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ORIGINAL ARTICLE

Activation of toll-like receptors 2 and 4 on CD34$^+$ cells increases human megakaryo/thrombopoiesis induced by thrombopoietin

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Abstract

Background: Platelet Toll-like receptor (TLR)2/4 are key players in amplifying the host immune response; however, their role in human megakaryo/thrombopoiesis has not yet been defined.

Objectives: We evaluated whether Pam3CSK4 or lipopolysaccharide (LPS), TLR2/4 ligands respectively, modulate human megakaryocyte development and platelet production.

Methods: CD34$^+$ cells from human umbilical cord were stimulated with LPS or Pam3CSK4 with or without thrombopoietin (TPO).

Results: CD34$^+$ cells and megakaryocytes express TLR2 and TLR4 at both RNA and protein level; however, direct stimulation of CD34$^+$ cells with LPS or Pam3CSK4 had no effect on cell growth. Interestingly, both TLR ligands markedly increased TPO-induced CD34$^+$ cell proliferation, megakaryocyte number and maturity, proplatelet and platelet production when added at day 0. In contrast, this synergism was not observed when TLR agonists were added 7 days after TPO addition. Interleukin-6 (IL-6) release was observed upon CD34$^+$ or megakaryocyte stimulation with LPS or Pam3CSK4 but not with TPO and this effect was potentiated in combination with TPO. The increased proliferation and IL-6 production induced by TPO + LPS or Pam3CSK4 were suppressed by TLR2/4 or IL-6 neutralizing antibodies, as well as by PI3K/AKT and nuclear factor-κB inhibitors. Additionally, increased proplatelet and platelet production were associated with enhanced nuclear translocation of nuclear factor-E2. Finally, the supernatants of CD34$^+$ cells stimulated with TPO+LPS-induced CFU-M colonies.

Conclusions: Our data suggest that the activation of TLR2 and TLR4 in CD34$^+$ cells and megakaryocytes in the presence of TPO may contribute to warrant platelet provision during infection episodes by an autocrine IL-6 loop triggered by PI3K/NF-κB axes.

KEYWORDS
interleukin-6, megakaryocytes, NF-kappa B, platelets, toll-like receptor 2/4

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1 | INTRODUCTION

Bacterial infections can be associated with thrombocytopenia or with reactive thrombocytosis.[1,2] The pathogenic mechanisms involved in thrombocytopenia are multiple and include the occurrence of disseminated intravascular coagulation during sepsis, the adhesion of platelets to the activated vascular endothelium or an increased consumption associated with the formation of neutrophil extracellular traps or mixed aggregates formation between platelets and neutrophils or monocytes.[3,4] On the other hand, the molecular bases involved in thrombocytosis are less known. Previous reports showed that the rise in platelet counts is mediated through the release of interleukin (IL)-6 and IL-11.[5,6] IL-6, in particular, can induce an increase in thrombopoietin (TPO) and the development of reactive thrombocytosis.[7] Moreover, IL-6 infusion in humans increases the number of circulating platelets and higher IL-6 levels were observed in patients with reactive thrombocytosis compared with healthy individuals.[8] It was reported that increased TPO transcription in hepatocytes accounts for the increase in platelets counts mediated by IL-6.[7] However, it has also been shown that the complex between IL-6 and its seric receptor can directly regulate megakaryopoiesis by acting on glycoprotein 130 on $CD34^+$ cells.[9]

Pathogen recognition is mediated by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). TLRs are transmembrane receptors that are found both in the plasma membrane and in endosomal vesicles. On ligation, TLR signaling triggers the expression of proinflammatory cytokines, chemokines, and costimulatory and adhesion molecules, leading to the priming of the adaptive immune system and initiation of inflammatory responses. Among the different TLRs, TLR4 and TLR2 are found on the cell membrane and mainly recognize wall-associated components of Gram-negative and Gram-positive bacteria, respectively, which are the main causes of nosocomial infections.[10,11]

Despite not being immune cells, platelets express all members of the TLR family, and their activation has proven to play a relevant role in the amplification of the innate immune response of the host against bacterial and viral infection.[12,13] However, and in contrast to several studies on murine cells or cell lines, the expression of TLRs in megakaryocytes or hematopoietic stem and progenitor cells (HSPCs) as well as their potential regulation of human megakaryo/thrombopoiesis is still poorly characterized. Early work has shown TLR4 surface expression on Meg-01 cells; however, the functional role of this receptor was not addressed.[14] Further confirmation of TLR4 on megakaryocyte cell surface and increased expression with maturation of the cells was demonstrated in murine megakaryocytes isolated from fetal livers.[15] Maratheftis et al[16] also identified TLR4 in human bone marrow megakaryocytes and $CD34^+$ cells and found that it was up-regulated in apoptotic $CD34^+$ cells from patients with myelodysplastic syndromes possibly contributing to the cytopenia of these patients. Interestingly, mice with deletion of a 74, 723-bp DNA fragment in the third exon of the TLR4 gene, exhibit decreased platelet number and turnover, highlighting the concept of TLR4 requirement for the regulation of platelet production from megakaryocytes.[17] The decrease in platelet numbers of mice deficient in TLR4 was also observed by Andonegui et al,[15] but not in the study of Aslam et al[18] however, these discrepancies could have been due to the different mice strains used in each study.

Essentials

- The role of Toll-like receptors (TLR) 2/4 on human megakaryo/thrombopoiesis is largely unknown.
- Cord blood-derived $CD34^+$ cells were used to study the expression and functionality of TLR2/4.
- TLR2 and TLR4 activation enhances thrombopoietin-induced megakaryocyte and platelet production.
- IL-6 from $CD34^+$ cells and megakaryocytes accounts for TLR2/4 effects via AKT and nuclear factor-κB pathways.

TLR2 on megakaryocytes was studied in the megakaryocytic cell lines Meg-01 and Dami. It was observed that stimulation of Meg-01 with Pam3CSK4 triggers the activation of nuclear factor (NF)-κB, PI3/AKT, and ERK1/2 pathways and up-regulation of transcription factors involved in megakaryocyte maturation.[19] In addition, stimulation of Dami cells with heat-killed lactobacillus, another TLR2 ligand, resulted in up-regulation of TLR2 together with cytokine secretion, mainly IL-6, which beyond being a TPO trigger is a positive regulator of megakaryocyte generation and CD41 expression.[20]

The expression of TLR2 and TLR4 has been observed in human HSPCs from mice[21,22] and human bone marrow[16,23] as well as in HSPC-derived from cord blood.[24] Evidence mainly from mice models points out that during inflammation/infection, direct activation of TLRs on HSPCs promotes the delivery of immune cells.[22,25] Because the findings concerning HSPCs and megakaryocyte biology obtained in mice or cell lines cannot always be extrapolated to humans and there is no report regarding direct activation of human TLR2 or TLR4 on progenitors and/or megakaryocytes, in this study we aimed to demonstrate that direct activation of human TLR2 or TLR4 on hematopoietic progenitors and/or megakaryocytes could be another mechanism that contributes to the increased number of platelets during bacterial infection. Human megakaryo/thrombopoiesis is generally studied using primary HSCPs isolated from bone marrow, peripheral blood, mobilized or not with granulocyte colony stimulating factor, or cord blood cells. Although it is known that there are differences between them, especially regarding their proliferative capacity or ploidy, all of these sources represent widely accepted models to study human $CD34^+$ cell biology. In this study, we evaluated the different stages of megakaryopoiesis and platelet production using $CD34^+$ cells from umbilical cord blood stimulated with TPO, PamCS34K, which is a synthetic triacylated lipopeptide that mimics the acylated amino terminus of bacterial lipopeptides and also a TLR2 ligand, or lipopolysaccharide (LPS), which is a wall component of Gram-negative bacteria as well as a TLR4 ligand. Our results show that activation of these TLRs on $CD34^+$ produce more megakaryocytes with a higher degree of maturation and a larger

number of platelets than TPO alone through activation of PI3/AKT and NF-κB signaling pathways and IL-6 release.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The study was approved by the Ethical Committee of the National Academy of Medicine, Argentina. All individuals provided written informed consent for the collection of samples and their subsequent analysis.

2.2 | Reagents

StemSpan™ serum free medium was purchased from StemCell Technologies; TPO, CD34, and CD61 purification kits were from Miltenyi Biotec. Fetal bovine serum (FBS) was obtained from GIBCO. Purified LPS derived from *Escherichia coli* O111:B4, and propidium iodide (PI) were purchased from Sigma-Aldrich. Click-iT® Plus EdU Alexa Fluor-488, TO-PRO-3, Alexa Fluor-488 anti-mouse, and Alexa Fluor-546 anti-rabbit were obtained from Invitrogen.

