Neutrophil “plucking” on megakaryocytes drives platelet production and boosts cardiovascular disease

Highlights
- Plucking of neutrophils on megakaryocyte extensions drives platelet production
- Neutrophils induce mechanosignaling in megakaryocytes via CXCR4 and NOX2
- Neutrophil plucking boosts thrombus burden in thromboinflammatory diseases

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In brief
The importance of megakaryocyte and neutrophil interactions within the bone marrow is insufficiently understood. Petzold and colleagues define neutrophil “plucking”-induced mechanosignaling in megakaryocytes as a regulator of platelet production that boosts cardiovascular diseases.
Neutrophil “plucking” on megakaryocytes drives platelet production and boosts cardiovascular disease

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SUMMARY

Intravascular neutrophils and platelets collaborate in maintaining host integrity, but their interaction can also trigger thrombotic complications. We report here that cooperation between neutrophil and platelet lineages extends to the earliest stages of platelet formation by megakaryocytes in the bone marrow. Using intravital microscopy, we show that neutrophils “plucked” intravascular megakaryocyte extensions, termed proplatelets, to control platelet production. Following CXCR4-CXCL12-dependent migration towards perisinusoidal megakaryocytes, plucking neutrophils actively pulled on proplatelets and triggered myosin light chain and extracellular-signal-regulated kinase activation through reactive oxygen species. By these mechanisms, neutrophils accelerate proplatelet growth and facilitate continuous release of platelets in steady state. Following myocardial infarction, plucking neutrophils drove excessive release of young, reticulated platelets and boosted the risk of recurrent ischemia. Ablation of neutrophil plucking normalized thrombopoiesis and reduced recurrent thrombosis after myocardial infarction and thrombus burden in venous thrombosis. We establish neutrophil plucking as a target to reduce thromboischemic events.
**INTRODUCTION**

Platelets and neutrophils cooperate as motile surveillants to maintain host integrity and to protect against sterile and infectious injury. In pathological conditions, however, this liaison of platelets and neutrophils may be harmful, driving thrombo-inflammatory processes that can lead to lethal thrombotic events, such as myocardial infarction (MI), venous thromboembolism (VTE), or stroke (Stark and Massberg, 2021).

Platelets are produced by megakaryocytes (MK) in the bone marrow (BM), which is also where neutrophils arise from myeloid precursors (Stegner et al., 2017). Inside the BM, platelet-releasing MKs reside in the perisinusoidal space, a communication hub that also provides exit and entry points for neutrophils traveling into the blood or returning from the blood to the BM compartment (Itkin et al., 2016). Direct stochastic cellular interactions between MKs and neutrophils in the BM are therefore predicted (Boisset et al., 2018) and were recently described in the rare setting of emperipolesis (Cunin et al., 2019). Yet, we know little about the biological relevance of interactions between MKs and neutrophils in the BM. In particular, it is unclear whether and, if so, how these interactions participate in adverse thromboinflammatory responses that eventually drive thrombotic complications.

Here, we show that circulating neutrophils homing back to the BM “pluck” on perisinusoidal MK extensions, the proplatelets (PPLs). PPLs are thin MK protrusions formed into BM vessels where they are cleaved off into smaller pre-platelets that will finally segregate into young reticulated (i.e., RNA-rich) prothrombogenic platelets (Machlus and Italiano, 2013). We demonstrate that neutrophil plucking accelerates PPL growth and shedding by direct force transmission and by reactive oxygen species (ROS)-transduced mechanosignaling in PPLs. This process depends on CXCR4-directed migration of neutrophils to BM MKs. Neutrophil plucking tunes the release of platelets in the steady state but exacerbates the production of prothrombotic immature platelets following MI, ultimately aggravating post-MI cardiovascular complications.

**RESULTS**

**Neutrophils interact with MKs inside the BM**

To characterize interactions between MKs and polymorphonuclear neutrophils inside the BM, we generated *Pf4-cre*+/+/*confetti/Ly22-eGFP dual reporter mice and visualized MKs (and their progeny) and neutrophils using multi-photon microscopy of the calvarian bone (Figure 1A). Neutrophils and MKs engaged in close and long-lasting physical contacts in the BM. On average, each MK was in immediate contact with four different neutrophils at any given time. Over 1 h, each MK accumulated a total of eight interactions with an average duration of approximately 13 min (Figures S1A and S1B).

