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The Feverfew plant-derived compound, parthenolide enhances platelet production and attenuates platelet activation through NF- κ B inhibition

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Abstract

Introduction—Few treatments are available that can safely and effectively stimulate new platelet production for thrombocytopenic patients. Additionally, recipients of transfused platelets may experience an inflammatory response due to stored platelets becoming unnecessarily activated, thus creating the need for suitable agents that will dampen undesirable platelet activation. We investigated the effect of the feverfew plant-derived compound, parthenolide on platelet production and platelet activation because of its well-studied ability to induce apoptosis or differentiation in some types of cancer.

Methods—Parthenolide was used to treat human megakaryoblastic cell lines, primary human and mouse megakaryocytes. Resulting platelet production and function was measured via flow cytometry. The two most common parthenolide signaling mechanisms, oxidative stress and nuclear factor- κ B inhibition, were assessed within the megakaryocytes using reactive oxygen species, glutathione and luciferase reporter assays. The influence of parthenolide on *ex vivo* platelet activation was tested with parthenolide pretreatment followed by collagen or thrombin activation. The resulting P-selectin surface expression and released soluble CD40 ligand was measured.

Results—Parthenolide stimulates functional platelet production from human megakaryocyte cell lines, and from primary mouse and human megakaryocytes *in vitro*. Parthenolide enhances platelet production via inhibition of nuclear factor- κ B signaling in megakaryocytes and is independent of the parthenolide-induced oxidative stress response. Additionally, parthenolide treatment of human peripheral blood platelets attenuated activation of stimulated platelets.

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Conclusion—Overall, these data reveal that parthenolide has strong potential as a candidate to enhance platelet production and to dampen undesirable platelet activation.

Keywords

thrombopoiesis; parthenolide; platelet activation

Introduction

Platelets play a fundamental role in hemostasis and have emerged as important contributors to inflammation and immunity [1–3]. Thrombocytopenia, a disorder of low platelet counts and sometimes abnormal bleeding, is a serious problem that has limited treatment options, the most widely used being platelet transfusion. There is great interest in discovery of new compounds to boost thrombopoiesis. We previously reported that 15-deoxy- $\Delta^{12,14}$ -Prostaglandin J_2 is capable of enhancing platelet production via oxidative stress [4]. In screening other thrombopoiesis-enhancing candidates, the anti-inflammatory feverfew plant-derived compound, parthenolide showed strong potential. Parthenolide is currently being investigated for its ability to induce apoptosis [5–8] or differentiation [9] in some types of cancer cells. The best known mechanisms of parthenolide are the inhibition of nuclear factor- κ B (NF- κ B) activity via inhibitor- κ B kinase [10] and the induction of oxidative stress [5,11]. Interestingly, both the increase of reactive oxygen species (ROS) and decrease of NF- κ B signaling have been correlated with the final stages of megakaryocyte maturation and thrombopoiesis [12,13]. We therefore chose to further investigate the mechanism of parthenolide enhancement of platelet production to better understand how parthenolide and other drug candidates could serve as thrombopoietic agents.

There are $150\text{--}400 \times 10^6$ platelets per μL of whole human blood, and these cells are important early responders to stress signals, playing an intricate role in the initiation and maintenance of inflammation. Activated platelets upregulate surface immunomodulatory proteins such as P-selectin and CD40 ligand (CD40L) [14] and release many inflammatory mediators from their α -granules and dense bodies such as platelet factor-4, prostaglandin- E_2 , serotonin, vascular endothelial growth factor, RANTES, and soluble CD40 ligand (sCD40L) [1,2,15]. These mediators can act to amplify an inflammatory or immune response, and contribute to diseases such as type-2 diabetes. Overabundance of inflammation can be detrimental to the vasculature or tissue where it is occurring. Thus, there is a rising demand for anti-platelet molecules that would decrease unwanted platelet activation, thus decreasing overall inflammation. Parthenolide has previously been reported to have some anti-platelet activity [16–18]. However, the exact mechanism of platelet inhibition by parthenolide has not been elucidated. We and others have recently demonstrated that functional NF- κ B family proteins are not only present in megakaryocytes and platelets, but play a role in their activation [19–21]. We therefore tested whether parthenolide would influence platelet activation via NF- κ B.

