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Corresponding Author: Jaesang Kim

Authors: Jusong Kim¹, Gaunghai Jim², Jisu Lee², Kyeong Lee², Yun Soo Bae¹, Jaesang Kim^{1,*}

Institution: ¹Department of Life Science, Ewha Womans University,
²College of Pharmacy, Dongguk University,

An article in the field of biomedical science

Title: 2-(trimethylammonium)ethyl (R)-3-methoxy-3-oxo-2-stearamidopropyl phosphate enhances thrombopoietin-induced megakaryocytic differentiation and plateletogenesis

Authors: Jusong Kim¹, Gaunghai Jin², Jisu Lee², Kyeong Lee^{2*}, Yun Soo Bae^{1*}, Jaesang Kim^{1*}

¹Department of Life Science, Ewha Womans University, Seoul 03760 Korea

²College of Pharmacy, Dongguk University, Goyang 10326, Korea

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*Corresponding authors:

Kyeong Lee, Tel: 82-31-961-5214; Fax: 82-31-961-5214; E-mail: kaylee@dongguk.edu

Yun Soo Bae, Tel: +82-2-3277-2729; Fax: +82-2-3277-3760; E-mail: baeys@ewha.ac.kr

Jaesang Kim, Tel: +82-2-3277-3414; Fax: +82-2-3277-3760; E-mail: jkim1964@ewha.ac.kr

Abstract

We have previously reported the effects of 2-(trimethylammonium)ethyl (R)-3-methoxy-3-oxo-2-stearamidopropyl phosphate [(R)-TEMOSPho], a synthetic phospholipid, on megakaryocytic differentiation of myeloid leukemia cells. Here, we demonstrate that (R)-TEMOSPho enhances megakaryopoiesis and plateletogenesis from primary hematopoietic stem cells (HSCs) induced by thrombopoietin (TPO). Specifically, we demonstrate at sub-saturation levels of TPO, the addition of (R)-TEMOSPho enhances differentiation and maturation of megakaryocytes (MKs) from murine HSCs derived from fetal liver. Furthermore, we show that production of platelets with (R)-TEMOSPho in combination with TPO is also more efficient than TPO alone and that platelets generated *in vitro* with these two agents are as functional as those from TPO alone. TPO can thus be partly replaced by or supplemented with (R)-TEMOSPho, and this in turn implies that (R)-TEMOSPho can be useful in efficient platelet production *in vitro* and potentially be a valuable option in designing cell-based therapy.

Introduction

Platelets are anucleate cellular fragments found in large numbers in circulating blood (1). They originate from MKs which have descended from HSCs and undergo endomitosis achieving polyploidy of upto 256N prior to apoptotic disintegration and release of platelets (2, 3). Thrombosis and hemostasis are both mediated by platelets whose well balanced activity is thus crucial for vascular integrity and ultimately for survival. Platelets, currently obtained from donated blood, are used in various medical procedures including cancer treatments and certain types of surgery. Not surprisingly, not only is there a widespread shortage problem, but side effects such as febrile and anaphylactoid reactions and blood-borne infections remain as unresolved risk factors (4).

Numerous efforts to generate platelets *in vitro* have been made, and most of recent attempts are based essentially on a sequential differentiation of embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) first to HSCs and then to polyploidy MKs which can subsequently undergo apoptosis and release platelets (5-8). The key reagent for both the generation of HSCs and the induction of megakaryocytic differentiation is TPO. The need for this humoral growth factor and its receptor, CD110 encoded by *c-mpl* gene, in generation of platelets via maturation of MKs is well demonstrated both *in vitro* and *in vivo* (9, 10). Indeed, virtually all procedures reporting *in vitro* platelet generation to date use TPO as the essential element (5-8).

Generation and purification of large quantities of TPO suitable for application in cell culture-based production is likely to be highly costly. Here, we describe an alternate strategy utilizing (R)-TEMOSPho, a synthetic chemical previously shown to induce megakaryocytic differentiation of myeloid leukemia cells, as a supplement to TPO (11, 12). Specifically, we show that (R)-TEMOSPho, which cannot by itself induce efficient MK differentiation of murine HSCs, can enhance TPO activity in megakaryocytic differentiation and plateletogenesis. This implies that (R)-TEMOSPho can potentially be a useful reagent in efficient *in vitro* production of platelets and possibly in therapeutic settings.

