REPORT

Rectifying the impairment of immune thrombocytopenia plasmas through photobiomodulation

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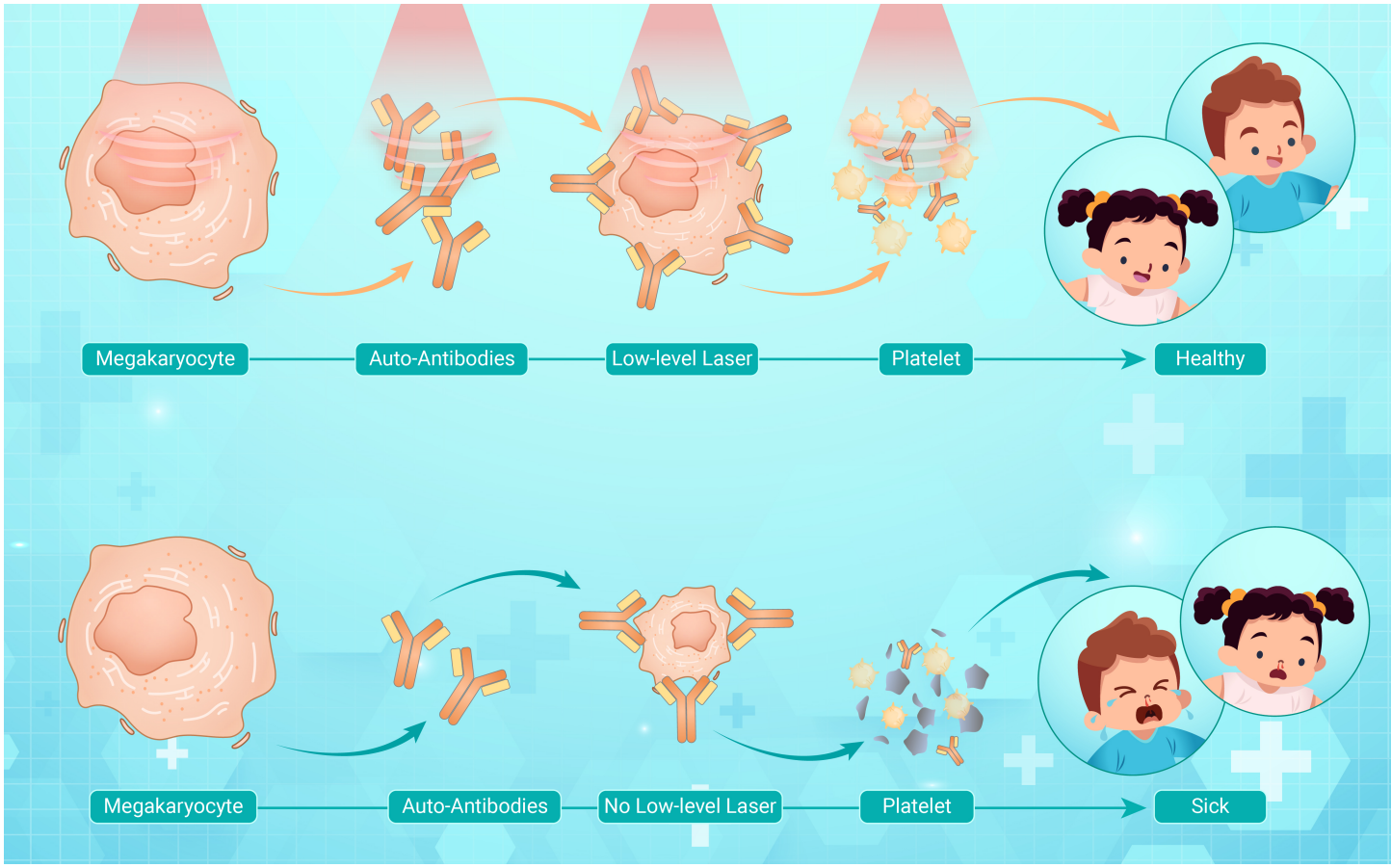
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Immune thrombocytopenia is an autoimmune hemorrhage disorder.
- Patients' plasmas hinder megakaryocyte differentiation and maturation.
- Photobiomodulation effectively mitigates the detrimental impacts.
- Photobiomodulation appears to uphold cellular mitochondrial functionality.

REPORT

Rectifying the impairment of immune thrombocytopenia plasmas through photobiomodulation

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Immune thrombocytopenia (ITP) is an autoimmune hemorrhage disorder. The first-line treatment of this disorder is corticosteroids, followed by thrombopoietin (TPO) receptor agonists such as Nplate, and/or splenectomy. Yet, the extended usage of corticosteroids or the expensive Nplate, coupled with the implications of splenectomy, raises concerns due to the array of associated side effects and an escalated vulnerability to subsequent complications. The current investigation shows that while anti-platelet antibodies and ITP plasmas hinder megakaryocyte differentiation and maturation and impair proplatelet and platelet formation in ex vivo culture of umbilical cord human CD34+ stem cells (cHSCs), low-level laser (LLL) treatment or photobiomodulation (PBM) effectively mitigates these detrimental impacts. PBM reinstated megakaryocyte differentiation and maturation, bolstering proplatelet and platelet formation in the presence of auto-platelet antibodies or ITP plasmas. The mitigating effects of PBM appear to pivot on its capacity to uphold cellular mitochondrial functionality and rectify the mitochondrial impairments engendered by anti-platelet antibodies or ITP plasmas. These findings underscore the potential of PBM as a safe and cost-efficient alternative for the management of a specific subset of ITP patients.

INTRODUCTION

Primary immune thrombocytopenia (ITP) is an autoimmune disorder characterized by a low platelet count resulting from platelet destruction and impaired platelet production. It can lead to an increased risk of bleeding, fatigue, and decreased quality of life. Current treatment options for ITP include corticosteroids, intravenous immunoglobulins, anti-D immunoglobulin, rituximab, thrombopoietin receptor agonists, fostamatinib, splenectomy, etc.[1-3] There are also some new targeted therapies under clinical trials, for instance, inhibitors for the neonatal Fc receptors, Bruton tyrosine kinase, and the complement pathway.[4] However, these therapies are commonly associated with a range of adverse effects or high expenses. In addition, even though occurring in a short period of time, neonatal thrombocytopenia places newborns at a high risk of intracranial hemorrhage, a disorder that adversely affects brain function and development, despite the fact that most of them survive with a normal level of platelets in a few weeks after birth.[5-7] No drugs or therapies are currently available for neonatal thrombocytopenia in general, apart from platelet transfusions. Hence, there is a need to develop a safe and cost-effective alternative to manage ITP in adults, children, and newborns.