Materials and methods

Reagents and antibodies

Parthenolide and the mouse anti-human heme oxygenase-1 (HO-1) antibody were purchased from Biomol (Plymouth Meeting, PA); BMS-345541, Tyrodes Salt Solution, fibrinogen and thrombin from Sigma (St. Louis, MO); Carboxy- H_2 DCFDA from Invitrogen (Carlsbad, CA); Collagen from Chrono-log Corporation (Havertown, PA); Cignal Lenti NF κ B Reporter (luc) Kit: CLS-013L from Qiagen (Frederick, MD); Mouse anti-human CD61:Alexa Fluor 647 from AbD Serotec (Raleigh, NC); Alexa Fluor 488 anti-human CD62P from Biolegend (San Diego, CA); BIT 9500 from StemCell Technologies (Vancouver, Canada);

Recombinant Human: IL-1 β , IL-6, thrombopoietin (TPO) and Stem Cell Factor (SCF) were purchased from R&D Systems (Minneapolis, MN); mouse anti actin from Oncogene Research (Cambridge, MA).

Cell line culture conditions

Meg-01 and MO7e cells were purchased from the American Type Culture Collection (Rockville, MD), and cultured in RPMI 1640 (Invitrogen) with 5% fetal bovine serum (Invitrogen), 10mM HEPES (Sigma), 2mM L-glutamine (Invitrogen), 4.5g/L glucose (Invitrogen), and 50 μ g/mL gentamicin (Invitrogen). MO7e cells were additionally supplemented with 100ng/mL of granulocyte monocyte-colony stimulating factor (R&D Systems). All treatments were diluted to a 10X concentration in Phosphate Buffered Saline (Invitrogen) before added to culture. Vehicle treatments contain equal volume of DMSO to the maximum concentration of drug treatments. NF- κ B luciferase reporter cell lines were obtained by transducing 1 $\times$ 10⁵ Meg-01 or MO7e cells with 1 $\times$ 10⁶ transducing units for 5 hours. Positively transduced cells were selected for and continuously maintained in media supplemented with 2 μ g/mL Puromycin (Invitrogen).

Primary Megakaryocyte differentiation and culture

Bone marrow was isolated from C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). 1 $\times$ 10⁶ cells/mL were cultured in IMDM (Invitrogen) with 20% BIT 9500 and 100ng/mL of rhTPO for 5 days, after which the cells were washed and treated with 10 μ M parthenolide. Adapted from previous procedures [4], human CD34⁺ cord blood cells were purchased from Allcells (Emeryville, CA) and first plated at 2.5 $\times$ 10⁵ cells/mL in IMDM supplemented with 20% BIT 9500, 0.8% Low Density Lipoprotein (L-8292, Sigma), 100ng/mL rhTPO, 50ng/mL rhSCF, 10ng/mL rhIL-6 and 10ng/mL IL-1 β . After 7 days of expansion, cells were cultured in the same conditions minus rhSCF, and on day 14 were used for treatment.

Megakaryocyte and platelet imaging, counting and analysis

Live megakaryocytes in culture were examined by phase-contrast microscopy with an inverted microscope (Olympus IX81), and images were captured and edited with SPOT RT Software 4.6 (New Hyde Park, NY). Cultures were stained with an anti-CD61 antibody and analyzed on an Accuri C6 flow cytometer (Ann Arbor, MI) for platelet counting and functional assays. Files were analyzed using FlowJo software (Treestar, Ashland, OR). Platelets were characterized by size gating, CD61 expression, and absence of DNA staining. Non-spun, unaltered culture was collected and stained; platelet counts were normalized to volume of analysis.

Human blood platelet isolation, treatment and analysis

Human blood was obtained from consenting donors in accordance with the Declaration of Helsinki under the University of Rochester IRB approval via venipuncture into tubes containing 3.2% sodium citrate solution (369714, BD Biosciences, Franklin Lakes, NJ). Tubes were centrifuged for 15 minutes at 250xg. Platelet rich plasma was removed centrifuged for 10 minutes at 1000xg. Platelets were washed with Tyrode's Salt Solution: acid-citrate-dextrose (25:3) supplemented with 1 μ g/mL PGI₂ (Cayman Chemical, Ann Arbor, Michigan), and spun again for 10 minutes, 1000xg. Platelets were resuspended in Tyrode's Salt Solution, counted, and used immediately for treatments at a concentration of 1–3 $\times$ 10⁷ platelets/mL. Treated and activated platelets were pelleted so the supernatant could be analyzed for CD40L via ELISA (R&D Systems). Platelets were then stained with CD61 Alexa Fluor 647 and CD62P Alexa Fluor 488 antibodies and analyzed on an Accuri C6. Alternatively, platelets were treated and spread for 45 minutes onto coverslips precoated overnight in 100 μ g/mL of fibrinogen and blocked for 1 hour with 0.5 mg/mL BSA (Sigma

A-8806). Coverslips were then washed with PBS and mounted onto slides (VWR) with Fluoromount-G (Southern Biotech, Birmingham, AL). Slides were analyzed with the Olympus BX51 light microscope and images were captured and analyzed with SPOT RT software.