When we stratified MKs by their morphology using the sphericity index (SI) to characterize MK maturation (Figure 1B), we found that mature MKs with an irregular shape (SI < 0.5) were less abundant but more likely to form PPLs compared to highly spherical (SI > 0.8) MKs (Figure S1C). Irregular-shaped, multilobulated MKs (SI < 0.5) established interactions with neutrophils more frequently, which were also longer last-
Figure 1. Neutrophil plucking on PPL-forming MKs

(A) Two-photon imaging of thrombopoiesis inside BM in dual reporter mice (Pf4-cre+/Confetti−/Lyz2-eGFP). Representative images from 4 independent experiments of MK (multicolor here shown in red) – neutrophil (green) interactions within the BM and a 3D image reconstruction of the magnified area are shown. Scale bars represent 40 μm or 10 μm (magnified area), respectively.

(B) MK characterization by sphericity index. Representative images from 4 independent experiments of low and high sphericity MKs are shown (bar represents 20 μm). Frequency distributions of MKs’ sphericity indices within the BM are shown (n = 4 animals per group, 29 MKs were observed and analyzed over the time frame of 1 h).

(C and D) In vivo video analyses quantifying MK-neutrophil interactions and interaction times by sphericity index. (C) Frequency distribution of MK-neutrophil (i.e. individual cells) interactions and (D) interaction times over 1 h are shown (n = 4 animals per group, 7 videos per group).

(E) Scheme of MK-neutrophil interactions during thrombopoiesis.

(F) Analysis of neutrophil interaction times at the PPL/budding region or Non-PPL/budding region of MKs, symbols indicate individual neutrophils (analysis from 4 animals).

(G) Image series and 4D reconstruction of neutrophil plucking on PPL-forming MK around the PPL budding site is shown. White arrows indicate plucking neutrophil. Scale bars represent 40 μm (overview image) or 5 μm (magnified area), respectively, timeline is indicated.

(H) Brightfield microscopy of MK-neutrophil co-culture is shown. Scale bar represents 20 μm.

(I) Neutrophil plucking during PPL elongation is visualized by time lapse imaging. Arrows indicate plucking neutrophil. Scale bar represents 5 μm. Bars represent mean ± SEM; symbols indicate individual animals; p values are indicated, *<0.05, **<0.01, ***<0.001, ****<0.0001, n.s. not significant. P values were determined using unpaired Student’s t test (F), one-way (C, D) ANOVA multigroup test. Please see also Figure S1.
Figure 2. Neutrophil plucking accelerates PPL growth and release during thrombopoiesis
(A–E) Analysis of MK-neutrophil interaction in Gr-1-treated neutropenic and control-antibody-treated dual reporter mice (P4-cre(+) /ConfettiLyz2-eGFP) by two-photon microscopy.
(A) Scheme of imaging setup in neutropenic mice.
(B) Localization of the interstitial, perisinusoidal, and intravascular BM compartment to further characterize MK-neutrophil interactions.
(C) Image sequence showing PPL formation under neutropenic conditions. White arrows indicate PPL-plucking neutrophil around the budding site. Asterisks indicate PPLs, dashed lines show vasculature, yellow box indicates magnified area. Scale bar represents 5 μm (magnified area) and 20 μm, timeline (min) is indicated.
(D) Quantification of interaction frequencies within BM compartments (control: n = 4 animals, ND: n = 3 animals, symbols indicate single MKs).

(legend continued on next page)
protected from neutropenia and showed normal platelet counts upon antibody treatment (Figures S2E and S2F). Pan-neutropic Mrp8-cre+/-/iDTR mice showed a similar decrease in platelet (i.e. platelet count: −25.7% vs. control) and reticulated platelet counts (i.e. reticulated platelet count: −20.8% vs. control) (Figure 3B). Similar to Gr-1 depletion, this was due to reduced platelet production, while platelet lifespan, MK numbers, size, and maturity (i.e. ploidy) were unaffected (Figures S2G−S2I). When we infused α-GPIb antibody to induce immune thrombocytopenia, subsequent platelet recovery was slower and incomplete in pan-neutropic mice compared to controls (Figure S2J).

To additionally determine whether alterations of the BM interstitial cytokine milieu account for the differences in platelet production in mice lacking circulating neutrophils, we quantified cytokine amounts following Gr-1 treatment. Yet, concentrations of known regulators of thrombopoiesis including thrombopoietin and IL-1α (Noetzli et al., 2019) were not altered in neutropenic compared to control mice (Figure S2K). Changes in interstitial cytokine composition are therefore unlikely to contribute to the regulation of platelet production by intravascular neutrophils.

**CXCR4-driven tropism orchestrates thrombopoiesis**

To resolve the molecular mechanism of platelet production by neutrophil plucking, we used in vitro co-culture. For quantification of in vitro thrombopoiesis we measured CD61+CD42d− platelet particles (PP) released from PPLs in the culture supernatants (Figure S2L). The presence of neutrophils, but not of CD3+ T cells, CD19+ B cells, or monocytes, boosted PP production to a similar extent as sphingosine-1 phosphate (Figure 3C), a known inducer of thrombopoiesis (Zhang et al., 2012). Analysis of CD3-deficient mice lacking T cells (Sommers et al., 2000), B-cell-depleted Cd19-cre iDTR mice (Demircik et al., 2019), Rag1−/− mice lacking B and T cells (Mombaerts et al., 1992), and monocyte-depleted mice following CCR2 depletion antibody (Mack et al., 2001) treatment for 5 days did not show altered platelet or reticulated platelet counts (Table 1). Together these data show that immune cell control of thrombopoiesis is cell type specific and restricted to neutrophils.

Neutrophils were able to trigger in vitro thrombopoiesis only when we allowed direct physical interaction with MKs, but not when we separated both cell types in a trans-well chamber (Figure 3C). Consistently, supernatants from phorbol 12-myristate 13-acetate (PMA) activated neutrophils did not affect PP release (Figure 3C). Time-lapse imaging suggested that plucking neutrophils applied force to PPLs to promote PP release. To test this, we used small molecule inhibitors to interfere with neutrophil cytoskeleton dynamics and myosin dependent force generation. Pretreatment of neutrophils with cytochalasin D and blebbistatin abolished PP release, indicating that neutrophil plucking requires neutrophil mechanotransduction (Figure 3D).

Next, we performed a receptor screening approach including a set of neutrophil receptors known to mediate platelet interactions. We found that CXCR4-deficient neutrophils isolated from Mrp8-cre+/+/Cxcr4−/− mice (Adrover et al., 2019; Casanova-Acebes et al., 2013) failed to increase PP yield compared to wild-type neutrophils (Figure 3E). Neutrophil-driven thrombopoiesis was also abolished following CXCR4 inhibition by AMD3100. In addition, we found an effect for beta1-integrins and integrin activator kindlin-III, required for neutrophil migration in vitro and neutrophil adhesion in vivo. In contrast, absence or inhibition of PSGL-1, β2 integrin, CD41, or CD40L did not affect neutrophil-induced thrombopoiesis (Figures 3E and S2M).

MKs express high amounts of the CXCR4 ligand stromal-cell-derived factor 1 (SDF-1/CXCL-12) on their surfaces (Massberg et al., 2006) (Figures S3A and S3B). We therefore focused on CXCR4 and analyzed whether ablation of Cxcr4 on neutrophils impacted their ability to pluck on PPL-forming MKs. First, we adoptively transferred CXCR4-deficient neutrophils isolated from Mrp8-cre+/-/Cxcr4−/−/GFP mice or Mrp8-cre−/-/Cxcr4−/−/GFP control neutrophils into C57BL/6 recipients (Figure 3F). Whole-mount confocal microscopy analysis showed that the majority of adoptively transferred wild-type neutrophils localized in close proximity to MKs (Figures 3F and 3G). In contrast, Cxcr4−/− neutrophils were less likely to enter the BM, as reported (Adrover et al., 2019), and were distributed randomly throughout the interstitial space without obvious preference for the MK microenvironment. Thus, to test whether defective homing of neutrophils into the perisinusoidal space translated into changes in thrombopoiesis in vivo, we analyzed Mrp8-cre+/-/Cxcr4−/− mice. BM imaging demonstrated that the pool of plucking neutrophils in direct proximity (<20 μm) to the PPL budding sites was drastically reduced in Mrp8-cre+/-/Cxcr4−/− mice, while neutrophil counts in peripheral blood were increased compared to control littermates (Figures 3H, 3I, S3C, and S3D). Reduced PPL plucking translated into reduced PPL growth speeds and prolonged release times without affecting PPL lengths (Figures 3J and S3E). In agreement with these findings, we observed reduced platelet and reticulated platelet counts in Mrp8-cre+/-/Cxcr4−/− compared to control mice (i.e. platelet