Low-level laser (LLL) or photobiomodulation (PBM) utilizes a red or near-infrared light with a wavelength ranging from 600 to 1100 nm, output power of 1-500 MW, and relatively low energy densities (0.04-50 J/cm^2), in either a continuous wave or pulsed mode. LLL has been safely applied to humans for wound healing, tissue repair, pain relief, alleviating neurodegenerative diseases and traumatic brain injury, and controls of inflammation in the clinics.[8,9-14] We have shown that LLL stimulates the expression of a group of genes involved in mitochondrial biogenesis in megakaryocytes (MKs), resulting in doubling the number of mitochondria and the rate of platelet production per MK, concomitant with MK enlargement.[15] In line with the *ex vivo* observations, noninvasive LLL treatment bolstered mitochondrial biogenesis, accelerated proplatelet formation and platelet production, and alleviated thrombocytopenia in several animal models including ITP.[15,16] In ITP models, LLL preserved mitochondrial functions in both MKs and platelets in the presence of the anti-CD41 antibody.[16] Mouse offspring of the timed pregnant mice receiving anti-platelet antibody daily at 0.5 mg/kg in the final 7 days of their pregnancy displayed signs of low platelet counts and internal hemorrhage, particularly in the head and intestines.[17,18] Strikingly, LLL admitted noninvasively twice a day for 3 consecutive days after birth elevated platelet counts significantly over sham controls on day 4 and completely normalized the counts on 7 after birth, in contrast to sham-control that took two weeks to normalize the platelet counts.[17] The signs of hemorrhage were no longer seen when examined on day 4 post-birth in LLL-pups, but still clearly presented in sham-treated pups.[17] While raising platelet counts robustly in thrombocytopenic mice, LLL treatment exhibited little influence on platelet counts in normal controls.[15,16]

In attempt to explore the clinical potential of this noninvasive, drug-free modality to treat ITP in adults, children, and newborns, we investigated the effect of LLL on human megakaryocytopoiesis and thrombopoiesis in the presence of anti-platelet antibodies as well as plasmas from ITP patients as autoantibodies are the part of the mechanisms underlying ITP.[1,19] Consistent with the findings in mice, LLL significantly reversed the hindrance of human MK maturation and platelet production caused by either autoantibodies or ITP plasma. These findings support LLL to be potentially a safe, convenient, and cost-effective modality to ameliorate ITP in a subset of ITP patients.

MATERIALS AND METHODS

Low-level laser (LLL) illumination

A 980 nm diode laser from OsTech, Berlin, Germany was employed with an energy density of 0.03 J/cm^2 for ex vivo treatment. The laser fluence was obtained by exposure of the megakaryocyte cultures for 30 seconds to 1 mW/cm^2 of 980 nm laser, measured by Handheld Laser Power & Energy Meter (Ophir Nova II) and verified in each experiment. The 980 nm laser beam was transmitted through an optical fiber with a diameter of 6 mm and held firmly by a clamp on a support stand. The distance between the light and the top surface of the plate was fixed. When subjected to 980 nm laser, each well with lid on was irradiated at 10 points/well in a 12-well plate, 5 points/well in a 24-well plate or 1 point/well in a 96-well plate to cover the entire area of the well. The sham light was administered similarly with a small soft white LED light bulb (3 W A15) from General Electric (GE). The temperature of the cell cultures remained unaltered with either light source or sham light during or after 30 seconds of illumination. LLL treatment began 1-2 hours after antibodies or ITP patient plasma was added to the MK culture to determine the effect of LLL on autoantibody-mediated impairment of platelet generation unless otherwise specified.

Human MK differentiation and platelet production ex vivo

Cryopreserved human umbilical cord blood (hUCB) CD34$^+$ cells were purchased from STEMCELL Technologies and differentiated in the serum-free expansion medium (SFEM) in the presence of recombinant human thrombopoietin (rhTPO) at a final concentration of 100 ng/ml (PeproTech).