Western Blotting

Whole cell lysates were prepared using ELB buffer (15mM HEPES (pH 7)), 250mM NaCl, 0.1% Nonidet P-40, 5mM EDTA, 10mM NaF, 0.1mM Na_3VO_4 , 50 μM ZnCl_2 , supplemented with 0.1mM PMSF, 1mM DTT and a mixture of protease and phosphatase inhibitors. Total protein was quantified with bicinchoninic acid protein assay kit (Pierce, Rockford, IL). 25 μg of total protein per treatment was loaded and run on 12% SDS PAGE gels, electroblotted onto a Immun-blot PVDF membrane (BioRad, Hercules, CA). Blots were blocked with 10% nonfat dry milk in 0.1% Tween 20 for 1 hour RT. In 2.5% milk, 1:1000 HO-1 or 1:10,000 actin primary antibodies, followed by washing and an appropriate type and amount of secondary antibodies. Blots were visualized using Immobilon Western chemiluminescence substrate (Millipore, Billerica, MA) and developed on Classic x-ray film (Laboratory Product Sales, Rochester, NY).

NF- κB TransAM assay

The TransAM assay Kit was purchased from Active Motif (Carlsbad, CA), and assays were performed as indicated by the manufacturer's protocol. Experiments were performed with duplicate samples.

Reactive oxygen species production

Meg-01 cells were treated in the dark with 10 μM carboxy-H₂DCFDA for 20 minutes, 37°C, washed with PBS, and distributed for further treatment in the dark for up to 3 hours and immediately analyzed on an Accuri C6 flow cytometer.

Measurement of Intracellular GSH

Meg-01 cells were treated with 10 μM parthenolide or BSO for 0.5, 1, 3, or 6 hours. Measurements of intracellular GSH were performed as previously described [22].

Data Representation

Experimental results represent the mean $\pm$ standard error bars. Graphs are representative of triplicates within one experiment, and all experiments were repeated independently at least 3 times. Statistical analyses are as described in figures and text.

Results

While screening multiple compounds during our investigation of thrombopoiesis-enhancing molecules, we found that the naturally occurring feverfew-derived compound, parthenolide, was capable of enhancing platelet production from the megakaryoblastic cell lines, Meg-01 and MO7e, within 24 hours (Figure 1). The morphology of a megakaryocyte undergoing thrombopoiesis involves changes in the plasma membrane, including formation of proplatelet extensions [23]. Changes in megakaryocyte morphology towards a typical platelet-producing cell are shown in Figure 1A and B, following parthenolide treatment in the Meg-01 and MO7e cells. The platelet-like particles produced from these cells share similar properties with freshly isolated primary platelets with expected morphology, surface markers such as CD61, and function [23].

We next determined whether this same enhancement of platelet production would occur in primary megakaryocytes after parthenolide treatment. Primary mouse megakaryocytes (PMM) were differentiated from bone marrow and treated with parthenolide. Compared to vehicle-treated PMM, parthenolide-treated PMM cultures had more megakaryocytes exhibiting a platelet-producing phenotype, such as proplatelet extensions (Figure 2A), and there were significantly more platelets in the 24 hour treated cells (Figure 2B). Similarly, primary human megakaryocytes (PHM) derived *in vitro* from human umbilical cord blood showed increased platelet-producing morphology after 5 hours of parthenolide treatment, (Figure 2C) and more platelets were produced in 24 hours compared to vehicle treated cells (Figure 2D).

In order to characterize the mechanism for the enhanced platelet production seen after parthenolide treatment, we investigated two major pathways reported to be influenced by parthenolide: oxidative stress and NF- κ B inhibition. Neither of these pathways has been studied during parthenolide treatment of megakaryocytes. Interestingly, megakaryocytes indeed undergo oxidative stress after exposure to parthenolide. Meg-01 and PHM cells increase cellular hemeoxygenase-1 (HO-1) levels in a dose and/or time-dependent fashion (Figure 3A). HO-1 combats oxidative stress [24], and its levels have previously been shown to increase in megakaryocytes when treated with an oxidative stress inducer, 15-deoxy- $\Delta^{12,14}$ -Prostaglandin J₂ [25]. Glutathione (GSH) is another molecule critical to the redox state of the cell [26]. Total GSH levels are also increased in a time dependent manner through 6 hours of parthenolide treatment (Figure 3B), and moreover, sustained elevation of total GSH occurs in parthenolide-treated cells through 48 hours (data not shown). BSO served as a GSH depletory control. Despite the noticeable increase in HO-1 and GSH levels, no significant reactive oxygen species (ROS) were detected in megakaryocytes at 3 hours (Figure 3C) or any timepoint tested (data not shown). Hydrogen peroxide (H₂O₂) was used as a positive control to induce oxidative stress and increased the levels of ROS in Meg-01 cells (Figure 3C). This concentration of H₂O₂ was not toxic to the cells (data not shown). H₂O₂ does not increase platelet production from Meg-01, MO7e, or PHM after 24 hours of treatment (Figure 4). Therefore, parthenolide's enhancement of platelet production does not appear to be caused by an oxidative stress response.

Parthenolide can inhibit NF- κ B function [10]. Whether it does so in megakaryocytes is unknown. We first assessed inhibition of NF- κ B using the TRANS-AM assay which quantifies the presence of specific nuclear proteins that bind to NF- κ B target DNA sequences. Parthenolide treatment led to a significant decrease in the levels of p65, p50, and RelB within the nucleus of Meg-01 cells (Figure 5A) or p65 within MO7e cells (Figure 5B) by 24 hours. To support these findings, an NF- κ B luciferase reporter was transduced into Meg-01 and MO7e cells. Parthenolide significantly reduced the basal NF- κ B activity at 6 hours. However at 24 hours, luciferase levels returned to normal (Figure 5C-D). A small molecule NF- κ B inhibitor, BMS-345542 (BMS), was used as a positive control in the luciferase assay. We also tested BMS on the two megakaryocyte cell lines to determine if this too, enhanced platelet production. BMS dose-dependently increased platelet production from both cell lines after a 24 hour treatment (Figure 5E-F).

Increases of platelet numbers are only clinically relevant if the platelets produced are functional. Therefore, we determined if platelets created from parthenolide-treated PHM were capable of becoming activated. Upon collagen treatment, normal human platelets demonstrate a decrease in size and increase in granularity, indicative of activation because they are releasing granules and changing morphology [27]. We isolated platelets from parthenolide-treated PHM and tested whether they were still able to be activated by collagen. Platelets formed from either vehicle- or parthenolide-treated PHM showed a similar ability to be activated by collagen, supporting their functionality (Figure 6).

We addressed the effects of parthenolide on freshly isolated human platelets because it was previously reported that parthenolide decreases serotonin release from activated platelets [16], therefore showing potential as an anti-platelet therapy. Herein, we tested whether parthenolide could decrease platelet activation. Upon endothelial damage, platelets respond by spreading over the damaged area to prevent blood from leaving the circulation. We mimicked this injury *in vitro* by coating glass coverslips with fibrinogen, causing the platelets to attach to the surface, extend filopodia, and fully flatten out with lamellopodia formation. Representative pictures show that parthenolide substantially decreased the number of platelets able to fully spread onto a fibrinogen coated coverslip (Figure 7A). CD62P is a marker that is highly upregulated on activated platelets, assisting in transendothelial migration of leukocytes, thus inflammation [2]. While parthenolide treatment did not affect the basal percent of CD62P positive unstimulated platelets, it did decrease the percent of CD62P positive platelets following collagen activation (Figure 7B). Soluble CD40L is a proinflammatory mediator abundantly released by activated platelets, and supernatant levels of platelet treatments were measured with ELISA. Parthenolide had no effect on basal secretion, but decreased soluble CD40L release when platelets were pretreated before collagen or thrombin activation (Figure 7C).