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(E) Analysis of PPL kinetics. Relative PPL length of individual PPLs (control: 3 PPLs; ND: n = 2 PPLs), PPL growth speed, and release time (control: n = 4 animals, ND: n = 3 animals) are shown.

(F−H) Neutrophil rescue by adoptive transfer in neutropenic Mrp8-cre+/-/iDTR mice.

(I) Treatment scheme is shown.

(G) Representative image series of 3 independent experiments and 3D reconstruction of PPL release in mice before and after neutrophil transfer, scale bars represent 15 μm. Analysis of PPL kinetics. Relative length of individual PPLs is shown before and after neutrophil transfer (before: n = 5 PPLs; after: n = 3 PPLs).

(I) Summarizing scheme of adoptive transfer experiments is shown.

(H) Quantification of PPL growth speed before and after neutrophil transfer (n = 3 animals per group). Bars represent mean ± SEM, symbols indicate individual animals or MKs; p values are indicated, *<0.05, **<0.01, ***<0.001, n.s. not significant. P values were determined using unpaired (E) or paired (H) Student’s t test and two-way (D) ANOVA multigroup test. Please see also Figures S1 and S2.
Figure 3. Neutrophil-expressed CXCR4 regulates neutrophil plucking and platelet production

(A and B) Quantification of platelet and reticulated platelet counts in two murine models of neutropenia. (A) Platelet and reticulated platelet counts in a model of Gr-1-antibody-induced neutropenia are shown (n = 7 animals per group). (B) Platelet and reticulated platelet counts in a model of diphtheria-toxin-induced neutropenia in Mrp8-Cre/Rosa26iDTR mice (n = 4 animals per group) are shown.

(C–E) In vitro thrombopoiesis in a co-culture of primary-fetal-liver-cell-derived MKs and BM-derived neutrophils. (C) Quantification of platelet particles (PPs) within supernatants by flow cytometry 6 h after addition of neutrophils, sphingosine-1-phosphate (10 nM), neutrophils separated by trans-well, pan CD3+ T cells, CD19+ B cells, monocytes, or neutrophil supernatants following PMA stimulation (no neutrophil: n = 6, neutrophil: n = 6, S1P: n = 5, trans-well: n = 3, CD3+ T cells: n = 5, CD19+ B cells: n = 4, monocytes: n = 4, neutrophil supernatant: n = 3).

(D) Analysis of PP production within co-culture supernatants after neutrophil treatment with actin polymerization inhibitor (cytochalasin D) or myosin inhibitor (blebbistatin)-treated neutrophils. PP release was determined by flow cytometry 6 h after co-culturing (n = 3).
count: –20.7%; reticulated platelet count: –17.4%; vs. control; Figure 3K). In contrast, platelet life span, MK numbers, MK maturity, and platelet neutrophil aggregates were not affected (Figures 3L, S3F, and S3G). Likewise, in Mrp8-cre+/−/Cxcr4+/− mice that do not express the CXC4R ligand CXCL12 on MKs, we found reduced neutrophil homing to MKs (Figures S3H and S3I) and reduced platelet production with lower numbers of platelets and reticulated platelets (i.e., platelets: –14.8%; reticulated platelets: –24.4%; vs. control), while platelet lifespan was unaffected (Figures S3J and S3K). This indicates that the CXC4R-CXCL12 axis mediates neutrophil tropism towards MKs and is essential for neutrophil-assisted thrombopoiesis in steady-state conditions. CXC4R–CXCL12 neutrophil–MK interactions supported platelet replenishment in situations of enhanced platelet consumption, as recovery of platelet counts was delayed and incomplete in Mrp8-cre+/−/Cxcr4+/−ΔΔ mice compared to controls in a model of immune thrombocytopenia (Figure S3L).

