAAGCTGCAAGGAATTTTGA	AACCAGGCACCAGGCATATT
Mff	TCGGGTCTGTCCTCCCCATA	CAACACAGGTCTGCGGTTTTCA
Pgc1a	GAGAACACTTGGGCTGTGAA	AGCTCACATCTAAAGGCATGAA
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA