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Accessibility
IL-1α induces thrombopoiesis through megakaryocyte rupture in response to acute platelet needs

Satoshi Nishimura,1,2,5,6 Mika Nagasaki,1,3 Shinji Kunishima,7 Akira Sawaguchi,8 Asuka Sakata,5 Hiroyasu Sakaguchi,9 Tsukasa Ohmori,5 Ichiro Manabe,1 Joseph E. Italiano Jr.,10 Tomiko Ryu,11 Naoya Takayama,12 Issei Komuro,1,2 Takashi Kadowaki,2,4 Koji Eto,12 and Ryozo Nagai5

1Department of Cardiovascular Medicine, 2Translational Systems Biology and Medicine Initiative, 3Computational Diagnostic Radiology and Preventive Medicine, 4Department of Diabetes and Metabolic Diseases, The University of Tokyo, Tokyo 113-8654, Japan
5Center for Molecular Medicine, Jichi Medical University, Tochigi 329-0498, Japan
6Japan Science and Technology Agency (JST), Precursory Research for Embryonic Science and Technology (PRESTO), Saitama 332-0012, Japan
7Department of Advanced Diagnosis, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya 460-0011, Japan
8Department of Anatomy, Ultrastructural Cell Biology, Faculty of Medicine, University of Miyazaki, Miyazaki 889-1092, Japan
9Web Solution Group, IMAGICA Imageworks, Tokyo 141-0022, Japan
10Division of Hematology, Department of Medicine, Brigham and Women’s Hospital, Vascular Biology Program at Boston Children’s Hospital, Harvard Medical School, Boston, MA 02215
11Internal medicine, Social Insurance Central General Hospital, Tokyo 105-8330, Japan
12Center for iPS Cell Research and Application, Kyoto University, Kyoto 606-8507, Japan

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Correspondence to Satoshi Nishimura: snishiriky@umin.ac.jp; or Koji Eto: kojieto@cira.kyoto-u.ac.jp

Abbreviations used in this paper: FRAP, fluorescent recovery after photobleaching; HSC, hematopoietic stem cell; MK, megakaryocyte; PPF, proplatelet formation; ROI, region of interest; TPO, thrombopoietin.

Introduction

Circulating platelet counts and thrombopoietic processes in BM megakaryocytes (MKs) are both tightly regulated. In vitro, thrombopoiesis occurs via proplatelet formation (PPF) in the presence of thrombopoietin (TPO), which entails microtubule-dependent extension of elongated pseudopodal structures that exhibit platelet-sized swellings arranged in tandem and containing platelet organelles (Patel et al., 2005; Thon et al., 2010; Machlus et al., 2014). In vivo studies using two-photon microscopy also confirmed the presence of PPF in mouse BM (Junt et al., 2007; Zhang et al., 2012). However, the estimated platelet number released from each MK cannot explain rapid platelet turnover, especially when the need is acute, such as during inflammatory reactions. We therefore suspected that there is another rapid thrombopoietic mode, in addition to PPF.

Although TPO has been identified as the most important regulator of platelet production (de Sauvage et al., 1994; Kuter, 2007), it was recently reported that MK maturation and platelet biogenesis can occur independently of TPO (Ng et al., 2014).
Figure 1. 
Proplatelet formation is the dominant mode of thrombopoiesis, but there is an alternative megakaryocyte rupture mode, which produces much larger numbers of platelet-like particles. (A–G) Time-lapse images of thrombopoiesis in living BM from 6-wk-old CAG-eGFP (green) mice under steady-state conditions (A, B, F, and G) or after treatment with TPO (C; 10 µg for 5 d; A, Video 1; B, Video 2; C, Video 3; F, Videos 4 and 5; and G, Video 6). Injected fluorescent dextran (red) shows the blood flow, and Hoechst (blue) labeled the nucleus. Slice views (top in A, C, and G), voxel views (bottom in B and F), and surface views (bottom row in A, B, C, and G) show MK surfaces and particle release at the single-platelet (triangle) level. (D and E) Numbers of particles released from MKs with proplatelet formation and MK rupture thrombopoiesis, which were calculated from visuals by automatic software. n = 50 cells from 5 animals in each group. Note that MK rupture thrombopoiesis is rapid and associated with much greater numbers of released particles. (H) Automatic software analysis of thrombopoiesis mode. Calculated changes in MK perimeters and cytoplasmic GFP signal intensities are shown. The long arm projections (>50% of the length of the mean MK diameter) were identified as proplatelets and divided into short (<100 µm) and long (>100 µm) proplatelet formation. Increases in the perimeter (deformity) and decreases in GFP intensity (during rupture) were identified as the MK rupture pattern.
However, no suggestions as to the detailed mechanism by which platelets are generated from MKs in the absence of TPO were provided. In addition, recent studies indicate that hematopoietic stem cells (HSCs) and MKs are in very close proximity within the hematopoiesis hierarchy, and that MKs and platelets can emerge directly from HSCs under stress conditions, e.g., after BM suppression by irradiation (Sanjuan-Pla et al., 2013; Yamamoto et al., 2013; Nakamura-Ishizu et al., 2014). Thus, the actual pathways of platelet biogenesis are not clear, and elucidation of unidentified thrombopoietic mechanisms, particularly under stressful conditions such as inflammation or acute thrombocytopenia, require direct visualization of the BM. We therefore endeavored to improve the capability of the two-photon microscopy technique such that we would be able to visualize platelet biogenesis from MKs at the single-platelet level and to trace the translocation of platelets into the blood circulation of the BM in living mice. Ultimately, we identified an alternative pathway entailing MK rupture–enhanced platelet release, which responded to acute platelet needs under regulation by IL-1α. Our results shed light on what appears to be a novel mode of platelet release from BM MKs.