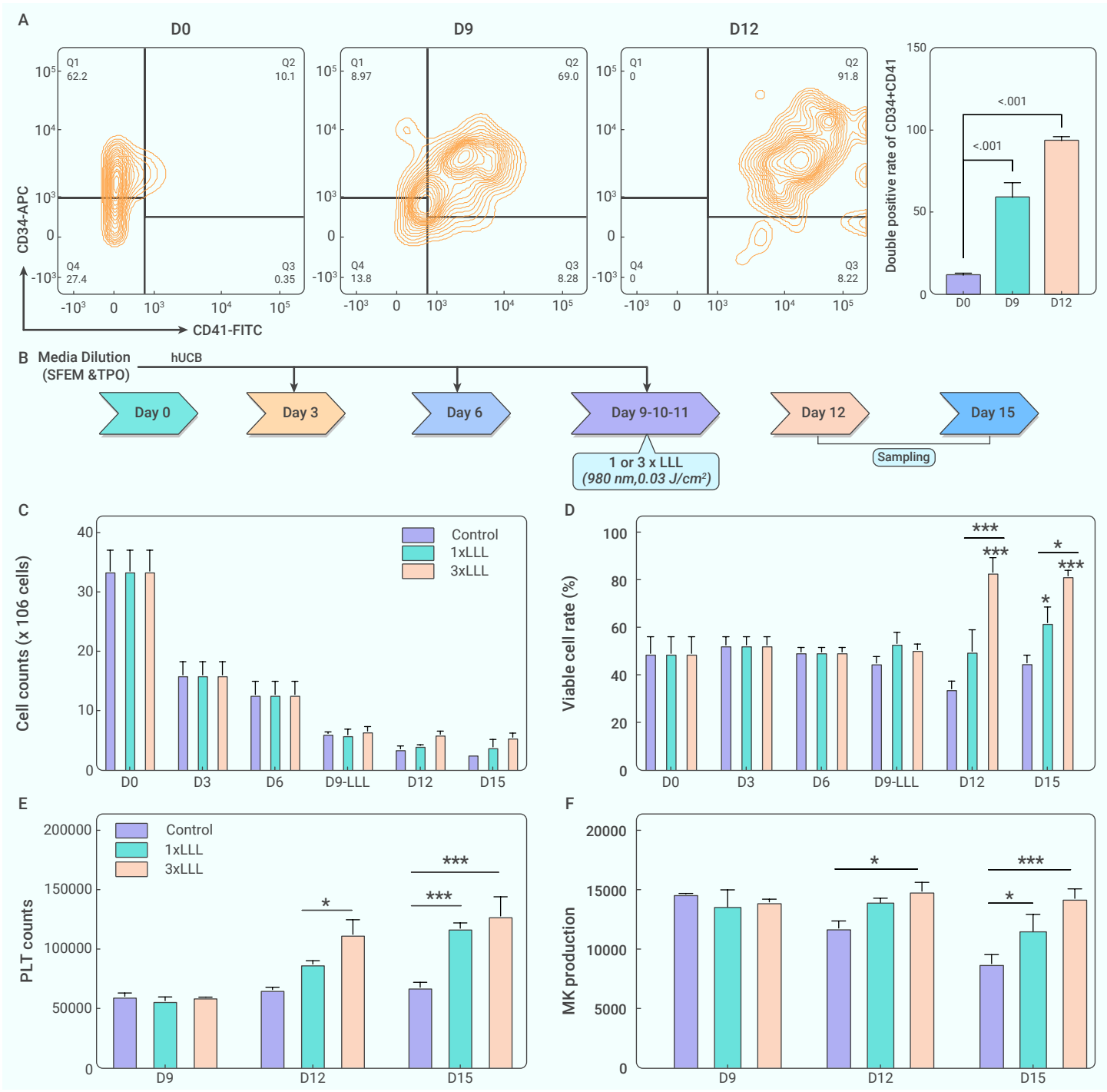

Figure 1. Enhanced differentiation of MKs and PLT production from hUCB34⁺ cells ex vivo by 980 nm laser at 0.03J/cm² (A) CD41 and CD34 double positive cells increase over time as shown by flow cytometry analysis. hUCB34⁺ stem cells were cultured ex vivo for 12 days and MKs were detected at high levels on days 9 and 12 by flow cytometric analysis of CD34 and CD41 expression on the cells. Shown are representative flow cytometric profiles (left) and mean percentages of CD34⁺CD41⁺ MKs. (B) A timeline of MK differentiation and LLL administration. LLL was administered once on day 9 only (1 x LLL) or 3 consecutive days from 9-12 (3 x LLL). C-F. LLL significantly augments cell viability as well as the PLT and MK production. Cell counts (C) and cell viability (D) were analyzed every three days during the 15 days of culture, while MKs (E) and platelets (F) were enumerated every three days starting from day 9 of the culture as depicted in B. Platelets were measured by flow cytometry in the basis of CD41 expression and forward/side scatter^{low}. n=6, *, p<0.05 **, p<0.01 and *** p<0.001, respectively, compared in the presence or absence of LLL treatments or between indicated groups.

MK differentiation stages during the culture were evaluated at indicated times by microscopy and/or CD41 expressions via flow cytometry. Phase contrast live cell images were taken by a time-lapse microscope (Zeiss Axio Observer Z1) using a 40x objective. The percentages ofproplatelet-forming (PPF) MKs were manually calculated from at least 9 fields randomly selected from triplicate cultures under a microscope.

ITP patient and control plasma samples

Plasma samples of nine de-identified patients with clinically identified ITP and 3 healthy volunteers were obtained from the Mass General Brigham (MGB) Biobank which collected samples from all consent subjects. In the patient groups, there were seven females and two males at the ages from 28 to 80 years. The clinical characteristics of these patients were not available, but they were diagnosed with ITP. As for the healthy control group, there were 2 females and 1 male in the similar age.

Flow cytometry analysis

MKs were quantified on the basis of CD41⁺ forward/side scatter (FSC)^{high}

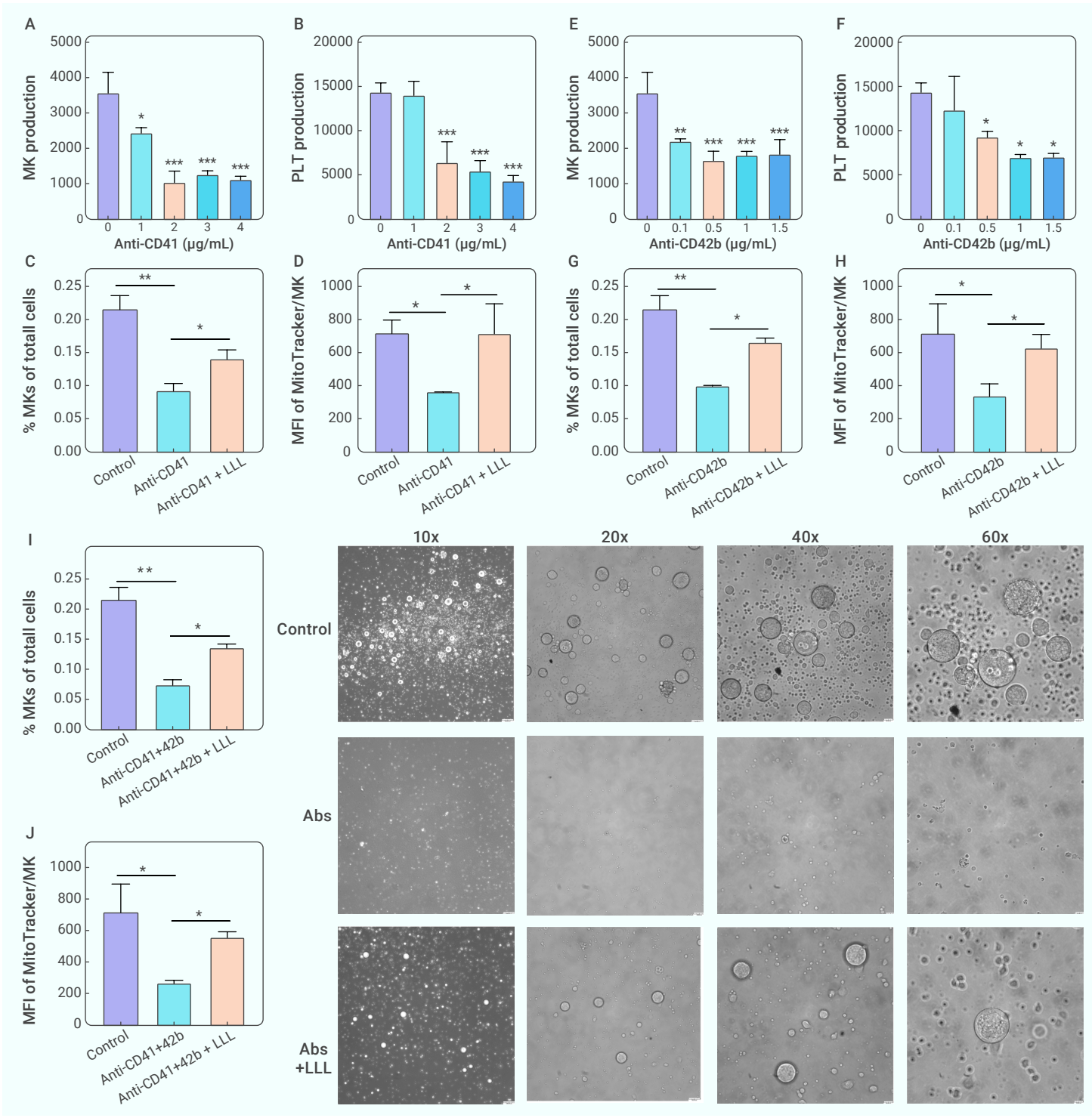

Figure 2. LLL treatment significantly mitigates antibodies-mediated impairment of MK maturation and PLT production (A-J) Antibodies-induced impairments of MK and PLT productions were significantly reversed by LLLT (C&G), as well as mitochondrial mass per MK cell (D&H).MKs and Platelets were maturated as Figure 1B and to the differentiation culture, anti-CD41 (A-D) or anti-CD42b (E-G) at indicated concentrations were added on day 9 of the MK differentiation, followed 1hr later with LLL and two more doses of LLL on days 10 and 11 (3xLLL). Anti-CD41 antibody at 3 ug/ml (C, D, I, and J) and anti-CD42b at 1 ug/mL (G, H, I, and J) were tested either alone or in combination (I and J) for their effects of MK differentiation and mitochondrial enhancement in the presence or absence of LLL. The MKs and platelets were analyzed on days 12 or 15, respectively, by flow cytometry. Mitochondrial mass was analyzed in MKs on day 12 by flow cytometry after staining with MitoTracker Deep Red (D, H, and J). L. Representative images of MK formation at day 12 in the presence or absence of anti-CD41 (3ug/ml) and anti-CD42b (1ug/ml) antibodies with or without LLL treatment. All data were represented by mean ± SEM. n=6. *p<0.05, **p<0.01, and ***p<0.001 compared in the presence or absence of LLL treatment or indicated groups.

after staining with anti-CD41-FITC and anti-CD34-allophycocyanin (APC) (BioLegend) by flow cytometry (FACSAria, BD Biosciences). To quantify platelets produced by MKs, platelets were collected from megakaryocyte differentiation cultures on indicated days and analyzed by FACSAria on the basis of CD41 expression and forward/side scatter^{low}. Mitochondria of MKs were stained with 200 nM MitoTracker Deep Red FM (Molecular Probes) at 37°C for 30 min. Isotype antibody controls were mouse FITC-IgG1 for anti-human CD41 antibody and APC-IgG2A for anti-human CD34 Antibody (BioLegend) during flow cytometric analysis of $CD34^{+}CD41^{+}$ cells. The relative mitochondrial content was determined by flow cytometric analysis of the mean fluorescence intensity at 660 nm. All flow cytometric data were analyzed by FlowJo software (Tree Star).

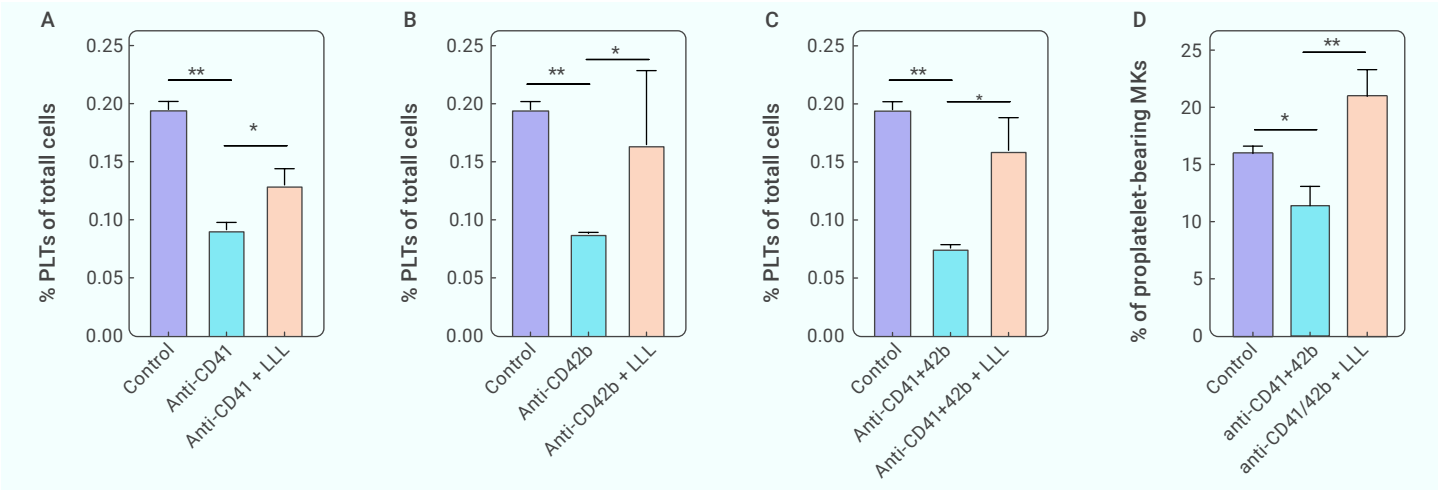

Figure 3. LLL counteracts autoantibody's adversary and increases PLT production and proplatelet bearing MK formation Sorted MK were differentiated in SFEM supplemented with either 3 μg/mL anti-CD41 (A) or 1 μg/mL anti-CD42b (B) or both antibodies (C), followed 2hr later by LLL or sham light treatment. Platelets were measured on day 3 by flow cytometry. Proplatelet-bearing MKs were counted manually on day 1 post-LLL under a microscope (D). All data were represented by mean ± SEM. n=6. *p<0.05, **p<0.01, and ***p<0.001 compared to in the presence or absence of LLL treatment.

Statistical analysis

Data are represented as mean± standard error of the mean (SEM). Statistical analysis was performed with 2-tailed student's t test for comparison between two groups or one-way ANOVA for multiple group comparisons. A value of P<0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA).

RESULTS

LLL promotes MK maturation and PLT production in human hematopoietic stem cells (HSCs) *ex vivo*

Human umbilical cord blood (hUCB) $CD34^+$ hematopoietic stem cells were cultured in serum-free expansion medium (SFEM) containing 100 ng/mL rhTPO with its replenishment every three days during the first 9 days of the differentiation (Figure 1B). The percentage of CD41 positive cells increased over time and $CD41^+CD34^+$ double positive cells reached 60% on day 9 or almost 100% on day 12 as shown by flow cytometric analysis (Figure 1A, right panel). The specificity of anti-CD34 and CD41 antibodies were verified relative to the isotype controls (Figure S1). The highest levels of platelet production were seen on day 15, in agreement with previous studies.[15] Our previous study showed that LLL affected primarily polyploid MKs that were attained in a relatively high number on day 9 of the differentiation (Figure 1A).[15] As such, the culture on day 9 was exposed to 980 nm diode laser at a power density of 0.03 J/cm^2 (1mW for 30 seconds) based on our previous report,[17] a laser density that showed similar effects on MK differentiation and platelet production as 3 J/cm^2 810 nm laser did.[15] The cells were collected every three days for measuring cell counts and viability (Figures 1C-D) or days 9, 12, and 15 for quantifying MKs and platelets (Figures 1E-F) as illustrated in Figure 1B. MKs were recognized based on $CD41^+$ and high forward scatter (FSC^{high}), whereas platelets were identified on the basis of $CD41^+$ and low forward scatter (FSC^{low}).[15] Cell counts and viability were measured by trypan blue staining under microscopy. LLL treatment on day 9 once significantly augmented cell viability and the production of MKs and platelets, especially when measured on day 15, despite no significant effect on the total count of the cells, which was decreasing over time (Figures 1C-D). Similar trends, albeit at lesser extent, were also obtained when the MK differentiation culture was treated similarly on day 6 (data not shown). LLL treatment on day 9 appears to be a better LLL intervention timepoint than on day 6.

It is well known that the differentiation and maturation of MKs are not synchronous and polyploid MKs are matured continuously in the culture for several days. Accordingly, the cell culture was exposed to LLL once a day for 3 consecutive days starting from day 9 to day 11 (3 x LLL) against a single dose of LLL administered on day 9 alone (1 x LLL) (Figure 1B). Once again, there was no evident difference in cell counts regardless of LLL treatment (Figure 1C). However, 3 x LLL significantly increased cell viability and the production of MKs and platelets when compared to 1 x LLL or sham controls (Figures 1C-D). The more viable the cells were, and the more MK counts and platelets were attained from multiple doses of LLL. The finding that 3 X LLL treatment resulted in the most effects on platelet production is likely ascribed to higher proportions of mature MKs involved, reinforcing a central role of mature MKs in response to LLL treatment.

LLL significantly mitigates antibodies-mediated impairment of MK maturation

It has been reported that platelet autoantibodies impede MK maturation, reducing platelet production as one of the primary mechanisms of ITP.[19,20] We previously showed that LLL mitigated ITP in mice with LLL administered 3-4 consecutive days.[15,16] To extend the mouse study to humans, hUCB CD34+ cells were differentiated in the presence of antiplatelet GPIIb/IIIa (anti-CD41) or anti-GPIb/IX (anti-CD42b) at varying concentrations (Figures 2A-B & E-F). Both anti-CD41 and anti-CD42b significantly lowered the numbers of MKs and platelets in a dose-dependent manner (Figures 2A-B & E-F) as previously reported.[19] The number of MKs in the presence of 3 μg/mL of anti-CD41 was not significantly different from a higher concentration of the autoantibody and 3 μg/mL of anti-CD41 was thus selected for the subsequent experiments. Likewise, 1 μg/mL of anti-CD42b was chosen in the parallel evaluation.

LLL treatments for 3 consecutive days from 9 to 11 significantly recovered MK production in the presence of 3 μg/mL of anti-CD41 antibody, evidenced with an increase of MK percentages when compared with sham treatment, although the production was inferior to autoantibody-free controls (Figure 2C). Similar effects of LLL on MKs were also observed in the presence of 1 μg/mL of anti-CD42b antibody (Figure 2G). We previously showed that mitochondrial biogenesis were bolstered by LLL in ex vivo MK differentiation from human bone marrow CD34+ hematopoietic stem cells.[15] Interestingly, a drastic decline in mitochondrial mass was observed in MKs in the presence of either anti-CD41 or anti-CD42b antibody in comparison with control cells as measured by the MitoTracker staining (Figures 2D & H). The mean mitochondrial mass per MK was however retained to nearly normal levels by the LLL regimen (Figures 2D & H).

ITP patients were sometimes found to have both anti-GPIIb/IIIa and -GPIb/IX antibodies in their blood, and thus a potential of 3 x LLL regimen in protection against the two antibodies-mediated impairment of megakaryocytopoiesis was assessed. A combination of the two antibodies significantly hampered MK differentiation, which was mitigated significantly by the LLL treatment (Figure 2I). Again, the mitigation concurred with an increased mitochondrial mass in the cells (Figure 2J). Representative microscopic results of MK maturation were shown in the presence or absence of the two

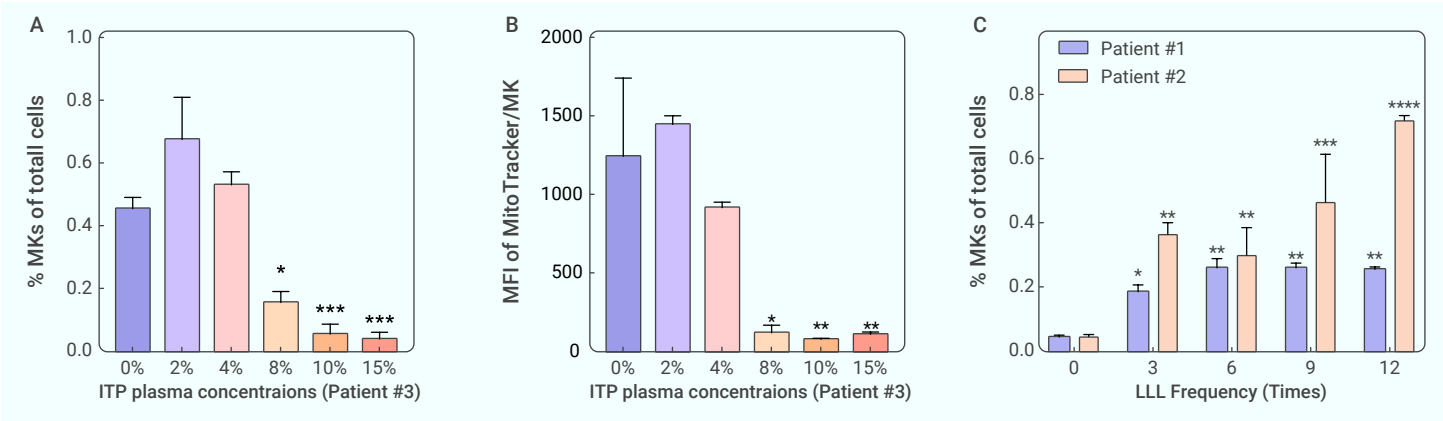

Figure 4. Plasmas from ITP patients severely reduce MK production and Mitochondrial mass The plasmas from ITP patient #3 were added, at indicated final concentrations, to the MK differentiation culture containing 10^5 hUCB CD34$^+$ cells on day 0. MK formation (A) and mitochondrial mass (B) were determined on day 12 by flow cytometry. MK differentiation cultures were also supplemented with 10% ITP plasma (from patient #1 or #2) as A and LLL was applied for 0, 3, 6, 9 or 12 consecutive days after 2 hrs of plasmas addition, followed by measuring MKs on day 12 (C), All data were represented by mean ± SEM. n=6. ⋆p<0.05, ⋆⋆p<0.01, and ⋆⋆⋆p<0.001 compared in the presence or absence of ITP plasmas (A &B) or LLL treatment (C).

antibodies (Abs) or 3x LLL treatment regimen (Figure 2L). The mature MKs were large and readily visible in the control (top) but barely seen in the presence of the two antibodies (middle). However, with LLL intervention, MKs were clearly seen (bottom) although the number of MKs was not as high as that of autoantibodies-free controls (Figure 2L). These findings demonstrate the ability of LLL to significantly prevent antibody-mediated hindrance of megakaryocytopoiesis in hUCB CD34$^+$ cells, suggesting a conservative effect of LLL in platelet regeneration between humans and mice.

LLL counteracts autoantibody's adversary and increases platelet production and the formation of proplatelet bearing MKs

The beneficial effects of the LLL regimen on MK differentiation resulted in corresponding increases of platelet production as measured on day 15 of MK differentiation irrespective of whether anti-CD41, anti-CD42b, or both antibodies were present (Figures 3A-C). The LLL regimen appeared to facilitated the final stages of platelet generation, a process that requires a large number of mitochondria. During the final stage of MK maturation, the cytoplasm was converted into a bunch of protrusions and branches defined as proplatelet-bearing MKs, which was hindered in the presence of the autoantibodies as suggested by the decline in percentages of proplatelet-bearing MKs (Figure 3D). The decline in proplatelet-bearing MKs was completely reversed by the LLL regimen (Figure 3D).

LLL enhances MK differentiation and platelet production in the presence of plasma from ITP patients

ITP is well documented to associate with autoantibodies that directly target at MK differentiation and platelet production.[19,20] Upon autoantibody binding to platelets, such as anti-glycoprotein IIb/IIIa (GPIIb/IIIa) and/or anti-GPIb/IX antibodies, platelets are easily destructed by the reticuloendothelial system. Several groups have demonstrated that plasmas from ITP patients aggravated the thrombocytopenia by disrupting megakaryocytopoiesis and thrombopoiesis as well.[19,20] To extend the beneficial effects of LLL on reversing ITP plasmas, plasmas from ITP patients, alongside control plasmas from healthy volunteers, were arbitrarily assigned to a number and added into MK differentiation culture in 24-well plates with 10^5 hUCB CD34$^+$ cells in each well. First, to determine an optimal concentration of ITP plasma, serial dilutions of plasma from ITP patient #3 (randomly selected) were added into the MK differentiation culture on day 0 of the differentiation mimicking *in vivo* in which autoantibodies are present throughout the entire differentiation process. There were no significant differences in megakaryocyte differentiation or mitochondrial mass in individual MKs in the presence of ITP plasma at final concentrations from 0% to 4% (Figures 4A-B). However, the plasma rigorously suppressed megakaryocytopoiesis when the concentration arose to 8% or higher, reaching a nadir at 10% of plasma (Figure 4A). The drastic suppression concurred with similar impairment of mitochondrial mass (Figure 4B). Accordingly, 10% of ITP plasma was used in subsequent investigation of LLL efficacy because this concentration of patient plasma could sufficiently suppress MK differentiation, while minimizing adverse effects of non-autogenous plasma on megakaryocytopoiesis considering that human leukocyte antigen (HLA) mismatched plasma was tested in the MK differentiation culture.

Next, the MK differentiation culture supplemented with 10% ITP plasma (patient #1 or #2, randomly selected) was exposed to LLL for 0, 3, 6, 9 or 12 consecutive days: i.e. LLL was given once a day starting on day 0, 3, 6, or 9 of the differentiation culture after 2 hrs of antibody addition so that autoantibodies' effects on various stages of MK differentiation could be all evaluated. As can be seen in Figure 4C, significant effects on MK production were observed after 3 x LLL treatment and further improved after 6 days' consecutive treatments in the presence of plasma from patient #1. However, this LLL treatment showed no accumulating effects with increasing frequencies of LLL treatments up to 12 treatments, implicating that the autoantibody in patient #1 may affect the early stage of MK differentiation. On the contrary, for patient #2, an increasing frequency of LLL treatments gave rise to an increasing number of MKs generated in a dose-dependent manner, meaning that autoantibodies in the patient may bind to MKs at both early and later stages via either single molecular target or multiple molecular targets (Figure 4C).

The impact of LLL on the yields of MKs and platelets generated from 10^5 hUCB CD34$^+$ cells were next investigated in the presence of plasmas from cohort ITP patients and healthy volunteers (Figure 5). To ensure the maximum efficacy, LLL or sham light was given once daily for 12 consecutive days throughout the initial 12 days of MK differentiation so as to minimize potential "false negatives" arising from insufficient LLL treatment. Percentages of MKs relative to a total cell count were not different significantly in the presence of all three healthy plasma controls, regardless of LLL treatments, in agreement with little effect of LLL on healthy or non-stressful cells. The average percentage of MKs attained in the control plasmas was arbitrarily set as the healthy controls (red dotted line) (Figure 5A). Under similar conditions, it was observed that ITP plasmas #1-5, but not #6-9, significantly lowered MK production, implicating variable effects of ITP plasmas on MK differentiations from HSCs. Strikingly, LLL treatments restored MK production to normal levels or higher only in the cultures containing 10% detrimental ITP plasma samples # 1-5, while leaving negligible effects on those cultures showing normal levels of MK production (plasma samples #6-8) (Figure 5A). Consistent with this finding, LLL markedly increased mitochondrial mass in MKs against the adverse effects of ITP plasmas #1-5 (Figure 5B), similar to LLL-bolstered mitochondrial biogenesis in mouse MKs.[15,16]