Materials and methods

Isolation and culture of HSC

The plan for this study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Ewha Womans University (No. 16-050). Timed pregnant mice were purchased from a commercial vendor (Orientbio Inc.). Mouse fetal livers were isolated from embryonic day (E) 12.5 and turned into single cell suspension by pipetting and passing through cell strainer. After removing red blood cells, HSCs were purified using The EasySep Mouse Hematopoietic Progenitor Cell Enrichment Kit (STEMCELL Technologies Inc.) following the manufacturer's protocols. Details are available upon request. Purified cells were seeded in 35mm dishes ($\sim 2 \times 10^5$) and cultured for 5 days in StemSpan™ Serum-Free Expansion Medium (STEMCELL Technologies Inc.). Murine TPO (Peprtech) and (R)-TEMOSPho were added at the indicate concentrations. (R)-TEMOSPho was synthesized following previously published steps with minor modifications (13). Details are available upon request.

Flow cytometry

Cells were incubated with FITC conjugated anti-CD41 antibody (BD biosciences) and analyzed by flow cytometry using a FACS Calibur (BD biosciences) and the BD Cell-Quest™ Pro version 6.0 software (BD bioscience). For DNA content analysis, cells were incubate for 30 min at room temperature (RT) in staining solution (50 μ g/ml Propidium Iodide, 100 μ g/ml RNase A, 0.1% Triton X-100) and analyzed by FACS Calibur.

Phalloidin staining

Staining for F-actin with phalloidin to delineate cellular boundaries and counterstaining with 4'-6-Diamidino-2-phenylindole (DAPI) were carried out as previously described (11).

Platelet functional assays

Platelet-rich plasma was prepared from mouse blood and resuspended in Tyrode buffer following a published protocol with minor modifications (5, 14). Details are available upon request. For preparation of culture-derived mouse platelets, culture medium was collected and centrifuged at 150g for 20min to isolate the supernatant to which 1 μ M PGE1 and 0.1 U/ml apyrase in 9X volume of ACD solution (Sigma-Aldrich) were added. After centrifugation at 900 g for 10 min, the pellet was resuspended and

washed with the washing solution containing 1 μ M PGE1 and 0.1 U/ml apyrase. The pellet containing platelets were finally isolated by centrifugation and resuspended in Tyrode buffer. To examine P-selectin expression, platelets were stimulated with 500 μ M AYPGKF (Pepton) and incubated for 15 min at 37°C prior to incubation with FITC conjugated anti-CD62p antibody (BD Bioscience) for 15 min at RT and analysis by FACS. For the fibrinogen binding assay, platelets were first activated with 500 μ M AYPGKF and subsequently incubated with the 3ul Alexa 488-labelled fibrinogen (Invitrogen) for 30 min in the dark. The mixture was then diluted with 1X PBS and analyzed by FACS. For the aggregation assay, platelets were activated with 100 μ g/ml fibrinogen (Merck Millipore), 1 mM CaCl₂, 1 mM MgCl₂ and 500 μ M AYPGKF and incubated for at RT for 10 min. The activated platelets were applied to 2.5 mg/ml fibrinogen coated slide and examined for platelet aggregation under a phase-contrast microscope.