**Results**

Proplatelet type thrombopoiesis continuously regulates the platelet supply but provides limited numbers of platelets from mature MKs

To address the mechanism by which rapid platelet turnover is regulated, especially under stress conditions, we visualized megakaryopoiesis and dynamic thrombopoiesis in three dimensions (3D) using an improved intraval visualization technique and focusing on BM MKs. The combined technologies of multicolor high-sensitivity GaAs detectors, resonance mirror high-speed scanners, and a piezo-drive electronically controlled stage were applied to CAG-eGFP and CD41-tandem(Td)Tomato mice, enabling us to monitor the behavior of single platelets shed from BM MKs (Fig. 1, A–C; Fig. S1; and Videos 1–3). MKs identified based on their large size, multinucleation, CD41 positivity, and strong GFP signals in CAG-eGFP mice were mainly located in the border area between BM vessel lumens and the stroma.

With no intervention under steady-state conditions, filamentous (elongated) proplatelets released small platelet-like particles from the tips of the protrusions within vessels (Fig. 1, A and B). The entire time course of this process was usually longer than the observation periods (1 h), during which release was intermittent, and the number of released particles was only 1.4 ± 0.3 per minute from a single MK. TPO administration increased PPF with development of longer (>100 µm) arm projections, but the numbers of released platelets (2.6 ± 0.5 per minute) were still small (Fig. 1, C and D), which prompted us to search for alternative modes of thrombopoiesis.

MK rupture, the alternative mode of thrombopoiesis, can release large numbers of platelets over a short period

Rigorous 3D and high-speed examinations and surface visualization revealed that there is another, minor, but distinct, mode of thrombopoiesis, which we named “MK rupture” thrombopoiesis (Fig. 1, D–G). During the rupture phenomenon, MKs exhibited ruffling and then irregular changes in cell shape, and GFP+ platelet-like particles were released from the cytoplasm primarily into BM vessel lumens (Fig. 1, F and G; and Videos 4–6). Thereafter, MKs showed a marked loss of the GFP signal from the cytoplasm along with an increase in the dextran signal. When we profiled the visuals of the thrombopoiesis process in MKs using automatic, software-based algorithms, we found that short PPF was the dominant mode of platelet biogenesis in the steady state (Fig. 1, H and I), but that MK rupture thrombopoiesis also occurred with increased particle number per a single MK (Fig. 1 D). Moreover, the MK rupture thrombopoiesis was distinct from typical FasL-induced apoptosis, which had a much longer time course and was associated with blebbing, large protrusions, and gradual GFP loss (Fig. 1, J and K).

MK rupture increases in response to acute platelet needs

We also found that MK rupture–dependent platelet formation increased after intraperitoneal administration of anti-CD42b blocking antibody, which induces acute platelet loss, or thioglycolate, which induces innate inflammation (Fig. 2, A–C; and Video 7). This suggests that the MK rupture phenomenon mediates rapid platelet release in response to acute platelet demand.