Neutrophil-derived ROS drive thrombopoiesis

To investigate the underlying mechanisms of how plucking neutrophils drive platelet production, we tested whether they were capable of transducing direct molecular signals to PPL-forming MKs, orchestrating platelet release. We focused on NADPH-derived ROS. To investigate the role of neutrophil-derived ROS, we transplanted BM from Cybb−/− mice (Pollock et al., 1995) into lethally irradiated wild-type recipient mice. Cybb−/− chimera had reduced platelet and reticulated platelet counts (Figures S4A and S4B) with unaltered platelet life span compared to control chimeras (Figure S4C). In addition to neutrophils, monocytes and B cells express NOX2; while it is not expressed by MKs (McCrann et al., 2009). However, NOX2-expressing monocytes and B cells did not induce thrombopoiesis (Table 1), suggesting that lack of NOX2 in neutrophils is responsible for the platelet phenotype in Cybb−/− chimera. In vivo visualization of platelet production revealed attenuated PPL production, while MK-neutrophil interactions were not affected (Figures S4D–S4G and Video S1). MK and neutrophil counts within the BM were increased in NOX2 null chimeras, indicating a regulatory function of NOX2 in MK and neutrophil production (Figures S4H and S4I). Mice treated with the ROS scavenger N-acetylcysteine recapitulated the findings obtained in Cybb−/− chimera (i.e., platelets: –13.1%; reticulated platelets: –18.1%; vs. control treatment) (Figures S4J–S4N). In vitro inhibition of NADPH oxidase by apocynin or genetic silencing of NADPH oxidase in Cybb−/− as well as Cyba−/− neutrophils (Nakano et al., 2008), which are unable to release superoxide anions and to generate H2O2 (Pollock et al., 1998), abolished neutrophil-driven thrombopoiesis. A similar response was found after degradation of extracellular ROS in the presence of catalase and superoxide dismutase (SOD; Figures 4A and S4O). We found that interactions with neutrophils promoted intracellular ROS production (Figures 4B and S4P) and activated MLC and ERK signaling by phosphorylation in MKs, both of which are required for MK maturation, PPL formation (Mazharian et al., 2009), and proper PP release (Chang et al., 2007) (Figure 4C). In addition, neutrophils showed a similar ROS response when we incubated with the CXC4R ligand CXCL12 (Figure S4Q). Inhibition of either ERK or MLC, even in the absence of neutrophils, abolished PP release (Figure S4R). To gain a better spatial resolution of p-MLC signaling within MKs, we used confocal microscopy. In analogy to immunoblotting, we found elevated p-MLC fluorescence intensity (MRI) values in the presence of neutrophils that were accentuated within PPLs compared to the cell body (Figures 4D–4F). This finding aligned with earlier data describing accumulation of p-MLC within the cytoplasmic swelling and at the PPL tip of the forming PPL (Chang et al., 2007).

To evaluate the potential relevance of neutrophil-driven thrombopoiesis in human probands, we analyzed a GWAS dataset obtained from 408,112 individuals (Vuckovic et al., 2020). Focusing on neutrophil migration and ROS production, we identified leukocyte-specific genetic variants for leukocyte integrin subunit LFA-1 (rs34114657) and myeloperoxidase (MPO; rs2526378), as well as leukocyte- and MK-expressed CXC4R4 (rs11679328), Slrpa (rs6045612), Lyn (rs12676105), JAK2 (rs7865719), Cyba (rs560088070), and SHP2 (rs11066283) that associate with platelet counts (Table S1). While correlative, these data pointed to similar mechanisms at play in humans.

In summary, the CXC4R4–CXCL12 axis mediates MK tropism of neutrophils that “pluck” on MK to drive PPL growth and release by direct force transmission and induction of ROS-dependent mechanosignaling (Figure 4G).

Neutrophils drive thrombopoiesis in MI

Constant delivery of platelets is essential for physiological blood cell homeostasis, yet it may be detrimental in the context of...
Table 1. Characterization of leukocyte-subpopulation-deficient mouse models (related to Figure 3)

| Cell Type          | Animal Model          | WBC (10^3/mL) | Platelet (10^3/mL) | Reticulated Platelet (%) | Neutrophil (10^3/mL) | Monocyte (10^3/mL) | B cell (10^3/mL) | T cell (10^3/mL) | Neutrophil (10^3/mL) | Monocyte (10^3/mL) | B cell (10^3/mL) | T cell (10^3/mL) | Neutrophil (10^3/mL) | Monocyte (10^3/mL) | B cell (10^3/mL) | T cell (10^3/mL) |
|--------------------|-----------------------|---------------|--------------------|--------------------------|----------------------|--------------------|------------------|------------------|----------------------|--------------------|------------------|------------------|----------------------|--------------------|------------------|------------------|
| Neutrophil         | Cd19_iDTR(+)           | 3.06 ± 0.23*  | 1113.25 ± 36.65    | 0.05 ± 0.04*             | 2.21 ± 0.05          | 0.16 ± 0.04*       | 0.41 ± 0.06*     | 1.13 ± 0.10      | 0.02 ± 0.005*       | 2.12 ± 0.31         | 1.44 ± 0.40       | 0.12 ± 0.07       | 0.02 ± 0.01*         | 0.03 ± 0.01*       | n.a.             | n.a.             |
| Monocyte           | Cd3e-KO                | 2.54 ± 0.49*  | 885 ± 43.87        | 0.83 ± 0.08*             | 1.13 ± 0.15          | 0.15 ± 0.04*       | 0.35 ± 0.06*     | 0.86 ± 0.61       | 0.01 ± 0.005*       | 2.12 ± 0.31         | 1.44 ± 0.40       | 1.32 ± 0.33       | 0.25 ± 0.09*         | 0.02 ± 0.01*       | n.a.             | n.a.             |
| B cell             | Isotype control        | 3.49 ± 0.45   | 999.75 ± 53.1      | 0.78 ± 0.37*             | 1.42 ± 0.11          | 0.16 ± 0.04*       | 0.37 ± 0.09*     | 1.13 ± 0.15       | 0.02 ± 0.005*       | 2.12 ± 0.31         | 1.44 ± 0.40       | 1.09 ± 0.09       | 0.25 ± 0.09*         | 0.02 ± 0.01*       | n.a.             | n.a.             |
| T cell             | CCR2 antibody          | 4.9 ± 1.27    | 942 ± 55.1         | 0.79 ± 0.37*             | 1.42 ± 0.11          | 0.16 ± 0.04*       | 0.37 ± 0.09*     | 1.13 ± 0.15       | 0.02 ± 0.005*       | 2.12 ± 0.31         | 1.44 ± 0.40       | 1.09 ± 0.09       | 0.25 ± 0.09*         | 0.02 ± 0.01*       | n.a.             | n.a.             |
| Neutrophil         | Cd19-iDTR(+)           | 3.06 ± 0.23*  | 1113.25 ± 36.65    | 0.05 ± 0.04*             | 2.21 ± 0.05          | 0.16 ± 0.04*       | 0.41 ± 0.06*     | 1.13 ± 0.10      | 0.02 ± 0.005*       | 2.12 ± 0.31         | 1.44 ± 0.40       | 0.12 ± 0.07       | 0.02 ± 0.01*         | 0.03 ± 0.01*       | n.a.             | n.a.             |
| Monocyte           | Cd3e-WT                | 4.85 ± 0.51   | 884 ± 12.67        | 0.78 ± 0.37*             | 1.42 ± 0.11          | 0.16 ± 0.04*       | 0.37 ± 0.09*     | 1.13 ± 0.15       | 0.02 ± 0.005*       | 2.12 ± 0.31         | 1.44 ± 0.40       | 1.32 ± 0.33       | 0.25 ± 0.09*         | 0.02 ± 0.01*       | n.a.             | n.a.             |
| B cell             | Rag1-WT                | 6.32 ± 1.05   | 1158 ± 148.9       | 0.79 ± 0.37*             | 1.42 ± 0.11          | 0.16 ± 0.04*       | 0.37 ± 0.09*     | 1.13 ± 0.15       | 0.02 ± 0.005*       | 2.12 ± 0.31         | 1.44 ± 0.40       | 1.09 ± 0.09       | 0.25 ± 0.09*         | 0.02 ± 0.01*       | n.a.             | n.a.             |
| T cell             | Rag1-KO                | 1.44 ± 0.29***| 954 ± 55.1         | 0.79 ± 0.37*             | 1.42 ± 0.11          | 0.16 ± 0.04*       | 0.37 ± 0.09*     | 1.13 ± 0.15       | 0.02 ± 0.005*       | 2.12 ± 0.31         | 1.44 ± 0.40       | 1.09 ± 0.09       | 0.25 ± 0.09*         | 0.02 ± 0.01*       | n.a.             | n.a.             |