Results and Discussion

We have previously shown that (*R*)-TEMOSPho can efficiently induce megakaryocytic differentiation of K562 and HEL cells which are myeloid leukemia cells (12). In order to examine its function in primary cells, we applied (*R*)-TEMOSPho to murine embryonic HSCs prepared from E12.5 fetal liver. Although a slight increase in the proportion of CD41⁺ cells was noted, the induction of megakaryocytic differentiation was overall negligible (Fig. 1A). Furthermore, the inductive effect on CD41 expression was not enhanced at higher levels of (*R*)-TEMOSPho. In fact, beyond 50 $\mu\text{g/ml}$ of the reagent, we noted cell death, a likely side effect of high dosage. This was in contrast to the effect of TPO which induced CD41 expression in a dose dependent manner maximally around 100 ng/ml (Fig. 1A). We were curious to see if (*R*)-TEMOSPho could nevertheless contribute to megakaryocytic differentiation by enhancing the effect of TPO. (*R*)-TEMOSPho and sub-maximal doses of TPO were applied in combination, and CD41 expression was examined. Interestingly, 25 $\mu\text{g/ml}$ of (*R*)-TEMOSPho in combination with 25 or 50 ng/ml of TPO could induce the proportions of CD41⁺ cells to the near maximal level achieved by 100 ng/ml of TPO (Fig. 1B). The bona fide induction of megakaryocytic differentiation was also confirmed by visible increase in cellular size (Fig. 1C). Another hallmark phenotype associated with megakaryopoiesis is endomitosis and resulting polyploidy. Indeed, TPO treatment readily induced increase in chromosomal content (Fig. 2A). As in the case of cellular enlargement, supplementing submaximal level of TPO (25ng/ml) with (*R*)-TEMOSPho promoted polyploidization. Notably, cells with higher chromosomal contents of 16N and 32N were relatively enriched among cells treated with both TPO and (*R*)-TEMOSPho even when compared to cells treated with 100 ng/ml of TPO. The increase in chromosomal content was also confirmed via fluorescence microscopy with DAPI-staining for chromosomes and F-actin staining to delineate cellular boundaries (Fig. 2B).

We next investigated whether culture-derived MKs can produce platelets and if (*R*)-

TEMOSPho can aid TPO in the process. Proplatelet-like projections were readily seen in TPO-treated cultures, and supplementing with (*R*)-TEMOSPho appeared to enhance the level induced by the sub-maximal concentration of TPO (Fig. 3A). In order to quantify the plateletogenesis, we followed a previously established protocol for isolating platelets from mouse sera based on forward and side scatter properties and assessing the platelet level based on CD41 expression (5, 12, 14). Using mouse blood-derived platelet preparation as the positive control, we isolated platelet populations with identical flow cytometric characteristics. The proportions of CD41⁺ cellular particles clearly indicated that supplementing with (*R*)-TEMOSPho significantly stimulated the plateletogenesis induced by TPO (Fig. 3B).

Next, we examined whether culture-derived platelets are functional. Mouse platelets described as above were used as the positive control. First, we investigated the expression of P-selectin, a cell adhesion molecule on the surface of activated platelets. Upon stimulation with the agonist peptide AYPGKF, platelets prepared from various conditions showed positive response to P-selectin by flow cytometric analyses consistent with activation of PAR4 receptor activation and translocation of P-selectin to the cell surface (Fig. 4A). We also examined fibrinogen-binding activity. Again, upon activation with the peptide AYPGKF, a strong binding to fluoroprobe-coupled fibrinogen was seen for culture-derived platelets as well as for mouse blood-derived platelets (Fig. 4B). Lastly, we visually examined the platelet aggregation upon activation. Indeed, phase-contrast microscopic examination showed clear aggregation post activation consistent with functionality of the platelets (Fig. 4C)

In sum, we have demonstrated that (*R*)-TEMOSPho promotes TPO-induced megakaryocytic differentiation and platelet production from primary hematopoietic stem cells. This makes it possible to use sub-saturation levels of TPO in generating platelets *in vitro* and could potentially make the process more cost-efficient and thus viable as an alternative to platelet transfusion. Furthermore, our results also suggests that (*R*)-TEMOSPho is potentially

applicable in TPO-based or c-mpl agonist-based therapeutic treatments as a supplement.

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Compliance with ethical standards

Conflict of interest: the authors declare that they have no conflict of interest.

Figure legends

Figure 1. Promotion of TPO-induced megakaryocytic differentiation by (R)-TEMOSPho. (A) Dose-dependent induction of CD41 as a megakaryocytic marker by TPO treatment for 5 days is shown. In contrast, (R)-TEMOSPho by itself has virtually no effect. (B) Combinatorial application of TPO and (R)-TEMOSPho efficiently induces CD41 expression. Note that at all concentrations (ng) of TPO tested, addition of 25 µg of (R)-TEMOSPho induces CD41 to higher levels than TPO alone. (C) Phase contrast micrographs of cells with indicated treatments. Note that the combination of TPO and (R)-TEMOSPho has more pronounced effect in inducing cellular enlargement, a hallmark feature of megakaryocytic differentiation than TPO alone while (R)-TEMOSPho by itself has little effect. Statistical significance, tested by Student's t-test is indicated (* $P < 0.05$, ** $P < 0.005$).