In contrast, LLL treatment did not augment MK and platelet production in the presence of patients #6-8 where the mitochondrial mass was not impaired by ITP plasma either, similar to the control plasmas (Figures 5A-B). The discovery that LLL specific rectifies the defects of mitochondrial functionality is unique, suggesting its potential use in combination with other modalities to potentially further ITP therapeutic efficacy. These observations also imply that ITP patients #6-8 may have alternative underlying mecha-

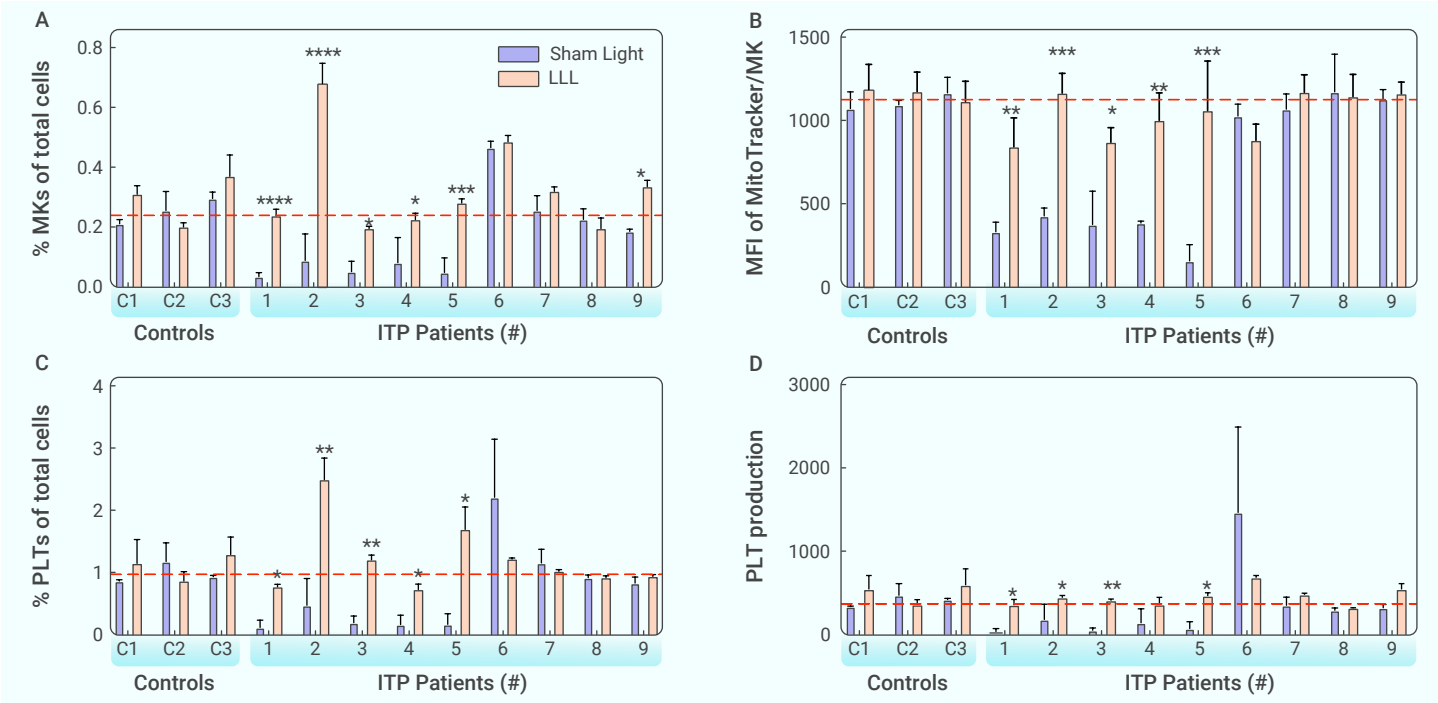

Figure 5. LLL enhances MK differentiation and PLT production in the presence of plasma from ITP patients Plasmas from 9 patients with diagnostic ITP were added into MK culture, 3 healthy plasma samples were set as controls, 12 x LLL or sham light treatments were given once a day for 12 consecutive days. MKs in individual MKs were measured on day 12 (A) and Platelets (C and D) on day 15 by flow cytometry. Mitochondrial mass was determined on day 12 with flow cytometry after MitoTracker Deep Red staining (B). All data were expressed by mean ± SEM of triplicate cultures. The study was repeated once with comparable results. *p<0.05, **p<0.01, and ***p<0.001 compared in the presence or absence of LLL treatment.

nisms at play, potentially unrelated to auto-anti-platelet or MK antibodies. ITP #9 may also predominantly involve mitochondrion-independent mechanisms too, since MK and platelet productions, as well as mitochondrial mass remained normal in the culture comprising plasma #9, despite a moderate enhancement in MK production due to LLL treatment. Collectively, these results suggest that the low "MFI of MitoTracker/MK" could serve as a potential biomarker for evaluating the effectiveness of LLL treatment in future clinical applications. In line with LLL's ability to counteract MK impairment induced by detrimental ITP patients' plasmas, there were corresponding increases of platelet production, as indicated by both the percentages of platelets and total platelet counts, in the cultures containing plasmas #1-5 (Figures 5C-D). The robust effects of LLL on mitigating ITP plasmas-mediated MK differentiation impairment argues persuasively for its clinical potential in treating a significant subset of ITP patients.

DISCUSSION

We showed that human MK differentiation and maturation, as well as proplatelet and platelet formation, were significantly impaired by anti-platelet autoantibodies. But the impairment was effectively rectified with 980 nm LLL at 0.03 J/cm^2 with more pronounced effects observed with three doses compared to a single dose in the presence of anti-CD41, anti-CD42b, or both. The LLL also reversed the defect in mitochondrial activity induced by autoantibodies or ITP plasma, a defect that appears to affect approximately 50% of the tested ITP patients. These observations suggest that LLL can be a promising alternative to this specific subset of TIP patients. The findings align with and extend prior investigations showing that LLL promotes the survival and differentiation of cryopreserved human CD34$^+$ cells, facilitating their differentiation into MKs, proplatelets, and platelets *ex vivo*.[15] Additionally, LLL also shown potential in ameliorating ITP and other forms of thrombocytopenia in several preclinical models.[15,16] Beyond its direct impact on platelet regeneration, LLL can also improve mitochondrial function and prevent apoptosis in hematopoietic stem cells and their progenitors and facilitate the engraftment of hematopoietic stem cells.[15,16] These additional benefits can significantly contribute to platelet regeneration in ITP patients as well.