IL-1α contributes to MK rupture thrombopoiesis in response to acute platelet need

To determine whether TPO is involved in MK rupture thrombopoiesis, or whether other intrinsic factors are involved, we next sought mediators in BM cell culture medium that positively influenced MK production in the presence of TPO. Using this screening assay, we identified seven candidate factors and determined that IL-1α increases platelet production from MKs to an even greater degree than TPO (Fig. 3, A and B). Moreover, serum IL-1α levels were acutely increased during platelet recovery after depletion using an anti-CD42b antibody, whereas increases in TPO levels were not seen until day 7 (Fig. 3 C). Increases in serum IL-1α levels induced in response to acute peritoneal inflammation evoked by thioglycolate injection were accompanied by transient increases in platelets (Fig. 3 D). In addition, neutralizing anti–IL-1 receptor (IL-1R) antibody...
IL-1α–dependent platelets were larger in size than those in TPO-treated mice (Fig. 4, J and K), and anti-CD42b antibody increased the large platelet populations in control mice but not in IL-1R1−/− mice (Fig. S2). IL-1α–dependent platelets also had shorter lifetimes, which was not affected by clodronate-mediated macrophage suppression (Fig. 4 L), confirming the intrinsic nature of the lifetime. The particles released in vivo stained positively with mitochondrial membrane potential dyes, indicating intact mitochondrial function (Fig. S3 A). Platelets isolated from IL-1α–treated mice showed less JONA binding, but overall platelet aggregation in vitro and thrombus formation in vivo were comparable to the TPO-mediated responses. Thus, IL-1α stimulation leads to production of larger platelets with minor impairment but intact thrombotic function (Fig. S3).

IL-1α–induced MK differentiation, maturation, and MK rupture–dependent platelet biogenesis with PPF inhibition in vitro

IL-1α–induced MK rupture thrombopoiesis was also observed in fetal liver MKs, which enabled us to examine its molecular mechanism and to clarify differences from FasL–induced apoptosis (Fig. 5, A–D). As mentioned, the particles released from IL-1α–treated MKs were larger in size than those induced by TPO (Fig. 5 E). IL-1α increased the ploidy and number of MKs (Fig. 5, F and G), as well as the release of platelet-sized CD41+CD42b+ particles (Fig. 5 H), indicating positive effects of IL-1α on MK differentiation, maturation, and platelet biogenesis, i.e., IL-1α increased MK rupture thrombopoiesis.
IL-1α stimulation induces caspase-3 activation without evident apoptosis

RT-PCR analysis of cultured fetal liver MKs showed that IL-1α increased expression of *Gata1*, *Bak1*, and *Bax*, which are known to be involved in thrombopoiesis and preapoptotic gene activation (Fig. 6 A; Kaushansky, 2003). Although there has been controversy regarding the caspase cascade in thrombopoiesis (De Botton et al., 2002; Clarke et al., 2003; Josefsson et al., 2014), IL-1α induced caspase-3 as well as p53 activation and AKT/ERK phosphorylation via IL-1R1 (Fig. 6, B–D; and Fig. S4). siRNA-mediated knockdown of IL-1R1 efficiently suppressed the pAKT–pERK signal (Fig. 6 C and Fig. S4), but *Thpo* and *Il1r1* expression in fetal liver MKs and TPO-R expression in isolated platelets were unaltered by IL-1α (Fig. 6, E and F). Immunofluorescence experiments also revealed the activation of caspase-3 and release of von Willebrand factor–positive granules, but TUNEL staining was negative, which makes this process different from typical FasL-induced apoptosis (Fig. 6, G–J). Caspase inhibition using Z-VAD (OMe)-FMK suppressed the effect of IL-1α on platelet counts in vivo (Fig. 6 K) and platelet-like particle releases in vitro (Fig. 5 H). Indeed, IL-1α–induced MK rupture thrombopoiesis was blocked in *Casp-3*−/− mice but not *Thpo*−/− mice (Fig. 6 L). These results indicate the rupture machinery is associated with caspase-3 activation via IL-1α/IL-1R1 but is distinct from TPO-dependent signaling and typical apoptosis.