Recombinant human stem cell factor (SCF) and FLT3L were obtained from PeproTech. Pam3CSK4, blocking antibodies (Abs) against TLR2 (TL2.1) and TLR4 (HTA125) and control immunoglobulin G (IgG) were obtained from InvivoGen. The Abs TLR2-PE (clone T2.1), TLR4-PE (HTA125), IgG2a-PE, CD11b APC/Cy7 (M1/70), DyLight™ 649 Streptavidin, biotin and LEAF purified anti-human IL-6 (clone MQ2-13A5), and LEAF purified mouse IgG_{κ} were from BioLegend. The mouse monoclonal antibody (mAb) anti-β Tubulin (D10) and rabbit polyclonal (p) Ab anti-NF-E2 (C-19) were obtained from Santa Cruz Biotechnology. The anti-CD34-FITC, anti-CD41-Pe, anti-CD61-FITC, anti-CD42b-FITC, anti-AKT-Alexa Fluor-488 (pSer473), Annexin V-FITC, goat anti-rabbit conjugated to horseradish peroxidase, mouse anti β-actin and irrelevant IgG_1-FITC, PE, or Alexa Flour 488 were purchased from BD Biosciences. Fixable Viability Dye eFluor™ 780 was from eBioscience. mAb anti-human TPO receptor (clone 167620) was from R&D. Rabbit mAb against IkB-α (E130) was from Abcam.

Inhibitors (Ly294002 and Bay11-7082) were purchased from Biomol.

TriReagent to obtain RNA was from Genbiotech, and 2X SYBR® Green master mix was from Bio-Rad.

2.3 | Purification of $CD34^+$ cells

Umbilical cord blood was collected during normal full-term deliveries of healthy mothers (n = 30). Blood was collected by puncturing the umbilical vein after delivery in a commercial stem cell collection bag containing citrate phosphate dextrose as anticoagulant. Samples were kept at room temperature and processed between 48 hours after collection. Cord blood samples were diluted with one volume of phosphate-buffered saline (PBS) and centrifuged 180 × *g* 15 minutes with the brake off. The upper phase containing platelets was removed. Low-density mononuclear cells were prepared by centrifugation of the remaining blood over a Ficoll Hypaque (1.077 g/cm^3) gradient. Cells collected from the interface were washed and resuspended in PBS containing EDTA (2 mmol/L) and bovine serum albumin (0.5% (w/v). $CD34^+$ cells were purified using a magnetic cell-sorting system (Miltenyi Biotec) in accordance with the manufacturer's recommendations. The purity of the $CD34^+$-enriched population was determined by immunolabeling the cells with FITC-conjugated anti-CD34 monoclonal antibody that reacted with an epitope other than the antibody used for separation. After two Mini-MACS column separations, the purity of cell suspension was determined by flow cytometry and ranged typically between 95 and 99% for $CD34^+$.[26]

2.4 | Cell culture

The $CD34^+$ cells were cultured in StemSpan medium in the presence of 1% FBS and antibiotics. To obtain megakaryocytes, TPO (50 ng/mL) was added at days 0 and 7 of culture and plates were placed in a humidified atmosphere of 5% CO_2 until day 17. For polymerase chain reaction (PCR) experiments, megakaryocytes were purified by immunomagnetic selection from day 11 cultures using anti-CD61 magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the final cell suspension was >97%. TLR2 or TLR4 agonists (Pam3CSK4 and LPS, respectively) were added at day 0 or 7 of culture. In selected experiments, inhibitors of PI3K/AKT (Ly294002) or NF-κB (Bay11-7082) pathways, neutralizing antibodies to TLR2, TLR4, IL-6, or their respective isotypic controls were added 1 hour before TLR stimulation.

2.5 | Flow cytometry studies

To evaluate megakaryocyte differentiation and maturation, cells at day 11 of culture were double stained with CD41-PE and CD42b-FITC. TLR2 and TLR4 expression was determined in freshly purified $CD34^+$ cells with an anti-TLR2-PE or TLR4-PE Abs and in day 11 megakaryocytes by double staining with CD61-FITC and anti-TLR2-PE or anti-TLR4-PE. c-MPL expression was determined in purified $CD34^+$ cells fixed with 4% paraformaldehyde and stained with mAb anti-human TPO receptor followed by a secondary Ab conjugated to Alexa-488. The percentage of positive cells and the median fluorescence intensity (MFI) were analyzed by flow cytometry (BD FACSCalibur™ or Sysmex-Partec CyFlow® Space) and FlowJo 7.6 software.

2.6 | Real-time PCR and quantitative PCR (qPCR)

RNA was isolated from cell pellets by using TriReagent. cDNA was synthesized using random hexamers (Biodynamics, Buenos Aires, Argentina) and MMLV reverse transcriptase (Promega). The primer sequences used to amplify the target fragments were: TLR2 sense 5′-ATCCTCCAATCAGGCTTCTCT -3′ and antisense 5′ACACCTCTGTAGGTCACTGTTG-3′; TLR4 sense 5′-ATAGCGAGCC

ACGCA TTCAC-3′ and antisense 5′-GGAACCACCTCCACGCAG-3′; and Elongation factor 1-alpha 1 (EeF1A1) as housekeeping gene and internal control, sense 5′-TCGGGCAAGTCCACC ACTAC -3′ and antisense 5′-CCAAGACCCAGGCATACTTGA -3′. The qPCR was carried out in CFX96 Connect real time PCR detection system (Bio-Rad), using 2X SYBR® Green master mix (Bio-Rad). The specificity of PCR products was confirmed by melt curve analysis. The comparative Ct method ($2^{-\Delta\Delta CT}$) was used to analyze the expression level of the target genes calibrated to $CD34^+$ cell expression.

2.7 | Annexin-V binding assay and EdU proliferation assay

$CD34^+$ cells were stimulated with different agonists and 36 hours later washed and incubated in binding buffer for 30 minutes with Annexin-V-FITC at 37°C. Then, PI was added and immediately analyzed by flow cytometry. For EdU assay, cells were cultured for 4 days and then incubated with EdU for 2 hours at 37°C. The detection system was performed following the manufacture′s protocol. Simultaneous cell viability was determined with Fixable Viability Dye eFluor™ 780 (eBioscience) and analyzed by flow cytometry.

2.8 | Proplatelet formation

The proplatelet formation was analyzed after 13 days of cell stimulation with TPO alone or in combination with TLR agonists. Cells were plated on fibrinogen-coated coverslips (100 μg/mL) in 24-well plates (1×10^5 cells per well) and cultured for 16 hours at 37°C and 5% CO_2. The total number of megakaryocytes producing proplatelets was counted by phase-contrast microscopy. The proplatelet formation was also evaluated by fluorescence microscopy by staining the cells with an anti-β tubulin mouse mAb and revealed with an anti-mouse Alexa fluor-488. Proplatelet-forming megakaryocytes were identified as large cells exhibiting long filamentous structures containing swellings. The extent of proplatelets formation was calculated as the percentage of proplatelets bearing cells by counting 200 cells per treatment.[27]

2.9 | Platelet production in culture

Platelets were counted after 17 days of culture. Cells were incubated with a CD61-FITC mAb or an irrelevant isotype and PI (1 μg/mL). Viable culture-derived platelets were counted as $PI^-/CD61^+$ events with the same scatter properties as human peripheral blood platelets. The number of platelets in culture was relativized with the number of megakaryocytes determined by flow cytometry as $PI^-/CD61^+$ events with the scatter properties of nucleated cells.[27]

2.10 | NF-E2 translocation assay

Cells were cytospin at day 9 of culture, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. After blocking, the cells were incubated with a rabbit anti-NF-E2 pAb and then with an Alexa-546 conjugated secondary goat anti-rabbit IgG. To identify megakaryocytes, CD61-FITC was used as a lineage marker. The nuclei were stained with TO-PRO-3. Slides were mounted with PolyMount and analyzed by fluorescence microscopy (BX60; Olympus). In the $C61^+$ cells, the fluorescence corresponding to nuclear or cytoplasmic NF-E2 staining was quantified using an image masks strategy related to TO-PRO-3 nuclear signal. The nuclear/cytoplasmic NF-E2 ratio for each cell was calculated with the ImageJ program in at least 200 cells per treatment. NF-E2 nuclear translocation was revealed by an increase in nuclear/cytoplasmic ratio of NF-E2.

2.11 | Determination of I-κBα degradation by immunoblotting

$CD34^+$ cells were expanded with stem cell factor (100 ng/mL), FLT3L (25 ng/mL), and TPO (12.5 ng/mL) for 8 days. Then, cells were washed with PBS and starved with StemSpan medium with 1% FBS overnight. Cells (1×10^6/treatment) were stimulated with TPO without or with the TLR2/4 agonists for 1 hour and lysed with loading buffer. Membranes were probed with mAb against IkB-α (E130) and reprobed with an Ab against β-actin to ensure equal loading. Protein bands were visualized by using enhanced chemiluminescence kit (Thermofisher). Immunoblotting results were semiquantitated using ImageJ software.

2.12 | Detection of AKT phosphorylation

Freshly purified $CD34^+$ cells were starved in StemSpan medium for 4 hours. Cells were stimulated for 30 minutes at 37°C, washed with ice-cold PBS. Then, were fixed and permeabilized according to the BD Phosflow protocol (protocol III). Next, cells were stained with Alexa Fluor-488-conjugated anti-phospho AKT (Ser473) or Alexa Fluor-488-IgG1κ isotype and subjected to flow cytometry.

2.13 | Measurement of IL-6 levels

The production of IL-6 was determined in the supernatants with a commercial ELISA kit according to the manufacturer's instructions (eBioscience). In selected experiments, cells were incubated for 1 hour with Ly249002 or BAY 11-7082 previous to TLR stimulation.