Autoantibodies targeting MKs have been shown to disrupt their maturation and differentiation, hinder proplatelet bearing MK formation, and induce apoptosis within these cells.[19,20] All these adverse processes are associated with compromised mitochondrial functions, which can be significantly repaired by 810 nm LLL as demonstrated in our previous works.[15,16] In the studies, it was found that anti-CD41 antibody activated caspase3/7 and induced apoptosis in platelets and their precursors, which was reduced substantially by LLL treatment.[16] Moreover, daily injection of anti-CD41 antibody led to 5.6-fold increases in the percentage of platelets expressing activated caspase-3. But the proportion of apoptotic platelets declined to a near normal level by two doses of noninvasive whole-body LLL treatment.[16] The prior and current observations suggest that LLL-mediated enhancement of mitochondrial functions protected from apoptosis induced by autoantibodies, which is at least part of the mechanism underlying the beneficial effects of LLL on platelet functions. It has been shown that mitochondrial cytochrome *c* oxidases in complexes III and IV of the mitochondrial respiratory chain act as the chromophores for 810 nm light and can be activated or protected by the laser in various cells under cellular stress conditions. However, for 980 nm light, there are no known chromophores within the mitochondrial respiratory chain or cells. This raises an intriguing question about how laser therapy at 980 nm facilitates megakaryocytopoiesis and platelet biogenesis. Wang et. al. has reported that the effect of 980nm laser, but not 810 nm laser, could be blocked by calcium channel blockers like capsazepine, a specific inhibitor for transient receptor potential vanilloid 1 (TRPV1) ion channel, or SKF96365, a non-selective inhibitor for transient receptor potential canonical (TRPC) ion channels.[21] In line with this, the 980 nm laser sharply elevated intracellular calcium levels 30s after 980 nm laser at 0.3 J/cm^2, concurrent with a precipitous decrease in mitochondrial calcium in adipose-derived stem cells.[21] LLL-mediated redistribution of intracellular calcium among different organelles appears to increase not only mitochondrial membrane potential and ATP production but also other calcium-dependent signaling pathways.[21] Moreover, inhibition of ATP synthase almost entirely abolished the effects of 980 nm laser on ATP synthesis, whereas inhibition of cytochrome c oxidase did not impede the increase in ATP levels in response to the 980 nm laser.[22] Similar photobiomodulation of 980 nm and 810 nm lasers has been also observed in various cell types, including fetal liver and bone marrow MKs, myoblasts, osteoblasts, periodontal ligament mesenchymal stem cells, keratinocytes, and fibroblasts.[17,22-24] Nevertheless, MKs seem much more

sensitive to 980 nm laser than 810 nm laser, making 980 nm laser or LED more appealing for clinical application. This is because it would be more practical to deliver such a low dose of 980 nm laser (0.03 J/cm^2) into internal organs noninvasively, in contrast to 810 nm laser, which requires a 100-fold higher energy density (3 J/cm^2). Another study showed that 635 nm laser enhanced TPO production via the ROS-dependent Src/ERK/STAT3 signaling pathway.[25] Whether or not 980 nm can activate other non-mitochondrial pathways remains to be determined.

Noninvasive LLL therapy would be particularly attractive for the potential treating mild to moderate neonatal thrombocytopenia, reducing the risk of brain development defects or other potential side effects associated with low platelet counts. In this regard, blue light-based neonatal phototherapy represents the most successful phototherapy and has successful treated jaundice in myriad newborns over four decades with a proven track record of safety. The phototherapy involves noninvasive exposure of newborns to blue light and cures the disorder without the need for injections or drug administration. Currently, oral agents eltrombopag and romiplostim (Nplate) are licensed for treating ITP in children and adults.[26,27] However, these agents are not approved for newborns, probably because most mild and moderate neonatal thrombocytopenia naturally resolve within 7 to 14 days after birth. Yet, the two agents promote the differentiation of MK precursors from HSCs and take 7~10 days to translate into increased circulating platelet counts, which is too slow to effectively address neonatal thrombocytopenia. On the contrary, LLL treatment targets MKs, rather than HSCs or MK precursors, resulting in a significant rise in platelet counts over controls within 24 hrs after the first treatment, as we previously shown in preclinical studies.[15,16] Furthermore, endogenous TPO levels are already present at high levels in neonates, potentially rendering MK progenitors less sensitive to TPO mimetics in newborns.[6] The ability of LLL to not only accelerate megakaryocytopoiesis but also increase platelet production per MK demonstrates that noninvasive LLL may serve as a potential alternative for mitigating mild to moderate thrombocytopenia in newborns and reducing the number of platelet perfusions in cases of severe thrombocytopenia.

Limitations of the study stem from the inherent complexity of the interaction between laser photonic energy and mammalian cells, a subject that remains poorly understood despite 50 years of research on photobiomodulation. Particularly, further investigation is warranted to unravel how LLL can reverse certain effects of ITP plasmas while leaving others unaffected, which may reveal specific pathways or cellular process that respond best to LLL. Additionally, the sample size of ITP plasmas used in this study is relatively small, potentially not fully representative of the broader population of ITP patients who could potentially benefit from LLL therapy. Moreover, to truly gauge the efficacy of LLL in treating neonates with thrombocytopenia, it would be essential to assess plasmas from newborns with thrombocytopenia, provided they are available for evaluation. Furthermore, our preclinical studies suggest that LLL may have potential in mitigating thrombocytopenia with different underlying causes, but this hypothesis requires further investigation in future research.

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AUTHOR CONTRIBUTIONS

L. W. designed and performed the research. L.W, J.K. Y, Z.C.W, and M. X. W analyzed the data and participated in drafting the paper. M.X.W designed and supervised the

research, secured the funds, and finalized the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

Plasma samples of nine de-identified patients with clinically identified ITP and 3 healthy volunteers were obtained from the Mass General Brigham (MGB) Biobank which collected samples from all consent subjects. For in vitro studies, hUCB was obtained from full-term deliveries. Informed consent was obtained from all healthy adult donors. The study was carried out in accordance with the Declaration of Helsinki and approved by the Partners Human Research Ethics Committee of Massachusetts General Hospital (MGH).

DATA AND CODE AVAILABILITY

Open resources: the plasma samples were publicly provided by the Mass General Brigham (MGB) Biobank (https://www.massgeneralbrigham.org/en/research-and-innovation/participate-in-research/biobank/for-researchers).

SUPPLEMENTAL INFORMATION

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