Figure 2. Promotion of TPO-induced polyploidy by (R)-TEMOSPho. (A) Flow cytometric analyses for DNA contents following indicated treatments. Note that the combination of TPO and (R)-TEMOSPho has more pronounced effect in inducing polyploidy, another hallmark feature of megakaryocytic differentiation than TPO alone. In the graph at the bottom, relative proportions of populations with distinct polyploidy are shown. Note the higher proportions of 16N and 32N populations among cells treated with both TPO and (R)-TEMOSPho than among cells treated with TPO alone. The results represent mean \pm SD from three independent assays. (B) Cells were DAPI-stained for nuclei and F-actin-stained (red) with phalloidin to delineate boundaries of single cells. Statistical significance, tested by Student's t-test is indicated (* $P < 0.05$, ** $P < 0.005$).

Figure 3. Promotion of TPO-induced plateletogenesis by (R)-TEMOSPho. (A) Phase contrast micrographs of proplatelet projections (white arrows) are shown. Cells were cultured for 10 days in vitro. Note that virtually no proplatelet projections are seen with (R)-TEMOSPho alone. In contrast, TPO treatment with or without (R)-TEMOSPho generated proplatelet projections which were readily confirmed by visual inspection. (B) Platelets isolation from mouse blood or culture media are shown. Platelets were isolated by forward and side scatter and quantitated based on forward scattered and CD41 expression. Results are shown in the graph below. Note higher proportions of CD41 positive platelets present in culture media of cells treated with both TPO and (R)-TEMOSPho than in the culture media cells treated with 25 ng/ml of TPO alone. The results represent mean \pm SD from three independent assays. Statistical significance, tested by Student's t-test is indicated (* $P < 0.05$).

Figure 4. Functional characterization of culture-derived platelets.

(A) Platelets from mouse blood plasma and culture media were activated with the peptide AYPGKF and examined for P-selectin (CD62p) expression by flow cytometry. Green and red histograms represent naïve and activated platelets respectively. Note that all culture-derived

platelets show similar level of P-selectin expression upon activation. The results represent mean \pm SD from three independent assays. (B) Platelets from mouse blood plasma and culture media were activated with AYPGKF and examined for fibrinogen binding by flow cytometry. Green and red histograms represent naïve and activated platelets respectively. Note that all culture-derived platelets show similar extents of fibrinogen binding upon activation. The results represent mean \pm SD from three independent assays. (C) Aggregation upon activation was examined by phase contrast microscopy. Note the aggregation in all platelet preparations. Statistical significance, tested by Student's t-test is indicated (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$).