2.14 | Intracellular IL-6

Freshly purified $CD34^+$ cells were cultured in StemSpam medium and stimulated for 20 hours with different agonists, then protein secretion was blocked with BD GolgiStop™ (BD, Bioscience) for 4 hours. Cells were collected, washed with PBS, and stained with Fixable Viability Dye eFluor™ 780, CD11b-APC/Cy7 and CD41-PE. Intracellular IL-6 staining was performed after fixing and permeabilizing cells using the BD Cytofix/Cytoperm kit (BD Biosciences) and biotin anti-human IL-6 and DyLight™ 649 Streptavidin. IL-6 expression was analyzed in viable cells for each population defining fluorescence minus one controls in a Sysmex-Partec CyFlow® Space flow cytometer and analyzed with FlowJo 7.6 software.

2.15 | Statistics

Results were expressed as means ± standard error of the mean from independent experiments. Significant differences ($P < .05$) were identified by one-way or two-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's multiple comparisons test or Wilcoxon nonparametric test using the GraphPad software package (PRISM Version 6.0, San Diego, CA).

3 | RESULTS

3.1 | TLR2 and TLR4 are expressed in freshly isolated human CD34$^+$ cells and megakaryocytes

Initially, we evaluated the expression and regulation of TLR2 and TLR4 on CD34$^+$ cells and megakaryocytes at the transcriptional and protein level. As depicted in Figure 1, both cells express messenger RNA (mRNA) for TLR2 and TLR4 (Figure 1A). To compare TLRs expression in progenitors and megakaryocytes their expression was further evaluated by qPCR in purified CD34$^+$ cells and megakaryocytes from the same culture 11 days after TPO stimulation. Although the amount of mRNA for TLR2 transcripts decreased, TLR4 mRNA levels were higher in megakaryocytes than in their precursors (Figure 1B). TLRs expression was also confirmed at the protein level in both cell types by flow cytometry (Figure 1C).

3.2 | The activation of TLR2 and TLR4 on CD34$^+$ cells enhances megakaryopoiesis

To test whether activation of these receptors modulates megakaryo/thrombopoiesis, we first evaluated CD34$^+$ cells expansion induced by TPO in the absence or presence of TLR2 and TLR4 ligands. As expected, CD34$^+$ cells stimulation with TPO increased cell proliferation, reflected by higher total cell number and EdU incorporation. However, the treatment of CD34$^+$ cells with LPS or Pam3CSK4 alone (without TPO) was not able to sustain cell survival, although at the highest concentrations both ligands induced a very low rate of cell proliferation (Figure 2A,B). Nevertheless, the addition of Pam3CSK4 or LPS significantly increased TPO-induced proliferation in a concentration-dependent manner (Figure 2A,B). To evaluate whether the increase in cell number was also associated with an antiapoptotic effect mediated by LPS or Pam3CSK4, Annexin-V$^+$ cells were analyzed by flow cytometry after 36 hours of cell stimulation. Despite the high percentage of apoptotic cells in control samples (1% FBS without TPO), both TLR ligands slightly inhibited cellular death in the absence of TPO but failed to further contribute to the antiapoptotic effect mediated by TPO (Figure 2C). Then, we also analyzed

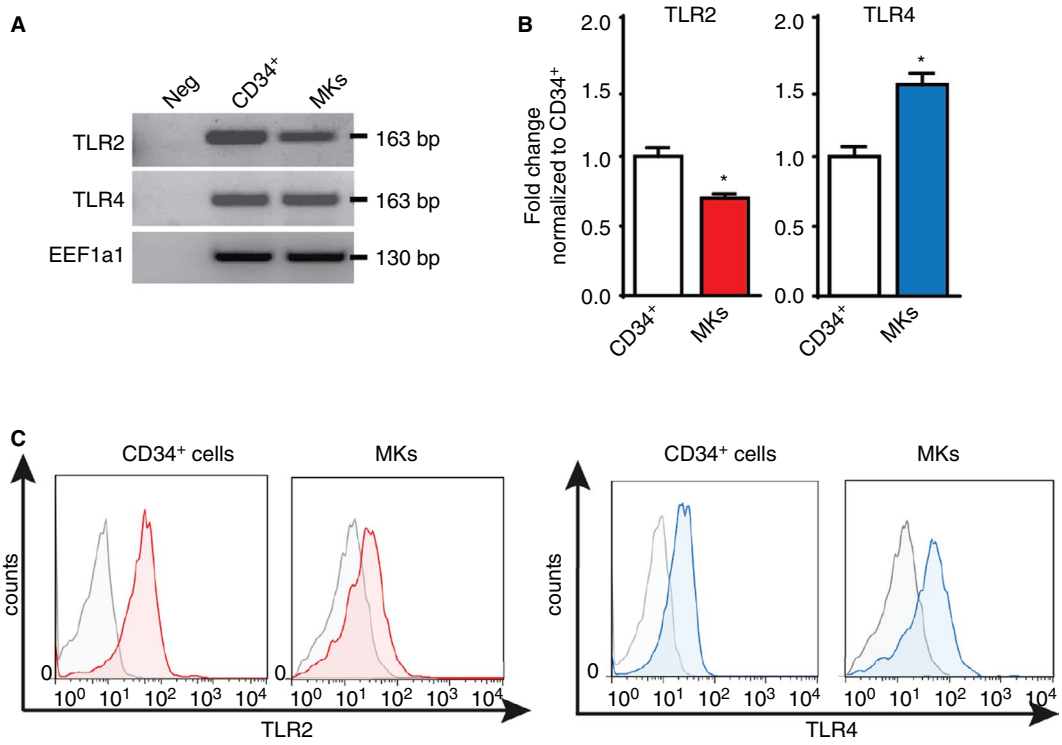
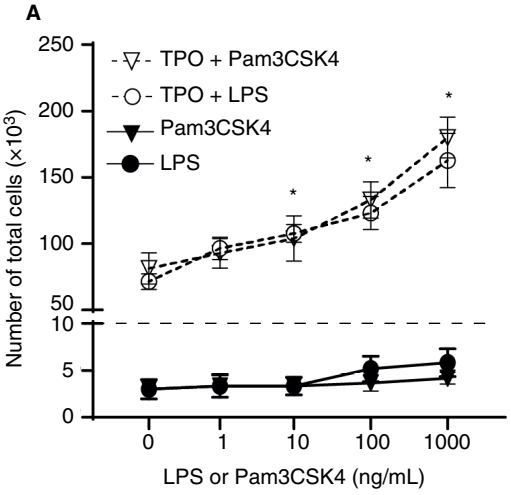
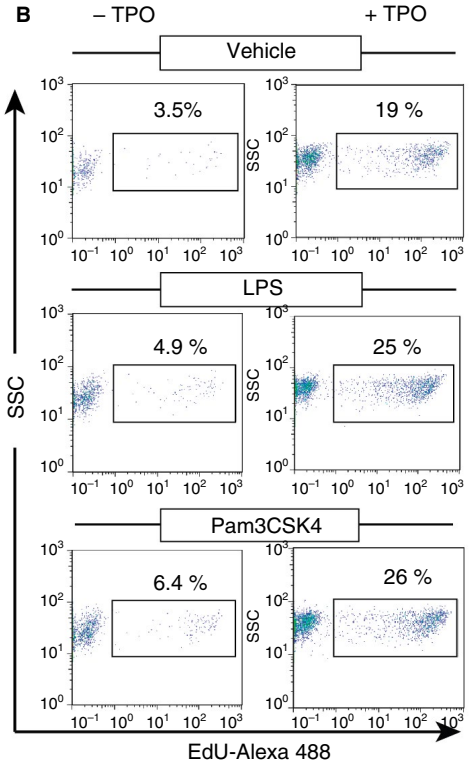
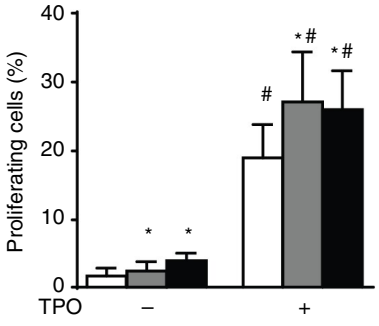
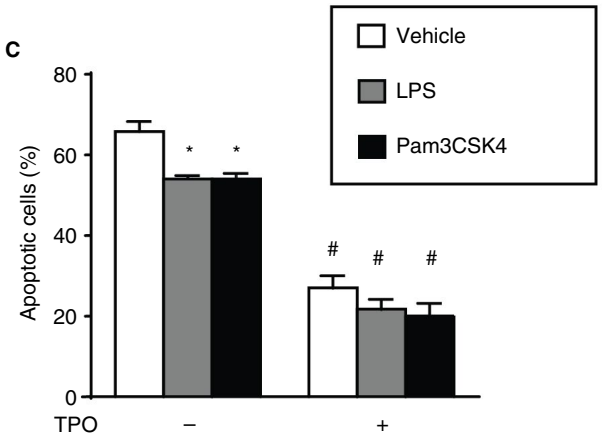