In order to partially address the mechanism of parthenolide involvement in the altered activation of stimulated platelets, we assessed if oxidative stress alone could cause similar effects as parthenolide-pretreated platelets. Using H₂O₂ as a positive control, we demonstrate that oxidative stress pretreatment of platelets before their stimulation with collagen did not affect the surface CD62P expression, and, in fact, increased the release of sCD40L (Figure 7).

Discussion

Platelets are vital to hemostasis and have a critical role in immunological and inflammatory processes within human circulation. Severe thrombocytopenia often leads to hemorrhage, creating a rationale for developing thrombopoietic drugs. On the other hand, continuous activation of platelets is a major contributor to chronic inflammatory vascular diseases such as atherosclerosis and type-2 diabetes [2,28], creating the demand for new anti-platelet drug development. Either condition is detrimental, further exemplifying the delicate balance of adequate platelet numbers, and the risks of excessive platelet activation. We demonstrate here that parthenolide is a potential candidate agent for treatment of both conditions, as it increases platelet production from megakaryocytes and attenuates platelet activation during stimulation. Specific delivery mechanisms would need to be implemented, depending on the condition needed to be treated.

Two megakaryoblastic cell lines, Meg-01 and MO7e, can spontaneously produce platelet-like particles in culture [23]. We demonstrated that parthenolide facilitated morphological changes indicative of thrombopoiesis, and increased production of platelet-like particles within 24 hours of treatment (Figure 1). Similarly, parthenolide enhanced platelet production within primary *in vitro* differentiated human megakaryocytes (Figure 2). Compared to 15-deoxy- $\Delta^{12,14}$ -Prostaglandin J₂, which we previously reported as an enhancer of platelet production [4], parthenolide showed a weaker, but still significant enhancement of platelet production (comparison not shown). However, platelet production enhancement in a clinical setting by parthenolide and similar novel agents has not yet been assessed. It is worthy of noting that these primary megakaryocytes were first differentiated and matured with thrombopoietin (see Materials and Methods) before treatment of parthenolide. A bone marrow-directed conjunctive therapy may need to be considered before transition to an *in vivo* setting.

ROS and other oxidative stressors have been shown to increase after parthenolide treatment [5,7,11,29]. The increase of ROS in some cell types was associated with a decrease in GSH [5,29,30]. Contrary to those cell types, Meg-01 cells increased their total GSH levels (Figure 3B), indicating they may be counteracting oxidative stress in this way, as the GSSG/2GSH couple is considered to be the “cellular redox buffer” [26]. Meg-01 cells are capable of GSH depletion, as we demonstrated using BSO, but parthenolide does not act in this manner. Increase of GSH and HO-1, another antioxidant enzyme [24], along with no detectable ROS increase, suggests that the cells are capable of equilibrating to the induced oxidative stress caused by parthenolide.

We showed that H₂O₂, which produces more ROS in megakaryocytes than parthenolide (Figure 3C), and therefore generating an imbalanced redox state, was unable to affect platelet production from Meg-01, MO7e, or PHM cells (Figure 4). Additionally, pretreatment of megakaryocytes with antioxidants before parthenolide treatment did not alter the increase of platelet production (data not shown). These data support the likelihood that oxidative stress does not play a major role in the enhancement of parthenolide-induced platelet production. This led us to investigate another targeted pathway of parthenolide: NF-κB [10]. We have previously shown NF-κB signaling to play a role in megakaryocyte activation after PMA stimulation, and Zhang et al. showed that a constitutive NF-κB signal within proliferating megakaryoblasts decreased at final stages of maturation [13, 19].

Using two different assays, we showed that parthenolide decreased NF-κB activity in megakaryocytes (Figure 5). The rebound of NF-κB activity, as measured by luciferase at 24 hours, indicates that parthenolide is not toxic at this concentration to these cells, and also that the stability and duration of the effects of parthenolide may be short lived. The more stable NF-κB inhibitor positive control, BMS, showed further reduction of luciferase activity. Additionally, this NF-κB inhibitor was also able to increase the number of platelets produced by megakaryocytes. While we cannot exclude the possibility that additional signaling pathways are also affected, we conclude that NF-κB inhibition is almost certainly a leading cause for the thrombopoietic effects of parthenolide observed in megakaryocytes. However, the short duration of parthenolide effectiveness may need to be addressed before transitioning into an *in vivo* model. More stable parthenolide analogs are already under investigation in the anti-cancer setting [31]. However, short lived efficacy of parthenolide may be advantageous if direct bone marrow delivery is feasible, as it may be harmful give an anti-platelet drug intravenously to a thrombocytopenic patient.