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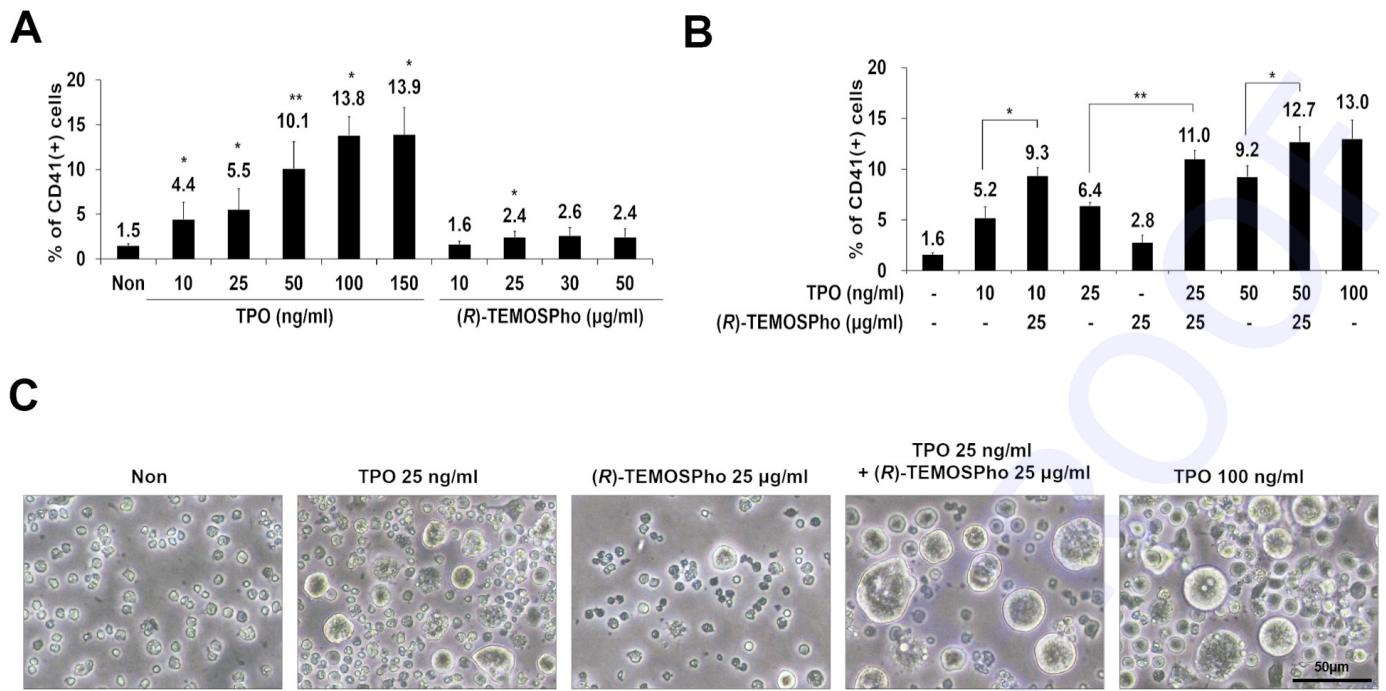