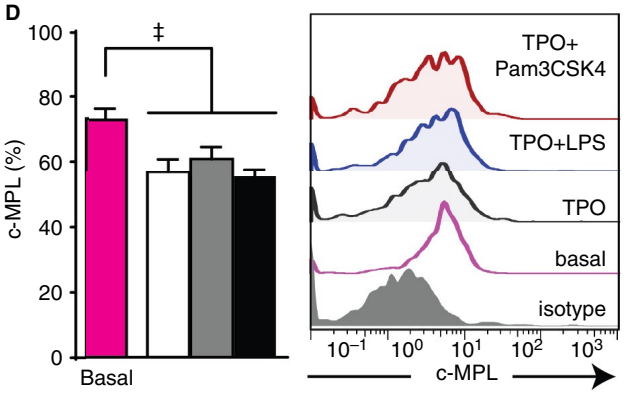
FIGURE 1 TLR2 and TLR4 are expressed in freshly isolated human CD34$^+$ cells and megakaryocytes. (A) TLR2 and TLR4 mRNA expression were initially evaluated in purified CD34$^+$ cells and megakaryocytes (MKs) by endpoint RT-PCR. EEF1a1 was used as a housekeeping gene. (B) Quantitative PCR (qPCR) was performed to compare the expression level of TLR2 and TLR4 in CD34$^+$ cells vs MKs. Relative quantification was calculated by $2^{(-\Delta\Delta CT)}$ fold change and normalized to CD34$^+$ cells. EEF1A1 housekeeping gene was used as internal control. (C) Representative histograms of TLR2 and TLR4 surface expression in CD34$^+$ cells and in the CD61$^+$ population at day 11 of culture. n = 3, *$P < .05$ vs CD34$^+$ cells, Wilcoxon test for paired samples

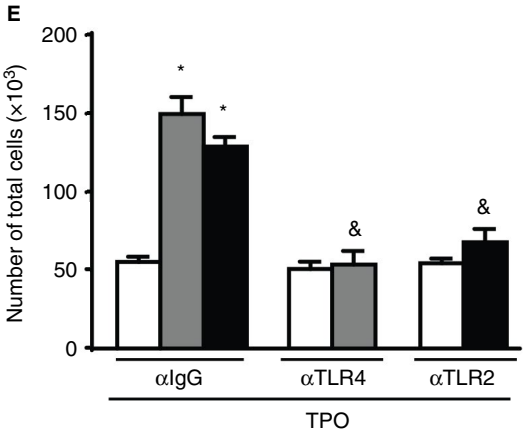

A
TPO + Pam3CSK4
TPO + LPS
Pam3CSK4
LPS
Number of total cells (×10³)
LPS or Pam3CSK4 (ng/mL)


B
– TPO
+ TPO
Vehicle
LPS
Pam3CSK4
3.5%
19 %
4.9 %
25 %
6.4 %
26 %
SSC
EdU-Alexa 488


Proliferating cells (%)
TPO
–
+


C
Vehicle
LPS
Pam3CSK4
Apoptotic cells (%)
TPO
–
+


D
c-MPL (%)
Basal
TPO+ Pam3CSK4
TPO+LPS
TPO
basal
isotype
c-MPL


E
Number of total cells (×10³)
αIgG
αTLR4
αTLR2
TPO

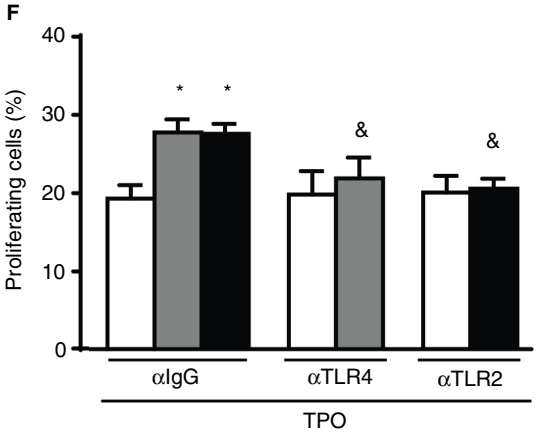

F
Proliferating cells (%)
αIgG
αTLR4
αTLR2
TPO

FIGURE 2 LPS and Pam3CSK4 synergize with TPO to induce proliferation of $CD34^+$ cells. $CD34^+$ cells (5×10^4/mL) were cultured in the presence or not of TPO (50 ng/mL) and stimulated with LPS or Pam3CSK4. (A) The total number of cells was determined by counting in a Neubauer chamber at day 11 of culture. (B) Cells were stimulated with TLR agonists (100 ng/mL) in the presence or not of TPO. The proliferation rate was determined after 4 days by measuring EdU incorporation in the viable subpopulation by flow cytometry. (C) $CD34^+$ cells were cultured with the same conditions as in B and Annexin-V/PI staining was determined 36 hours later by flow cytometry. (D) Surface c-MPL expression was determined by flow cytometry in freshly isolated $CD34^+$ cells (basal) and after 24 hours of stimulation. (E) Cells were pretreated with neutralizing Abs against TLR2, TLR4 or their respective isotypes (5 μg/mL) and then cultured in the presence of TPO and TLRs agonists (100 ng/mL). Numbers of total cells were determined after 11 days of culture. (F) Cells were pretreated as in panel E and the proliferation rate in the presence of neutralizing antibodies was determined by EdU incorporation after 4 days of culture. $^*P < .05$ vs vehicle, $^{\#}P < .05$ vs same treatment without TPO, n = 7, two-way ANOVA. $^{\&}P < .05$ vs αIgG, n = 7, $^{\ddagger}P < .05$ vs basal, one-way ANOVA

the expression of c-MPL, the TPO receptor, and found that as it was previously reported,[28] basal c-MPL surface expression was significantly downregulated in $CD34^+$ cells after 24-hour stimulation with TPO. This receptor endocytosis was not modified by TLRs activation indicating that the proliferative effect of Pam3CSK4 and LPS was not due to an increase in c-MPL expression (Figure 2D).

When $CD34^+$ cells were pretreated with blocking antibodies against TLR2 and TLR4, the enhanced cell proliferation triggered by Pam3CSK4 or LPS was completely suppressed, confirming a TLR-specific proliferating response (Figure 2E,F).

To evaluate whether activation of TLRs also regulates megakaryocyte commitment and maturation, we monitored CD41 and CD42b expression, specific markers of these cellular processes. The stimulation of $CD34^+$ cells with high concentrations (100 and 1000 ng/mL) of TLR agonists reduced TPO-mediated expression of CD41 and increased CD42b levels (Figure 3A). However, because of the enhanced proliferative effect of TLR2 or TLR4 agonists on $CD34^+$ cells, the net outcome was a higher number of megakaryocytes with a higher degree of maturation compared with TPO alone.

Because megakaryocytes express TLR2 and TLR4, it is conceivable that the observed synergistic effect of LPS or Pam3CSK4 was due to a combined action of TLR activation in both $CD34^+$ cells and megakaryocytes. However, when TLR2 or TLR4 agonists were added 7 days after TPO stimulation (40 ± 5% of $CD41^+$ cells) no changes were observed in megakaryocyte proliferation, differentiation, or maturation compared with cultures treated only with TPO indicating that the effects of Pam3CSK4 and LPS were mainly due to the activation of TLRs on $CD34^+$ cells (Figure 3B).

3.3 | TLR2 and TLR4 activation promotes proplatelet and platelet production

Having demonstrated that activation of TLR2 and TLR4 on $CD34^+$ cells promotes TPO-induced megakaryopoiesis, we then evaluated whether a similar effect was exerted in the last stages of this process including platelet biogenesis. As shown in Figure 4A, LPS or Pam3CSK4 increased proplatelet generation compared with cells cultured with TPO alone. Accordingly, the number of platelets produced in culture by megakaryocytes was higher in these cultures independently of the megakaryocyte yield (Figure 4B). Similar to the proliferative effect, platelet production was not modified when megakaryocytes were treated with the TLR agonists (Figure 4C).

3.4 | TLR2 and TLR4 signaling pathways involved in the potentiation of TPO-mediated megakaryo/thrombopoiesis

TLR2 and TLR4 downstream signaling mainly include the activation of PI3K/AKT and NF-κB axes.[10,29,30] To determine whether these signaling pathways participate in the LPS- and Pam3CSK4-mediated proliferative effect, $CD34^+$ cells were treated with Ly294002 or Bay11-7082 specific inhibitors of PI3K and NF-κB, respectively, at concentrations that did not or moderately suppress TPO-induced cell proliferation. Preincubation with Ly294002 or Bay11-7082 prevented LPS or Pam3CSK4 mitogenic action (Figure 5A). The involvement of AKT and NF-κB axis was further demonstrated by analyzing the phosphorylation of AKT and the degradation of I-κBα, the inhibitor of NF-κB. Both TPO and TLR agonists triggered AKT activation; however, when $CD34^+$ cells were stimulated with TPO in combination with TLR2 or TLR4 agonist, AKT phosphorylation was higher than each stimulus alone (Figure 5B). On the other hand, although $CD34^+$ cells stimulation with TPO failed to modulate I-κB levels, a significant decrease was detected in LPS or Pam3CSK4-treated cells. Furthermore, the reduction of I-κB signal was more pronounced when $CD34^+$ cells were stimulated with a combination of TPO and either TLR2 or TLR4 ligand (Figure 5C).