We previously identified that the NF-κB pathway functions in normal human platelet activation [19], which is why we chose to investigate whether NF-κB inhibition within parthenolide-treated megakaryocytes affects the function of daughter platelets. Had this been the case, platelets created from parthenolide-treated megakaryocytes would have decreased activation ability. To the contrary, the platelets derived from parthenolide-treated megakaryocytes appear to be fully functional cells capable of activation (Figure 6). This again, could be due to the short-lived effectiveness of parthenolide, therefore, only inhibiting NF-κB activity in mature megakaryocytes while it is not stable enough to affect daughter platelets. Importantly, this demonstrates that parthenolide enhances the production of functional platelets, making it a potential thrombopoietic drug candidate if it can be targeted to the bone marrow. Parthenolide is currently being investigated as an anticancer agent, but it may also have the potential to mitigate thrombocytopenia resulting from current therapies.

Anti-platelet drug therapies are increasingly sought as platelets are recognized as contributors to local and systemic inflammatory conditions such as atherosclerosis [2,28]. Anti-platelet therapies have also been shown to decrease platelets' ability to mediate tumor cell invasiveness [32]. We found that when used as a pretreatment on platelets *ex vivo*,

parthenolide decreased, but did not abolish the activation of stimulated peripheral blood platelets (Figure 7). This evidence marks parthenolide as a potential drug candidate for anti-platelet therapy. Intravenous injections should be considered as a means of drug delivery for an *in vivo* setting, allowing parthenolide to quickly contact and dampen activated platelets before losing effectiveness.

Oxidative stress alone cannot be the mechanism of decreased platelet activation after parthenolide treatment because H₂O₂ did not affect platelet activation (Figure 7). We previously reported that NF-κB inhibitors can attenuate platelet activation [19], and our data suggest that the inhibition of this pathway by parthenolide led to the decrease in platelet activity reported here. Another proposed mechanism of parthenolide attenuation of platelet activation may include alteration of sulfhydryl groups [17], as it has been established that platelet activation involves sulfhydryl-dependent pathways, particularly through inhibition of the glycoprotein IIb/IIIa [33]. Additional mechanisms of parthenolide-mediated anti-platelet activity include changes in arachidonic acid metabolism and interactions with protein kinase C [16,17]. Parthenolide is already used as an herbal medicine to treat inflammatory conditions such as arthritis and migraines [34]. Our new evidence corroborates previously reported literature of parthenolide-driven reduction of platelet activity [16,17,35], and likely acts through multiple pathways including NF-κB inhibition.

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Abbreviations

NF-κB	nuclear factor-κB
ROS	reactive oxygen species
CD40L	CD40 ligand
sCD40L	soluble CD40 ligand
IL	interleukin
GSH	glutathione
PMM	primary mouse megakaryocytes
PHM	primary human megakaryocytes
HO-1	heme oxygenase-1
H₂O₂	hydrogen peroxide
BSO	Buthionine sulfoximine
BMS	BMS-345542

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Figure 1. Parthenolide enhances platelet production from megakaryoblastic cell lines
 Megakaryocytes were treated with vehicle (Veh) or 10 μM PTL. (A) Meg-01 (5h) and (B) MO7e (3h) cells were imaged using an inverted microscope. PTL increased the formation of proplatelet extensions compared to vehicle-treated. Black arrows indicate proplatelet extensions, which are increased in PTL-treated cells. Graphs represent mean counts of platelet-like particles per μL of (C) Meg-01 and (D) MO7e cultures at 24 hours post PTL treatment measured by the Accuri C6 Flow Cytometer. (***) indicates $p < 0.001$ in a post-test of one-way ANOVA)

Figure 2. Parthenolide enhances platelet production from primary human and mouse megakaryocytes treated *in vitro*

Images of live megakaryocyte cultures taken from an inverted microscope of (A) primary mouse megakaryocytes (PMM), or (B) primary human megakaryocytes (PHM), after 2 or 5 hours, respectively, in the presence of vehicle (Veh) or $10\mu\text{M}$ PTL. Black arrows indicate proplatelet extensions, which are increased in PTL-treated cells. Graphs show mean CD61 positive platelet-sized events per μL of (C) PMM or (D) PHM culture at 24 hours post PTL treatment, measured by an Accuri C6 Flow Cytometer. (* indicates $p < 0.01$ in a two-tailed Student T test)