**IL-1α–treated MKs show impairment of proportional microtubule assembly**

We next asked how IL-1α–IL-1R1 signaling with preapoptotic gene activation changes the mode of platelet generation from PPF to rupture (Fig. 7). It was previously shown that PPF-mediated platelet generation is microtubule dependent (Patel et al., 2005; Thon and Italiano, 2010; Kunishima et al., 2014; Machlus et al., 2014). To determine whether IL-1α influences microtubule dependency, two microtubule inhibitors (colchicine or paclitaxel), were administered to mice or fetal liver MKs with or without subsequent IL-1α treatment, after
Figure 4. **IL-1α-induced MK rupture yields larger platelets.** (A–C) Time-lapse images of thrombopoiesis in living BM from 6-wk-old CAG-eGFP mice treated with IL-1α (10 µg/mouse s.c. daily for 5 d). [A, Video 8; B, Video 9; and C, Video 10.] (D) Quantification of MK dynamics and numbers and platelet counts in 6-wk-old CAG-eGFP mice treated with TPO (10 µg/mouse s.c. daily for 5 d [TPO10]), or with 70 µg/mouse daily for 3 d [TPO70]) or IL-1α (10 µg/mouse s.c. daily for 5 d). The BM was visualized and platelet counts were analyzed 7 d after the first administration. *n* = 50 high-power fields from 5 animals for each group. *, *P* < 0.05 versus vehicle treated mice. (E and F) Platelet counts in isolated blood (E) and the CD41 + CD42b + MK fraction among Lin− BM cells (F) treated with vehicle (CTRL), low-, or high-dose TPO, IL-1α, anti-IL-1α neutralizing antibody (IL-1Ab), anti-IL-1R neutralizing antibody (IL-1RAb), or isotype-matched control antibody (IgG1 for IL-1Ab, IgG2 for IL-1RAb). All antibodies were used at 100 µg/mouse administered i.p. daily for 3 d. *n* = 8 animals in each group. (G) Identification of newly produced MKs using MX-Cre-GFP mice. GFP-labeled cells were analyzed among Lin− CD41 + CD42b + BM cells 2 d after PIPC injection. The data shown are from a single representative experiment from among three repeats. (H) Quantification of thrombopoiesis under physiological conditions in 6-wk-old IL-1α+/+, IL-1α−/−, IL-1R1+/+, and IL-1R1−/− mice. (I) MK dynamics in chimeric mice. *n* = 50 high-power fields from...
which we examined whether the dynamics of platelet-like particle formation and release were suppressed by either drug in the presence of IL-1α (Fig. 7, A and B). Examination of tubulin immunofluorescence in fetal liver–derived MKs showed that IL-1α promoted formation of small vesicles in the periphery of MKs that all stained positive for β1-tubulin, but some were negative for α-tubulin (Fig. 7, C and D). However, TPO accelerated PPF, with the well-coordinated distribution of α- and β1-tubulin within MKs. TPO also similarly increased expression of both Tuba1c and Tubb1. In contrast, IL-1α induced a 10-fold increase in Tubb1 expression, and a 50-fold increase when combined with TPO, while having no effect on Tuba1c, or Tuba4a expression (Fig. 7 E and not depicted). We found the formation of a demarcation membrane system and development of platelet territories in IL-1α–treated MKs before membrane rupture (Fig. 7 F and Fig. S5). Platelets from Thpo−/− mice treated with IL-1α showed an absence of proper tubulin distribution, suggesting β1-tubulin oversupply, and those from IL-1α–treated WT mice are mostly spheroid shape rather than elongated shape by WT or TPO-treated platelets (Fig. 7, G–I; and Fig. S5). We concluded that IL-1α–induced disorganization of microtubule assembly accounts for the dysregulated α- and β1-tubulin synthesis, which should contribute to the PPF inhibition and MK rupture as a result.