Considering that the main transcription factor driving thrombopoiesis is NF-E2,[31] we evaluated if the increased proplatelet formation after TLR2 or TLR4 activation was driven by enhanced NF-E2 nucleus translocation. Cells were harvested at day 9 of culture, cytospinned, and stained for NF-E2 and CD41 as lineage marker. As shown in Figure 5D, higher levels of nuclear NF-E2 were observed in those megakaryocytes generated from $CD34^+$ cells treated with TPO plus LPS or Pam3CSK4.

3.5 | IL-6 is the soluble mediator involved in the augmented LPS and Pam3CSK4-induced proliferation

Because elevated IL-6 serum levels are associated with reactive thrombocytosis resulting from hepatic TPO synthesis,[7] we then tested whether IL-6 was a mediator involved in the increased growth of $CD34^+$ cells induced by TLR agonists. The levels of IL-6 in the supernatants of the different cell treatments were determined by ELISA at day 11 of culture. Although no IL-6 was detected in the TPO-treated cultures, IL-6 levels were significantly increased in

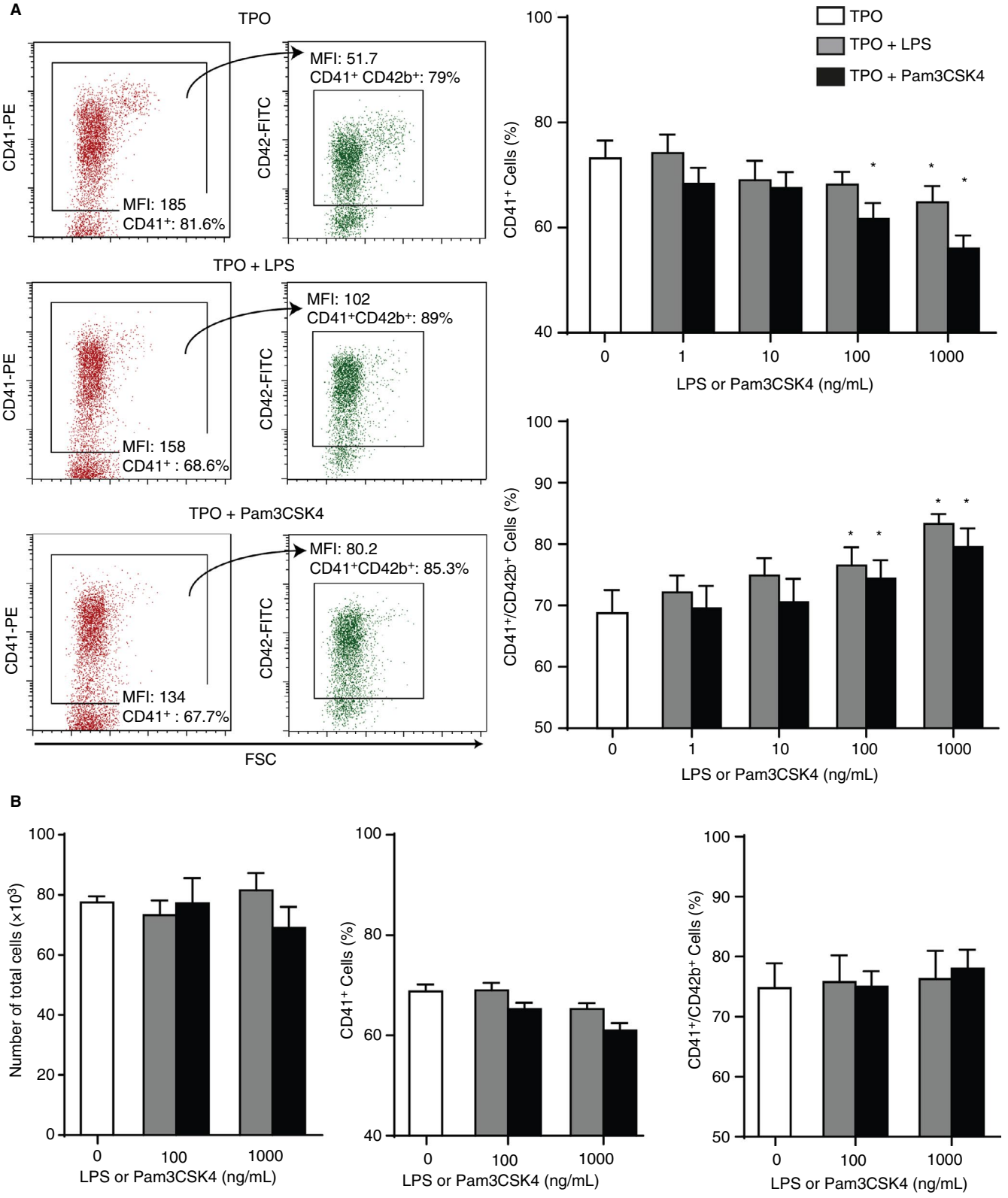


FIGURE 3 The activation of TLR2 and TLR4 on $CD34^+$ cells enhances megakaryopoiesis. (A) $CD34^+$ cells (5×10^4/mL) were stimulated with TPO alone or in combination with LPS or Pam3CSK4 at the indicated concentrations and the percentage of megakaryocytes ($CD41^+$ cells) and their maturity ($CD41^+/CD42b^+$ cells) were determined 11 days later by flow cytometry. Representative dot plots with LPS or Pam3CSK4 at 100 ng/mL. (B) Cells from 7-day TPO-treated cultures (25×10^4/mL) were stimulated with LPS or Pam3CSK4 and 4 days later, the number of total cells, megakaryocytic differentiation ($CD41^+$ cells) and mature megakaryocytes ($CD41^+/CD42b^+$ cells) were determined. *$P < .05$ vs TPO alone, n = 7, ANOVA

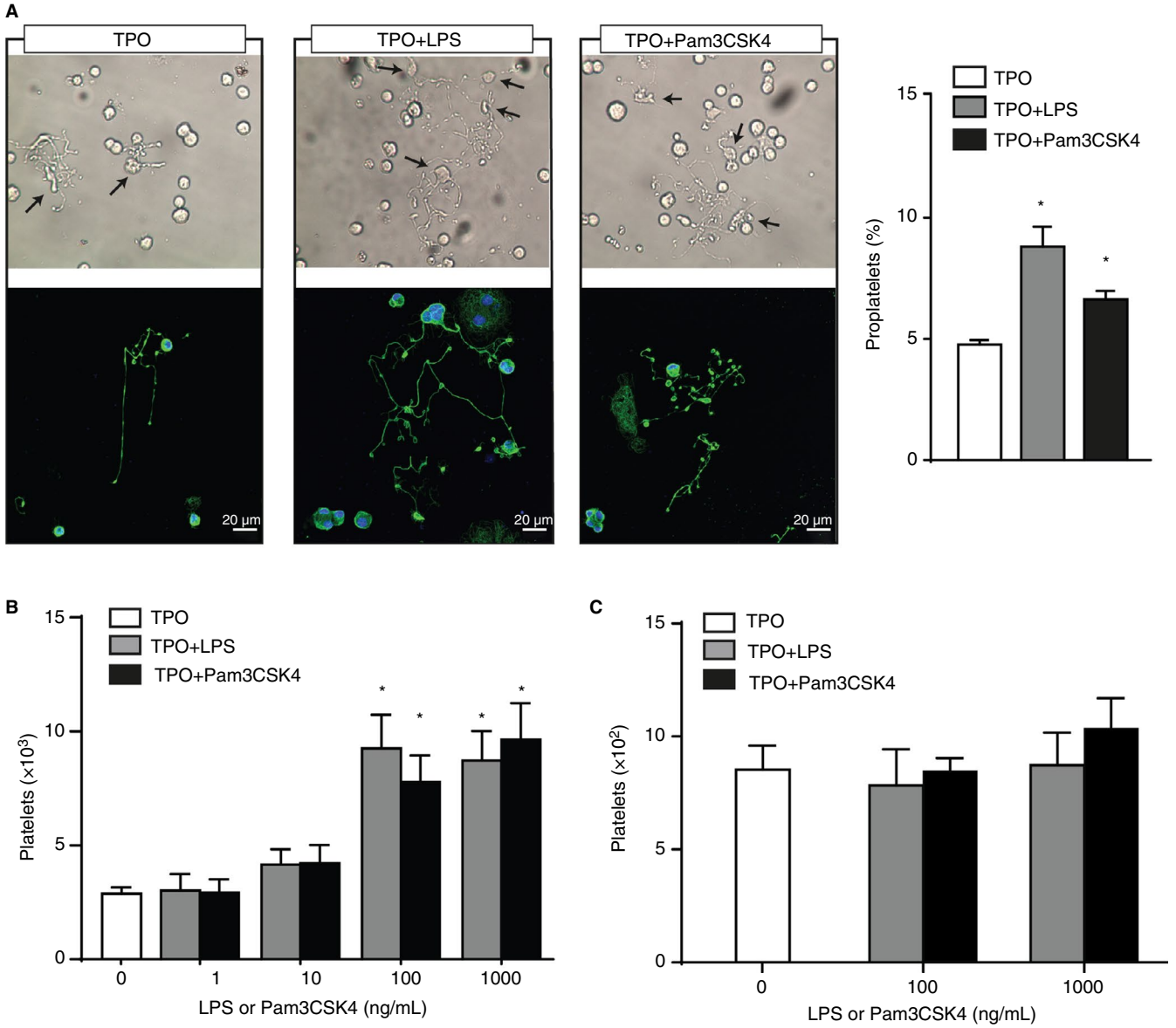


FIGURE 4 LPS and Pam3CSK4 increase TPO-induced proplatelet and platelet formation. (A) $CD34^+$ cells (5×10^4/mL) were stimulated with TPO alone or in combination with LPS or Pam3CSK4 (100 ng/mL); proplatelet generation was evaluated 13 days later. Arrows indicate proplatelet-forming megakaryocytes. The proplatelet formation was quantified by fluorescence microscopy (green = β1-tubulin; blue = TO-PRO-3 staining of nuclei, scale bar: 20 µm). (B) $CD34^+$ cells were cultured as in panel A and platelets were enumerated at day 17 of culture by flow cytometry as viable (PI^-) and $CD61\text{-}FITC^+$ events with the same scatter properties than blood platelets and relativized to megakaryocyte number in culture. (C) Cells from day 7 TPO-treated cultures (25×10^4/mL) were stimulated with TLR agonists for 10 days and culture-derived platelets were counted by flow cytometry as in B. *$P < .05$ vs TPO alone, n = 4-7, ANOVA