Figure 3. Parthenolide generates an oxidative stress response in megakaryocytes

(A) Western blots of whole cell lysates from 6 and 24 hour PTL or 24 hour vehicle (Veh)-treated Meg-01 cells (left) or PHM (right) for hemeoxygenase-1 (HO-1) with actin as a loading control. (B) Meg-01 cells were exposed to vehicle, a known GSH depletor, buthionine sulfoximine (BSO), or PTL for the indicated times. Mean micrograms of total glutathione levels (GSH) normalized to total protein from each treatment are shown in the graph. PTL-treatment over time showed an increase of total cellular glutathione beginning at 3 hours ($p < 0.01$ from a post-test of one-way ANOVA). (C) Meg-01 cells were prestained with H₂DCFDA, which detects ROS, or unstained (US) and then treated for three hours with vehicle (Veh), 10 μ M PTL, or a positive control ROS inducer, 50 μ M H₂O₂. Cells were immediately run on an Accuri C6 flow cytometer and histograms represent H₂DCFDA fluorescence. (The vehicle and PTL curves may be hard to distinguish as they exactly overlap.)

Figure 4. The oxidative stress response from Parthenolide does not contribute to its enhancement of platelet production

Graphs illustrate mean counts of platelet-like particles per μL of (A) Meg-01, (B) MO7e, or (C) PHM culture at 24 hours post treatment of vehicle (Veh), 10 μM PTL, or 50 μM H_2O_2 , measured by an Accuri C6 Flow Cytometer. (***) indicates p < 0.001; * indicates p < 0.05 from a post-test of a one-way ANOVA)

Figure 5. Parthenolide enhanced platelet production correlates with the decreased endogenous NF-κB signaling in megakaryocytes

(A) Meg-01 or (B) MO7e cells were treated with vehicle (Veh) or 10μM PTL for 1, 6, or 24 hours. A TransAM assay was performed with nuclear lysates from treated cells to measure p65, p50 and RelB DNA-binding activity. The results are graphed as the OD₄₅₀ ratio of PTL and vehicle-treated cells. The y-axis values represent the OD from PTL treated cells divided by vehicle treated cells. (***) indicates $p < 0.001$ in a Student T test comparing Veh vs. PTL treatment performed individually for each time point). (C) Meg-01 cells and (D) MO7e cells were stably transduced with a NF-κB Luciferase Reporter. Luciferase activity at 2, 6 or 24 hours of the indicated treatments are shown. BMS-345542 (BMS) (10μM) was used positive control for NF-κB inhibition. (*) indicates $p < 0.05$ from a two-tailed Student T test). Graphs of mean counts of platelet-like particles per μL of (E) Meg-01 and (F) MO7e cultures at 24 hours post treatment measured by the Accuri C6 Flow Cytometer. (***) indicates $p < 0.001$ in a post-test of one-way ANOVA).

Figure 6. Platelets derived from Parthenolide-treated primary human megakaryocytes are functional

PHM were treated with vehicle (veh) or 10 μ M PTL for 24 hours and the produced platelets were then isolated and exposed to 10ng/mL of collagen (Col) (1h). Activation of the CD61 positive platelets was measured using an Accuri C6 flow cytometer. Compared to unactivated (top), collagen-activated platelet scatter plots (bottom) revealed an increase in granularity and decrease in size. PHM-derived platelets are activated similarly whether produced from vehicle or PTL-treated PHM (compare bottom two panels).

Figure 7. Parthenolide decreases activation of human platelets isolated from peripheral blood (A) Platelets were spread onto a fibrinogen coated coverslips after a 15 minute pretreatment with either vehicle (Veh) (left) or 10 μ M PTL (right). Spreading status is indicated by the arrows. PTL-treated platelets have more partially spread and unspread platelets than vehicle-treated. (B, C) Platelets were not treated (NT) or pretreated with 10 μ M PTL, or 50 μ M H₂O₂ for 30 minutes before activation with either 5 μ g/mL of collagen (Col) or 0.4U/mL of Thrombin (Thr). (B) There was no effect on surface CD62P from any of the treatments without collagen activation. CD62P was only attenuated on activated platelets that were pretreated with PTL. (C) Soluble CD40L in activated platelet supernatant was lower in the PTL-pretreated samples. (* indicates $p < 0.05$ according to a two-tailed Student T test).