Figure 5. IL-1α–induced MK differentiation and rupture-dependent platelet biogenesis in vitro. (A–C) Time-lapse images of cultured MKs. Liver cells were collected from fetal CAG-eGFP mice on embryonic day 13 and cultured with TPO. On day 7 of culture, the cells were washed and incubated with anti-CD41 and Hoechst 33342. MKs were identified in the cultures as multinucleate and staining positive for CD41. MKs were then treated with TPO, IL-1α, or TPO plus Fas ligand 1 h before the experiments. Proplatelet production was observed in the presence of TPO, whereas MK rupture was seen in the presence of IL-1α. (D and E) Quantification of MK dynamics (D) and released particle size (E). n = 20 low-power fields (D) and n = 20 particles (E). Some cells were cultured with Z-VAD (OMe)-FMK (100 µM) 1 d before the experiments. (F) Fetal liver cells were isolated and cultured with TPO for 6 d. Differentiated MKs were washed and then incubated for 1 d with TPO, IL-1α, and anti-IL-1α neutralizing antibody. Ploidy was analyzed in CD41+CD42b+Lin− MKs. The data shown are from a single representative experiment from among three repeats. (G and H) BM cells isolated from 6-wk-old WT mice were cultured, and CD41+CD42b+Lin− MKs were counted (G) in each well after 7 d. The percent value was normalized to that of a control well. n = 5 experiments. (H) Release of CD41+CD42b− particles into the culture medium. n = 5 experiments. *, P < 0.05.
Figure 6. IL-1α–induced atypical apoptosis with caspase-3 activation in fetal liver MKs. (A and B) Liver cells were obtained from fetal WT mice on embryonic day 13, cultured with TPO or IL-1α, and analyzed on day 7. (A) RT-PCR analysis of gene expression in the harvested cells. The values were normalized to a vehicle-treated control. n = 5 experiments. (B) Flow cytometric analysis of caspase-3 activation in Lin− CD41+CD42b+ MKs. Fetal liver cells were cultured with TPO. siRNA-mediated knockdown was performed on day 4. On day 7, cells were washed, incubated with TPO, IL-1α, or IL-1β for an additional 1 d, and pAKT-pERK signaling in Lin− CD41+CD42b+ MKs was analyzed on day 8. The data shown are from a single representative experiment from among three repeats (B and C). (D) Western blotting of p53 and phospho-p53 in fetal liver cells cultured with TPO and IL-1α from day 0 to 7. MKs were enriched with discontinuous albumin density gradient centrifugation. (E) Fetal liver cells were differentiated using TPO for 7 d, and then stimulated with vehicle, TPO, IL-1α, or IL-1β for an additional 1 d, after which gene expression was analyzed by RT-PCR. (F) Flow cytometric analysis of T. orange+ platelets isolated from WT mice treated with TPO or IL-1α. NC denotes a negative control. The data shown are from a single representative experiment from among five repeats. (G-J) Immunofluorescence analysis of fetal liver MKs, which were cultured and differentiated from day 0 to 7 with TPO or IL-1α. Some cells were treated with Fas-ligand from day 6 to 7 after differentiation with TPO (FasL). Note that IL-1α–treated MKs were caspase-3–positive with release of von Willebrand factor–positive granules, but TUNEL staining was negative, which was different than typical FasL-induced apoptosis with blebbing. (K) Blood cell counts in WT mice treated with TPO, IL-1α, and/or Z-VAD (OMe)-FMK. (L) MK dynamics and platelet counts in WT, Casp3−/−, and Thpo−/− mice treated with vehicle or IL-1α. n = 3–8 mice. Bars, (red) 20 µm. * P < 0.05 versus CTRL group.
Figure 7. **IL-1α inhibited regulated tubulin assembly and proplatelet formation.** (A) Quantification of MK numbers and dynamics and platelet counts in 6-wk-old CAG-eGFP mice treated with IL-1α (10 µg for 5 d), colchicine (5 mg/kg i.v. once 6 h before experiments), and/or paclitaxel (10 mg/kg i.v. once 6 h before experiments). The BM was visualized and platelet counts were analyzed 7 d after the first administration. n = 24 high-power fields from 8 animals in each group. *, P < 0.05 versus control (WT) (B–D) Immunofluorescence analysis of fetal liver MKs cultured and differentiated from day 0 to 7 with TPO or IL-1α. In addition, the cells were treated with colchicine (2.5 µM) or paclitaxel (2.5 µM) from day 6 to day 7 (B). Some cells were also treated with Fas-ligand with TPO from day 6 to 7 (FasL). On day 7, the cells were fixed and stained (C and D). (E) RT-PCR analysis of gene expression in harvested cells. The values were normalized to vehicle-treated control. n = 8 experiments. (F) Electron microscopy of isolated BM MKs from TPO or IL-1α mice. Note that demarcation membrane system was similarly developed in two mice (1 and 2), before rupture, and fragments indicated several platelets (3 and 4). Bars, 2 µm. (G) Immunofluorescence study of tubulin distribution in platelets from WT, Thpo−/− mice, or Thpo−/− mice treated with IL-1α. (H) Electron microscopy of isolated platelets from WT mice treated with vehicle (WT), TPO, or IL-1α. (I) The short and long axis length was measured in randomly selected individual platelets, and ratio (short/long) was evaluated in 40 cells for each groups. Bars, 2 µm. *, P < 0.05.
et al., 2007; Takayama et al., 2010). However, although recent in vivo imaging-based analyses confirmed the presence of PPF (Junt et al., 2007; Zhang et al., 2012), we found that the number of platelets produced from each MK was both consistent with previously reported numbers (Thon et al., 2012; Avanzi and Mitchell, 2014) and too low for an adequate response to acute platelet needs or inflammatory stimuli.

Herein we demonstrated an alternative MK rupture–type thrombopoietic process, which can provide 20 times more platelets than PPF, released primarily into BM vessels within 1 h (Fig. 1 D), and which we visualized for the first time using high-speed two-photon microscopy with multicolor high-sensitive GaAs detectors (Fig. 1). The novel MK rupture–dependent pathway is primarily regulated by the inflammatory cytokine IL-1α, and is morphologically and mechanically distinct from PPF and typical FasL-induced apoptosis, as well as from the recently reported cytoplasmic elongation of large platelet progenitor structures (Thon and Italiano, 2010; Kowata et al., 2014), as summarized in Table S1 and Fig. 9.

Ng et al. (2014) recently reported that MK maturation and final platelet shedding can both be independent of TPO signaling, but they did not address alternatives to TPO. In this study, we found that IL-1α–IL1R1 signaling positively influences the MK differentiation phase, as well as platelet shedding through rupture, which compensated for the deficiency of TPO signaling in MKs (Ng et al., 2014). In addition, a shear-dependent

**IL-1α induces mechanically and functionally weaker plasma membrane structure**

The multiple functional changes seen in IL-1α–treated MKs before membrane rupture during thrombopoiesis were also examined. Using atomic force microscopy to evaluate MKs’ ability to push a bead-attached cantilever or exert contractile force to pull up a cantilever, it was found that IL-1α–treated MKs were less stiff and thus exerted less force (Fig. 8, A and B). Fluorescent recovery after photobleaching (FRAP) analysis further confirmed that membrane stability was reduced in IL-1α–treated mature polyploid MKs, but not in mononuclear CD41 cells (immature MKs), and that it was restored by caspase inhibition (Z-VAD [OMe]-FMK; Fig. 8, C and D; Ahn et al., 2002).

These results strongly indicate that IL-1α reduces plasma membrane potential, which leads to a mechanically and functionally weaker membrane structure and drives MKs toward MK rupture thrombopoiesis.

**Discussion**

**Novel MK rupture thrombopoiesis**

In vitro platelet biogenesis from fetal liver MKs, human CD34 cells, embryonic stem cells, or induced pluripotent stem cells all consistently show the presence of PPF and release of fragments from the tips of elongated pseudopodal structures under steady-state and TPO-stimulated conditions (Italiano et al., 1999; Eto et al., 2007; Takayama et al., 2010). However, although recent in vivo imaging-based analyses confirmed the presence of PPF (Junt et al., 2007; Zhang et al., 2012), we found that the number of platelets produced from each MK was both consistent with previously reported numbers (Thon et al., 2012; Avanzi and Mitchell, 2014) and too low for an adequate response to acute platelet needs or inflammatory stimuli.

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Megakaryocyte rupture by IL-1α • Nishmura et al. 463

Mechanism may also promote TPO-independent platelet generation (Jiang et al., 2014).

**MKs respond to acute platelet demand via IL-1α**

Under acute inflammatory or cytopenic conditions, rapid elevation of IL-1α promoted a change in cell programing to MK rupture thrombopoiesis (Fig. 2 and 3). Reductions in platelet counts during acute inflammation induced by neutralizing anti-IL-1α antibody indicate that an urgent requirement for platelet recruitment or recovery as a host defense may be associated with inflammation-mediated elevation of IL-1α levels, as reported previously (Rider et al., 2011). However, serum levels of multiple inflammatory cytokines, including IL-1β, IL-2, IL-6, and IL-11, were also elevated and may contribute to the increase in platelet production (Fig. 3). Although IL-1β, produced from platelets during acute inflammation, reportedly stimulates MK polyploidization (Yang et al., 2000; Denis et al., 2005; Beaulieu et al., 2014), the levels of IL-1β were not associated with elevation of platelets after administration of anti-CD42b antibody (Fig. 3 C). This result is consistent with the fact that the phenotypes of IL-1α and IL-1β knockout mice differ from one another (Nambu et al., 2006).

IL-1α–treated MKs showed clear changes to their cell membrane before MK rupture (Fig. 8). The plasma membrane was functionally and mechanically less stable in IL-1α–treated MKs. Instability of membrane structure after IL-1α treatment may also be associated with platelet shape in generation. Platelets by IL-1α appeared to display more spheroid but PPF-dependent platelets were longitudinal (Fig. 7, G–I; and Fig. S5), as demonstrated previously (Thon and Italiano, 2010). Interestingly, this membrane instability was dependent on caspase activity in only mature MKs (Fig. 8 D). That the action of IL-1α in MK rupture thrombopoiesis was caspase-3 dependent (Fig. 6, K and L) suggests caspase activation may occur downstream of the IL-1α–ILR1 axis in association with alteration of the cell membrane structure. The caspase-dependent thrombopoiesis theory has been discussed for a long time (De Botton et al., 2002; Clarke et al., 2003; Josefsson et al., 2014). Our data provide evidence of caspase dependency in platelet biogenesis; however, the caspase involvement is independent of TPO-mediated PPF, as recently demonstrated (Josefsson et al., 2014). But the detailed mechanism of caspase activation or p53 up-regulation in MK rupture thrombopoiesis must be elucidated further.