supernatants from TPO plus LPS- or Pam3CSK4-stimulated cultures (Figure 6A). Moreover, IL-6 blockade by a specific neutralizing Ab completely prevented cell proliferation triggered by TLR2 or TLR4 agonists (Figure 6B).

Although megakaryocyte activation with LPS or Pam3CSK4 did not modify proliferation or platelet production (Figure 3B,C), it could be possible that they were able to trigger IL-6 release. To determine whether the source of IL-6 production observed at the end of the culture was $CD34^+$ cells, megakaryocytes, or both, we next compared IL-6 levels in supernatants from freshly $CD34^+$ cells and megakaryocytes stimulated or not with TLRs agonists with or without TPO for 24 hours. We found that while in no circumstances did TPO induce IL-6 release, LPS or Pam3CSK4 stimulation of $CD34^+$ cells and surprisingly also megakaryocytes, resulted in IL-6 release that was significantly potentiated in the presence of TPO (Figure 6C,D). Because myeloid cells are the major source of IL-6, to determine the contribution of each $CD34^+$ cell subpopulation, intracellular IL-6 production was measured in $CD34^+CD11b^+$, $CD34^+CD41^+$, and $CD34^+CD11b^-CD41^-$ cells 24 hours after LPS or Pam3CSK4 stimulation with or without TPO. Surprisingly and in contrast to IL-6 released in cell culture supernatants, all $CD34^+$ cell subpopulations produced IL-6 under basal conditions and it was

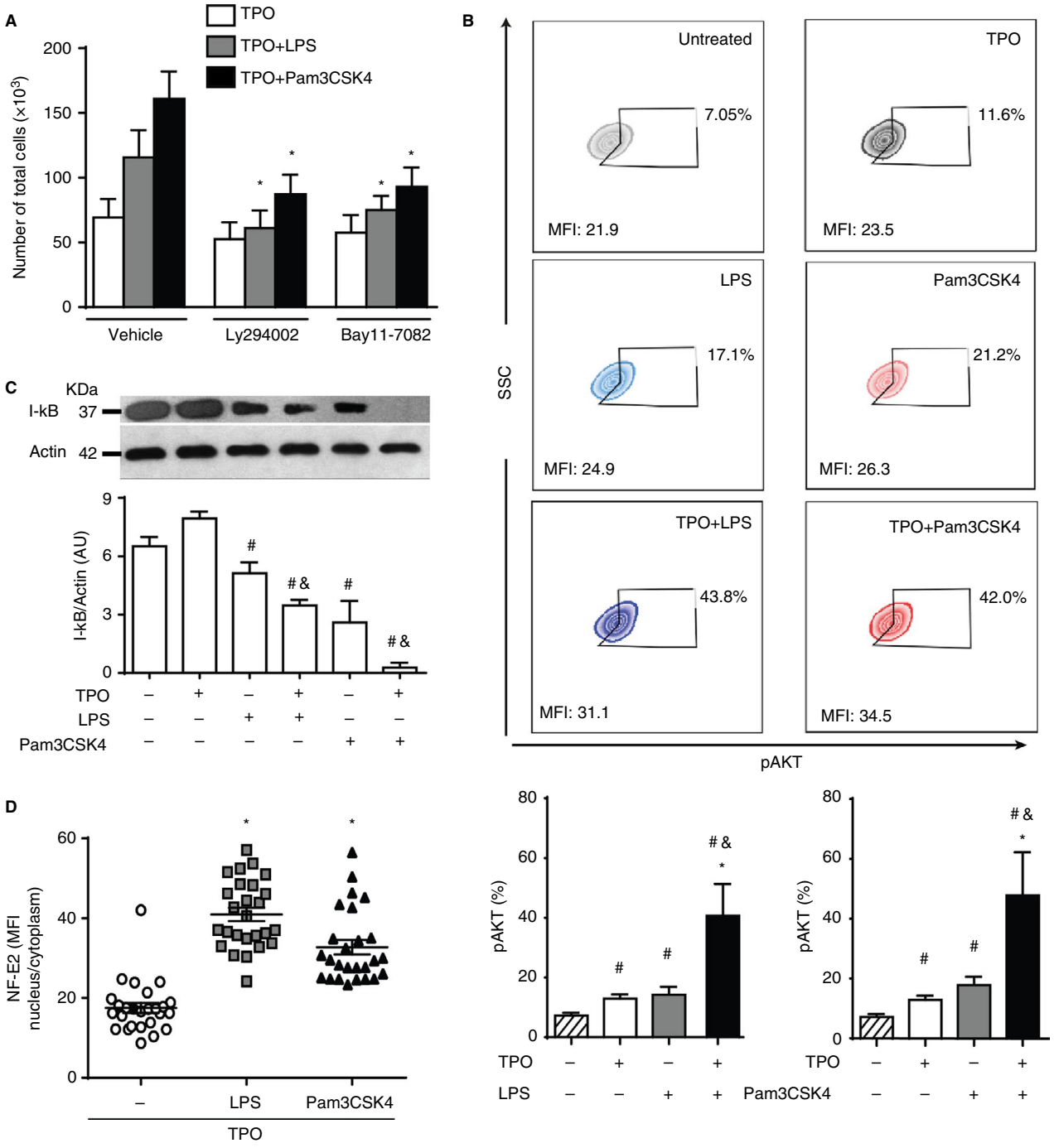


FIGURE 5 Signaling pathways involved in LPS and PAM3CSK4 potentiation of TPO-induced megakaryo/thrombopoiesis. (A) $CD34^+$ cells (5×10^4/mL) were pretreated for 1 hour with pharmacological inhibitors of PI3K/AKT (Ly294002) or NF-κB (Bay117082) (2.5 μg/mL) then treated with TPO (50 ng/mL) and TLR agonists (100 ng/mL) and proliferation was monitored after 11 days. (B) $CD34^+$ cells were starved and then stimulated for 30 minutes with each stimulus, at the same concentrations used in panel A, and the phosphorylation of AKT was determined as the percentage of positive cells by flow cytometry. (C) $CD34^+$ cells were starved and then stimulated for 1 hour. Stimulation was stopped with loading buffer and NF-κB pathway activation was evaluated by quantitation of I-κB degradation by Western blot. (D) NF-E2 expression was assessed in cells harvested at day 9 of culture by double staining with NF-E2 and CD61 Abs. Cells were analyzed by fluorescence microscopy and the nuclear translocation was estimated by using the ratio of MFI in the nuclear or cytoplasmic compartments. $^*P < .05$ vs TPO alone, $^{\#}P < .05$ vs untreated, $^{\&}P < .05$ vs LPS or Pam3CSK4, n = 4-7, ANOVA

significantly increased upon cell activation with LPS, Pam3CSK4, or TPO, being higher with the combination of TPO with either TLR agonist (Figure 6E).

Interestingly, PI3K/AKT axis and NF-κB pathway were not only responsible for the induction of proliferation mediated by TLR agonists but also for IL-6 release, as preincubation with their specific

inhibitors significantly suppressed IL-6 release from both $CD34^+$ cells and megakaryocytes (Figure 6F).

The results showing that activation of TLR2/4 in the presence of TPO, either in $CD34^+$ cells and megakaryocytes induced the release of IL-6 together with previous studies demonstrating that activation of TLRs on HSPC triggers the release of several cytokines,[25] raised the question whether the conditioned media from TPO- and TLRs-treated $CD34^+$ cells could affect the growth of other hematopoietic lineages. To answer this question, $CD34^+$ cells were first incubated for 24 hours with conditioned media from 11-day cultures and then placed in MethoCult methylcellulose without cytokines. Although conditioned media from TPO-derived cultures did not induce colony growth, monocyte/macrophage colonies were observed with conditioned media from LPS– (CFU-M: 7.2 ± 1.2 colonies/20,000 $CD34^+$ cells) but not from Pam3CSK4-treated cultures.