Fig. 1. Figure 1

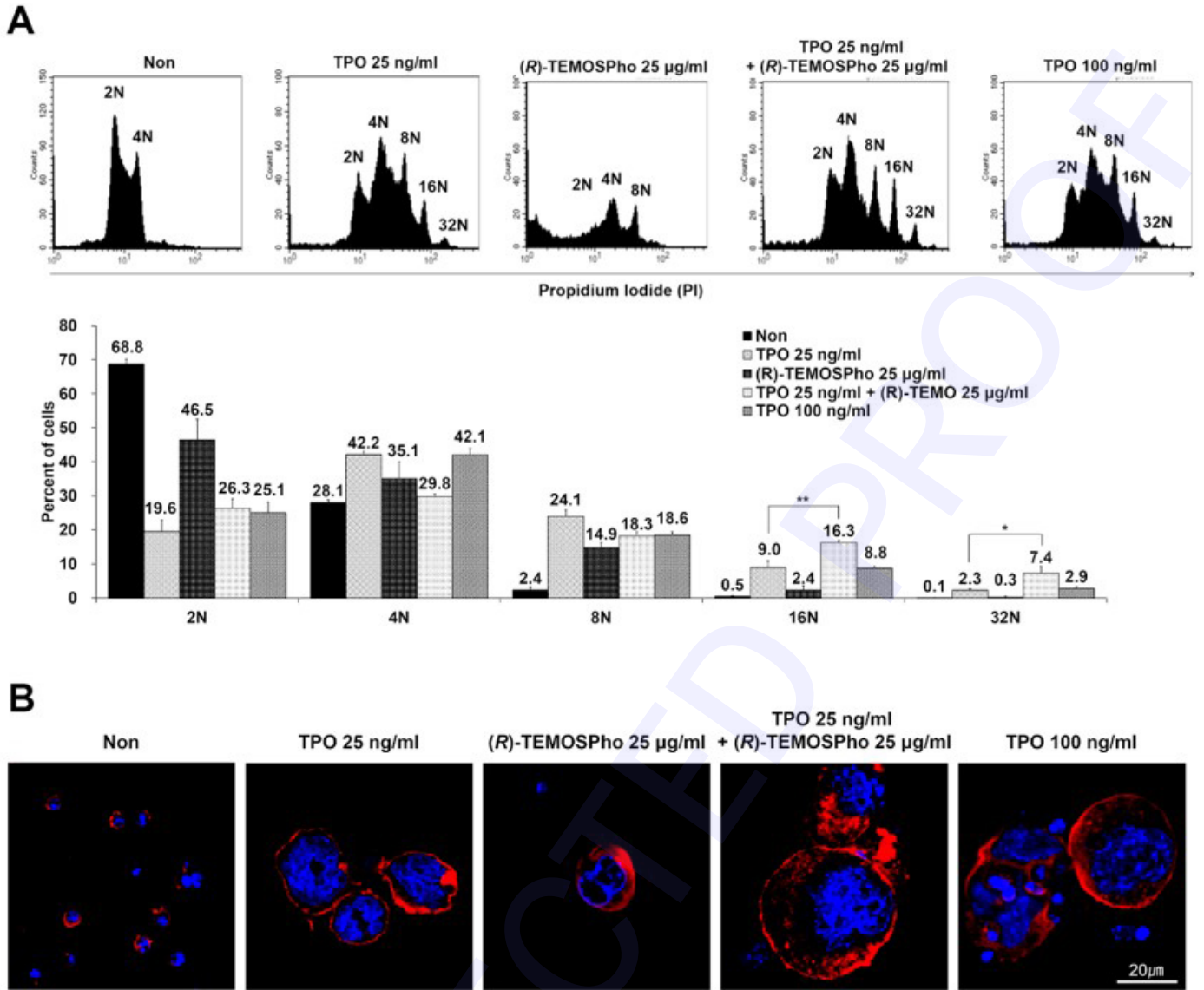
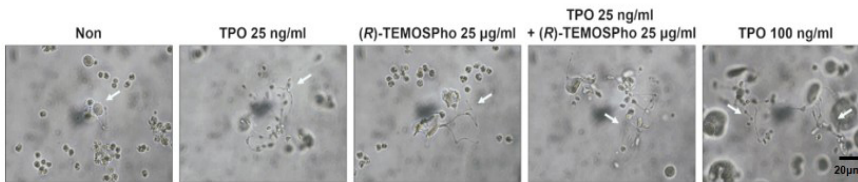