In conclusion, our findings provide direct evidence that IL-1α is an acute platelet releasing factor that induces enhanced platelet release through rupture of the mature MK membrane. This novel mechanism may enable rapid restoration in platelet numbers, but with insufficient microtubule organization. It appears the balance between TPO and IL-1α determines the cellular programming of MKs for thrombopoiesis in response to acute and chronic platelet needs.
Sau3AI and KpnI sites in exon 5 and intron 5, respectively, was replaced by a floxed neo cassette were a gift from Riken (Matsumura et al., 2004). A null mutation in which a 2.4-kb EcoR1–Pst1 fragment encompassing two exons was replaced with a PGK-neo cassette. B6.Cg-Tg(CAG-floxed Neo-EGFP)REP08Osb mice carrying the transgene for the CAG promoter and EGFP cDNA separated by a floxed neo cassette were a gift from Riken (Matsumura et al., 2004). IL-1x+/− mice were provided by Y. Iwakura (Tokyo University of Science, Tokyo, Japan; Horai et al., 1998). A null mutation in IL-1α was generated using a replacing vector in which a 1.5-kb DNA fragment between the Sau3AI and KpnI sites in exon 5 and intron 5, respectively, was replaced with a PGK-neo cassette. B6.129S1-Casp3tm1(J) (Casp3−/−) mice were obtained from Japan Charles River Laboratories. A targeting vector was designed to replace the caspase protease-conserved catalytic site of the endogenous gene with a PGK-neo cassette. Thy1KO mice were generated using a targeting vector to remove exon 3 by inserting a neo cassette, and were provided by B. Heissig (The University of Tokyo, Tokyo, Japan; de Sauvage et al., 1996).

Animal models

To assess the contributions made by humoral factors, 5- to 6-week-old CAG-eGFP mice were treated with TPO (Sigma-Aldrich) or IL-1r1−/− (R&D Systems or BioLegend) at dosages of 10 µg/mouse subcutaneously (SC) daily for 5 or 70 µg/mouse SC daily for 3 d. The mice were treated and observed at 6-week-old, 7 d after the first treatment. Some mice were also treated with neutralizing antibodies against IL-1α (R&D Systems or Biolegend), IL-1R (Biologend), isotype-matched control antibody (BioLegend), or Fas Ligand (5 µg/mouse i.v.; R&D Systems). All antibodies were administered at 100 µg/mouse i.p. daily for 3 d.

To assess the effect of acute inflammation, thioglycolate (Sigma-Aldrich) was intraperitoneally administered i.p. (3 ml of 3% solution/mouse once). To examine the effect of caspase inhibition, mice were treated with the pan-caspase inhibitor Z-VAD (OMe)-FMK (3 mg/kg IP daily for 5 d; Merck Millipore). To assess the effect of acute inflammation, mice were treated with RPE-conjugated rat anti-CD41 (MWReg30) and Hoechst 33342. Differentiated MKs, identified as multinucleate and staining positively for CD41, were visualized at 37°C in DMEM using a confocal microscope (Nikon A1R) with a 100× (N.A. 1.25) oil immersion objective lens (Nikon).

Immunofluorescence study

For immunofluorescence analyses, the cells were fixed in 4% paraformaldehyde for 45 min and permeabilized with 1% Triton X-100 (EMD Millipore) for 10 min. Thereafter, the specimens were blocked and incubated first for 12 h with a primary antibody (rabbit anti-β-tubulin [Patel-Hett et al., 2008], rat anti-CD41 [MWReg30; Biolegend], rabbit anti-μ chain [Emfler], or rabbit anti-active caspase-3 [Millipore]). They were then incubated for 1 h with an RPE-conjugated rat anti-CD41 (MWReg30), Alexa Fluor-conjugated mouse anti-α-tubulin (Invitrogen) and/or goat secondary antibody (Invitrogen). Finally, the cells were counterstained for 1 h with Hoechst 33342, and images were captured using a confocal microscope (Nikon A1R). The cells were excited using three laser lines (405 nm, 488 nm, and 561 nm), and the emission was collected using appropriate narrow band-pass filters and GaAs detectors.

Electron microscopy

For electron microscopy, MKs were fixed in a mixture of 0.5% glutaraldehyde and 2% paraformaldehyde, washed, and post-fixed with 1% osmium tetroxide. After dehydration, the collected pellets were embedded in Epon. Ultrathin sections (60–90 nm thick) were then stained with 2% uranyl acetate and Reynolds’ lead citrate. Samples were observed in a transmission electron microscope (HT7700; Hitachi) operating at 80 kV.