4 | DISCUSSION

The association between thrombocytosis and inflammation is widely recognized; however, the molecular mechanisms of inflammation-induced megakaryopoiesis are not completely elucidated. Several lines of evidence have shown that HSPCs not only respond to inflammatory cytokine signals, but also directly sense pathogens through their TLRs responding to the increasing demand of leukocytes during infection by augmenting leukocyte number.[22,25] In this study, we demonstrate that activation of TLR2 and TLR4 on human $CD34^+$ cells enhances TPO-induced megakaryo/thrombopoiesis by inducing IL-6 release that can regulate stress-induced megakaryo/thrombopoiesis. We found that $CD34^+$ cells and megakaryocytes express both receptors. The functionality of these receptors was demonstrated by showing that activation of TLR2 or TLR4 by Pam3CSK4 or LPS in the presence of TPO results in increased cell proliferation, megakaryocyte, proplatelet, and platelet formation. The abrogation of these effects by blockade of either receptor indicated that the proliferative effect was specifically associated with the activation of TLR2 and TLR4. Moreover, our results pointed out a significant role of TLR2 and TLR4 in HSPCs for the increased megakaryo/thrombopoiesis.

In contrast to previous evidence indicating that HSCs and progenitors may directly recognize bacteria through TLRs and promote myeloid commitment and proliferation[23,32] we did not find a direct effect of these TLR ligands on HSCs when used without TPO. One possible explanation for the failure of TLR agonists to exert an effect per se could be related to the experimental conditions used in each study, such as the origin of $CD34^+$ cells, the number of cells or TLR ligand concentrations. In fact, a differential TLR expression pattern between $CD34^+$ cells derived from cord blood or bone marrow was previously reported.[23,24] Because TLR agonists trigger the release of several growth factors and cytokines from progenitor cells, the higher cell concentration used in the system, the stronger the likelihood that the produced cytokines/growth factor can complement TLR signaling to induce proliferation/differentiation in response to TLR stimulation. Of note in our experimental conditions, and contrary to previous studies, both the number of cells used in the experiments (5×10^4/mL) and the minimal concentration of LPS or Pam3CSK4 that elicited a functional response (10 ng/mL) were low. Under these conditions and in the presence of a lineage-specific growth factor such as TPO, the main role of TLRs activation on HSPCs is to increase the cell proliferation rate.

Signaling pathways downstream the TPO receptor, c-MPL, mainly involve JAK2, STAT, and PI3K/AKT[33] whereas MyD88, PI3K, and NF-κB are the major pathways involved in TLR2 and TLR4 signaling.[10,29,30] Available data on NF-κB activation in human TPO-

stimulated HSPCs is very limited. Interestingly, it was reported that TPO-mediated activation of ERK1/2 and NF-κB pathways in HSPCs, are mainly required for a TPO-mediated effect on DNA repair.[34] In our hands, although activation of TLR2/4 but not c-MPL resulted in activation of NF-κB pathway, PI3K/AKT axis was activated by both TLR2/4 and c-MPL activation and both signals were potentiated by the simultaneous activation of TLRs and the TPO receptor. In concordance, the proliferative enhancement of LPS and Pam3CSK4 on TPO-induced $CD34^+$ cell growth was dependent on both PI3K and NF-κB activation but not on enhanced c-MPL expression. Together, these data suggest that in addition to being critical for DNA repair, a robust activation of NF-κB pathway, only reached by the cooperation between TPO and TLR2/4 activation, is necessary to warrant optimal levels of TPO-induced megakaryopoiesis under stress conditions. We also found that LPS and Pam3CSK4 but not TPO triggered IL-6 release from $CD34^+$ cells and the combination of either TLR ligand plus TPO produced sufficient amounts of IL-6 in an NF-κB and PI3K activation-dependent manner that were capable of promoting an increase in megakaryocytes. Our findings are in agreement with previous studies showing that LPS or Pam3CSK4 stimulation of cord blood progenitor cells results in a fast and broad secretion of cytokines, mainly IL-6, facilitating myeloid cells generation in an autocrine loop.[25,32] We now further extended this notion showing that a similar mechanism allowing the generation of greater number of megakaryocytes and platelets is operative upon TLR activation of HSPCs in the presence of TPO. Remarkably and in support of this concept, it was recently shown in a murine model that inflammatory signaling instructs a rapid megakaryocyte maturation program at distinct levels of megakaryopoiesis to regenerate the lost platelet pool.[35] Interestingly, the observation that all subpopulations of $CD34^+$ cells produce IL-6 upon stimulation with TLR2/4 or c-MPL agonists but it is only released by LPS or Pam3CSK4, indicates that TLRs ligands promote not only the synthesis but also the secretion of IL-6, the latter process not being exerted by TPO. Because the conditioned media from cultures treated with TPO and LPS induced the growth of monocyte/macrophages colonies, it could be conceivable that TPO plus TLR4 activation on HSPC could exert autocrine as well paracrine effects in the bone marrow niche enabling an emergency efficient hematopoiesis during inflammatory conditions. However, more studies are required to identify the cytokines involved in this effect.

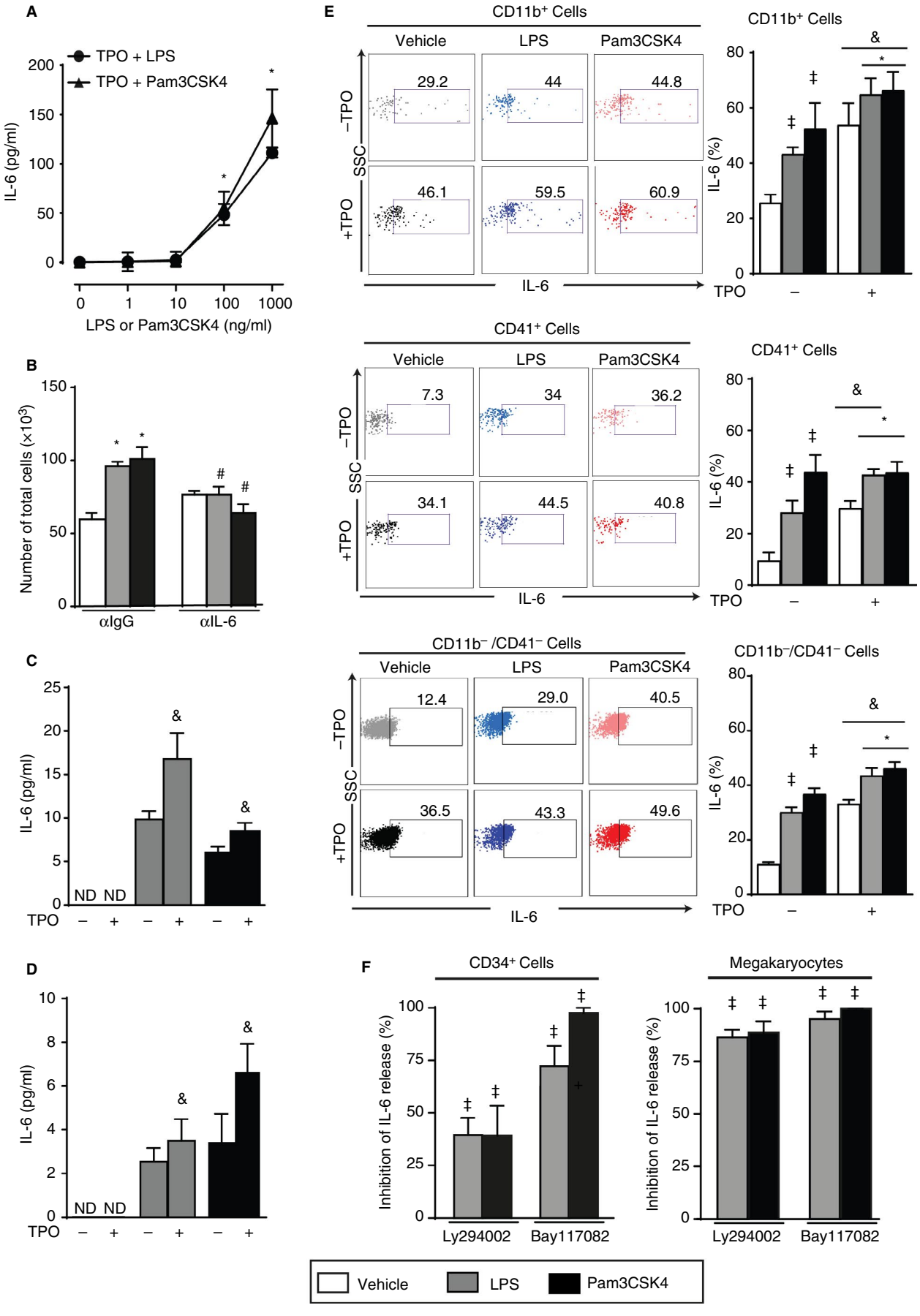

A
TPO + LPS
TPO + Pam3CSK4
IL-6 (pg/ml)
LPS or Pam3CSK4 (ng/ml)
B
Number of total cells (×10³)
αIgG
αIL-6
C
IL-6 (pg/ml)
ND ND
TPO
D
IL-6 (pg/ml)
ND ND
TPO
E
CD11b⁺ Cells
Vehicle
LPS
Pam3CSK4
–TPO
+TPO
SSC
IL-6
29.2
44
44.8
46.1
59.5
60.9
IL-6 (%)
TPO
CD41⁺ Cells
7.3
34
36.2
34.1
44.5
40.8
CD11b⁻ /CD41⁻ Cells
12.4
29.0
40.5
36.5
43.3
49.6
CD11b⁻/CD41⁻ Cells
F
CD34⁺ Cells
Megakaryocytes
Inhibition of IL-6 release (%)
Ly294002
Bay117082
Vehicle
LPS
Pam3CSK4