Fig. 2. Figure 2

A



B

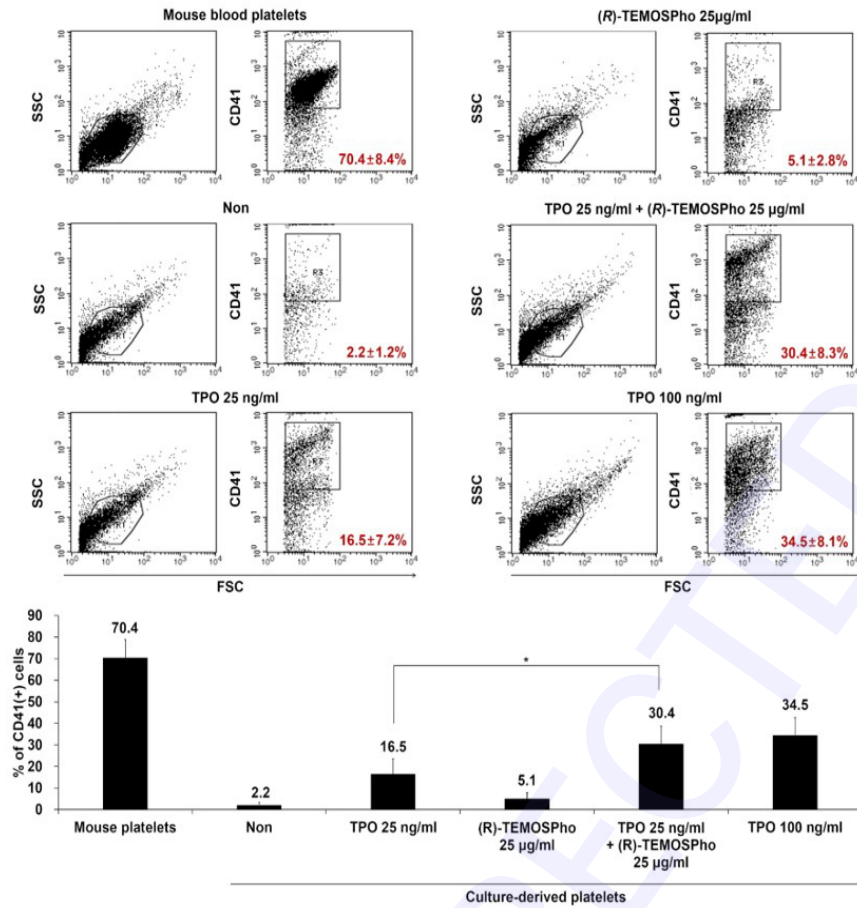


Fig. 3. Figure 3

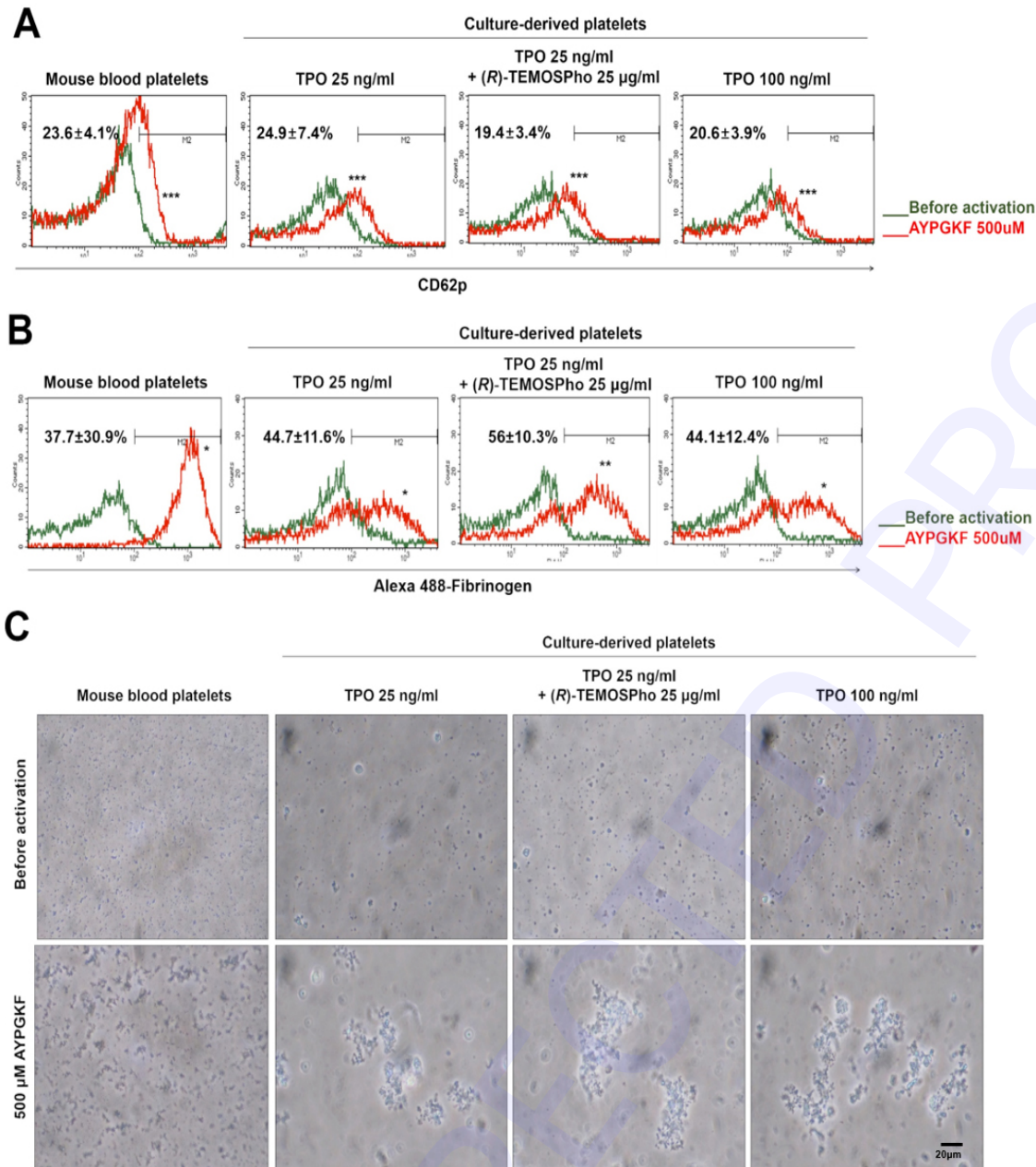


Fig. 4. Figure 4