Preparation of cells and flow cytometry

As previously described (Nishimura et al., 2009), we isolated BM cells from the scalp and femurs by flushing them with PBS, and collected blood samples from tail veins or by cardiac puncture under anesthesia. The cells were washed twice with PBS, incubated for 8.5 min in erythrocyte lysing buffer, and finally suspended in PBS supplemented with 3% fetal bovine serum. The isolated cells were then incubated with FcBlock antibody (BD) for 15 min on ice, labeled with dye-conjugated antibodies, and analyzed by flow cytometry using a Canto II flow cytometer (BD), spectrum analyzer SP6800 (Sony), and FlowJo 7.2.2 software (Tomy Digital Biology). Propidium iodide (Invitrogen) or a Zombie NIR kit (Biolegend) was used to exclude dead cells. Conventional approaches, including Western blotting, using an automatic algorithm in NIS-Elements (Nikon) and custom designed software (IGAMICA Imageworks), Slice, voxel, and maximum intensity projection views are provided in the figures and videos. Also calculated by the software were the particle release, time-dependent changes in MK perimeters, and the cytoplasmic GFP signal. After measuring the proplatelet long axis, a >50% increase in perimeter (defornity) and a decrease in the cytoplasmic GFP signal intensity (rupture) were identified as the rupture pattern. Alternatively, MKs showing long arm projections (>50% of the length of the average cell diameter) were deemed to contain proplatelets, and we categorized long (>100 µm) and short (<100 µm) proplatelet formation based on the arm length. The total numbers of rupture-type and proplatelet-type MKs detected during the observation periods were counted.

Fetal liver cell cultures

Liver cells were collected from fetal WT mice on embryonic day (E) 13. Single-cell suspensions, prepared through successive passaging, were cultured in DMEM supplemented with 10% FCS without (CTRL) or with TPO (50 ng/ml), SCF (50 ng/ml), Fas ligand (1 µg/ml), and/or IL-1α (50 ng/ml). On day 7 of culture, the cells were washed and analyzed. MKs were enriched by discontinuous albumin density gradient centrifugation before analysis. Caspase activation was evaluated using NuView Caspase-3 (Biotium; Bosch and Franklin-Tong, 2007). The mechanical properties of cultured fetal liver MKs were evaluated using atomic force microscopy (NanoWizard JPK Instruments AG) with bead-attached or sharp-end cantilevers.
cannot be applied to assess intracellular signals in MKs isolated from mice due to the small sample size. We therefore used flow cytometry for signal analysis.

Flow cytometry–based platelet aggregation assays were also performed using previously reported methods with some modification (De Cuyper et al., 2013). Platelet-rich plasma and whole blood were obtained and incubated with RPE-conjugated anti-CD41 (MWReg30; Biolegend) or FITC-conjugated anti-CD41 [Luc.H11] EμFre2 antibody, after which the two populations of labeled cells were mixed, 1:1, and incubated at 37°C. The cells were then activated with thrombin (Sigma-Aldrich; 5 U/ml), fixed in 0.5% formaldehyde at the indicated times thereafter, and analyzed using a Canto II flow cytometer (BD). The appearance of two-color events was considered to reflect aggregated platelets.

Western blotting
Western blotting was conducted with fetal liver cells enriched through discontinuous albumin density gradient centrifugation using rabbit anti-p53 (Santa Cruz Biotechnology, Inc.), or rabbit anti–phospho-p53 (Ser15; Cell Signaling Technology) antibodies.

Real-time quantitative PCR
For real-time PCR, total RNA was isolated using TRIzol (Invitrogen) and relative mRNA levels were determined using a Superarray kit (Superarray).

Statistical analysis
Statistical analysis was performed using JMP (SAS) and Excel 2013 (Microsoft) software. Results were expressed as means ± SEM. The statistical significance of differences between two groups was evaluated using Student’s t test. Differences between more than two groups were evaluated using ANOVA followed by post hoc Bonferroni tests. Correlations were evaluated using the Pearson correlation coefficient test. Values of P < 0.05 were considered significant.

Online supplemental material
Fig. S1 shows intravital visualization and validation study of BM MKs. Fig. S2 shows MK phenotype of IL-1α−/− mice, and serum TPO levels after IL-1α treatment. Fig. S3 shows thrombus formation function of IL-1α−/− induced platelets. Fig. S4 shows intracellular signal analysis of IL-1α−/−–induced platelets. Fig. S5 shows electron microscopic analysis of TPO- and IL-1α–induced platelets. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201410052/DC1.

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References


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Megakaryocyte rupture by IL-1α • Nishimura et al. 465