FIGURE 6 IL-6 is the mediator involved in the proliferative effect mediated by LPS or Pam3CSK4. (A) $CD34^+$ cells (5×10^4/mL) were cultured with different concentrations of LPS or Pam3CSK4 and IL-6 was measured in the supernatants 11 days later. (B) $CD34^+$ cells were pretreated and every 3 days with neutralizing Ab against IL-6 or its respective isotype and cultured with TPO in the absence or presence of TLR agonists (100 ng/mL). The total number of cells was determined after 11 days. (C) $CD34^+$ cells (5×10^5/mL) were cultured with TLR agonists (100 ng/mL) without or with TPO (50 ng/mL) and IL-6 levels were measured 24 hours later by ELISA. (D) Cells (5×10^5/mL) at day 10 of culture with TPO (85% $CD41^+$ cells) were treated as in panel C and IL-6 levels determined by ELISA. (E) $CD34^+$ cells were treated with TLR agonists with or without TPO and 20 hours later treated with Golgi Stop for 4 hours. Then cells were surface stained with CD11b-APC-Cy7 and CD41-PE, fixed, permeabilized, and stained with IL-6-biotin and DyLight™649-Streptavidin to determine by flow cytometry intracellular IL-6 levels in myeloid cells ($CD11b^+$ population), megakaryocytic progenitors ($CD41^+$ cells), and in the $CD11b^-/CD41^-$ population. Representative dot plots of three independent experiments. (F) Cells were pretreated for 1 hour with pharmacological inhibitors of PI3K/AKT (Ly294002) or NF-κB (Bay117082) (25 μg/mL) and then cultured with TPO and TLR agonists. IL-6 levels were measured 24 hours later, not detectable (ND). $^*P < .05$ vs TPO alone, $^{\#}P < .05$ vs αIgG, $^{\&}P < .05$ vs without TPO, $^{\ddagger}P < .05$ vs vehicle, n = 4-7, ANOVA

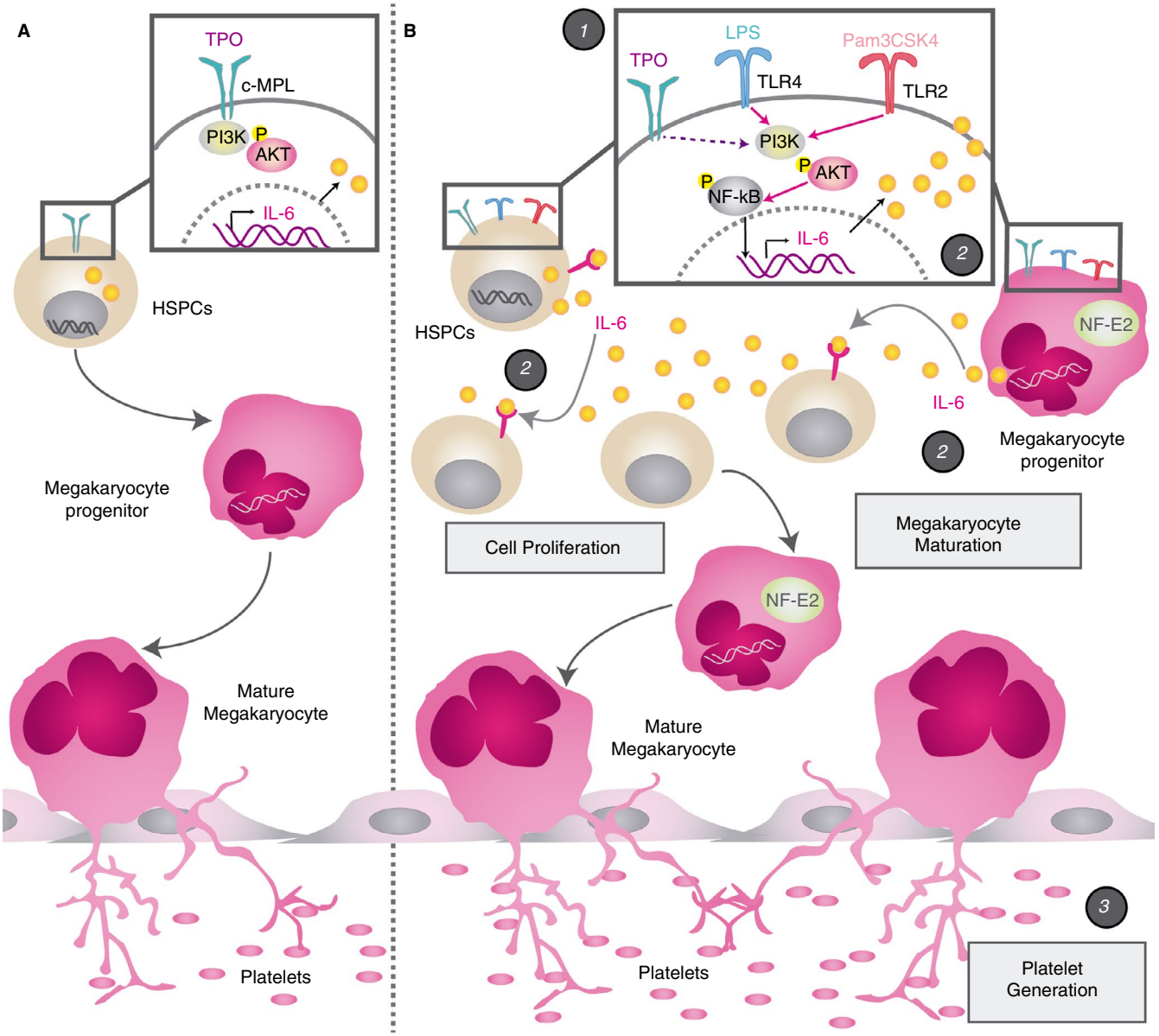


FIGURE 7 Model of TLR2 and TLR4 activation during megakaryopoiesis. (A) Under normal conditions, TPO binds to c-MPL receptor and trigger IL-6 expression, but not release, through PI3K/AKT signaling pathway resulting in megakaryocyte maturation and release of platelets. (B) The presence of LPS or Pam3CSK4 increases TPO-mediated megakaryo/thrombopoiesis by activation of TLR2 and TLR4 in hematopoietic stem and progenitor cells (HSPCs) and megakaryocytes (1). Both stimuli together enhance activation of PI3K/AKT and NF-κB that promote IL-6 production and release (2), creating an autocrine and paracrine loop that might contribute to warrant platelet provision during infection episodes (3)

Despite that TLR2/4 activation in megakaryocytes did not further modify their proliferation or maturation state of megakaryocytes or their ability to produce platelets we found that they induced IL-6 release that was potentiated by TPO. Of note, IL-6 is well known not only for its hematopoietic but also proinflammatory activities. In this sense, it is worth mentioning that increased expression of cellular fibronectin EDA isoform (EDA-FN) was recently observed during bone marrow fibrosis progression and that that EDA-FN-TLR4 axis induced megakaryopoiesis through the profibrotic IL-6 release.[36] Overall, these data and our current results indicate that activation of TLR4 not only by PAMPs but also by DAMPs promotes megakaryopoiesis through IL-6 synthesis and release in HSPCs and megakaryocytes. It is clear that, depending on the context and the persistence of the stimuli, it can be beneficial for the host or detrimental as in clinical conditions associated with thrombocytosis.

In conclusion, we demonstrated that activation of TLR2 and TLR4 in HSPCs and megakaryocytes reinforces TPO-induced megakaryo/thrombopoiesis through activation of PI3K/AKT and NF-κB signaling pathways and IL-6 release (Figure 7).

Knowledge of the functional consequences of TLR ligation on HSCs and megakaryocytes may be therapeutically exploited and applied to the treatment of platelet abnormalities in the setting of infection and other inflammatory conditions as well as in the context of cord blood-derived HSCs expansion for allogeneic hematopoietic stem cell transplantation.

AUTHOR CONTRIBUTIONS

L.P. D'Atri performed and designed experiments, interpreted results, and wrote the manuscript; C.S. Rodriguez and C.P. Miguel performed experiments; J.M. Ortiz Wilczyñski performed RT-PCR studies; R.G. Pozner performed immunofluorescence assays; E.A. Carrera-Silva performed qPCR studies and interpreted results; S. Negrotto and P.G. Heller contributed to the design and analysis of the study and M. Schattner designed the study, interpreted results, formulated discussion, wrote the manuscript, and assisted with manuscript preparation and editing. All the authors contributed to the analysis and/or interpretation of data and to the revision of the intellectual content of the manuscript. All the authors gave their approval to the final version of the manuscript.

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DISCLOSURE OF CONFLICT OF INTERESTS

The authors state that they have no conflicts of interest.

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