* Indicate a p-value < 0.05, ** indicate a p-value < 0.01, *** indicate a p-value < 0.001.

WBC: white blood cells; RBC: red blood cells; Platelet: platelets; Neutrophil: neutrophils; Monocyte: monocytes; B cell: B cells; T cell: T cells; Neutrophil: neutrophils; Monocyte: monocytes; B cell: B cells; T cell: T cells.

Platelet counts +50.7%, returning to the amounts found in patients undergoing percutaneous coronary intervention (PCI; baseline characteristics are shown in Table S2) revealed a transient increase in reticulated platelet counts (i.e. reticulated platelet counts +50.7%), returning to the amounts found in patients with stable coronary artery disease at day 5 (Figure 5A). CXCR4 expression on peripheral blood neutrophils showed a similar transient increase (Figure 5B).

To characterize the role of neutrophil-driven thrombopoiesis after MI we used a mouse model of myocardial ischemia reperfusion (I/R) injury closely resembling the situation in human MI patients. When we adoptively transferred neutrophils, we found a preferential localization of transferred neutrophils around BM-resident MKs in MI mice compared to sham (Figure 5C). Imaging of murine BM 48 h post MI, we observed increased numbers of PPL-plugging neutrophils in the perisinusoidal space associated with augmented PPL growth speeds and faster PPL release times compared to sham treatment (Figures 5D–5F and Video S2). Consistent with our observation in MI patients, we found elevated platelet and reticulated platelet counts 48 h after mouse MI (i.e. platelet counts: +13.1%; reticulated platelet counts: +29.6%; vs. sham treatment) (Figure 5H), while MK numbers and maturity within the BM were unchanged (Figure 5C). MI mice revealed elevated peripheral neutrophil counts with increased CXCR4 surface expression similar to MI patients, paralleled by increased ROS amounts (Figures 5D, 5I, and S5). These data show that neutrophil plugging contributes to disproportionate, prothrombogenic platelet production during MI.

**CXCR4-driven thrombopoiesis drives recurrent arterial thrombosis and VTE**

To study the consequences of increased numbers of circulating prothrombotic immature platelets, we induced MI in mice and analyzed subsequent thrombus formation in distant vascular beds outside the coronary arteries. We used a dual-labeled antibody assay to distinguish younger (<12 h of age) from older platelets. We found that already in the absence of a previous MI, young, reticulated platelets were preferentially recruited into microvascular mesenteric artery thrombus with higher fractions when compared to total blood. A preceding MI boosted the number of immature platelets in the blood and to a similar extent also within the distant thrombus (Figures 6A and 6B and Video S2). When we induced arterial thrombosis of the common carotid artery, the rate of vessel occlusions (Figure 6C and Video S2), thrombus size, and overall thrombus burden...
Figure 4. Neutrophil-derived reactive oxygen species drive thrombopoiesis

(A) PP release from in vitro MK cultures following co-culture with neutrophils in the presence or absence of NADPH inhibitor apocynin, superoxide dismutase (SOD) (50 U/ml), and catalase (50 μg/ml; n = 3), Cyb8(-/-) or Cybb(-/-) neutrophils (n = 4 per group).

(B) Visualization of ROS in co-cultured MKs. ROS (DCFDA, green) mean fluorescent intensity in MKs with or without neutrophil interactions (Ly6G positive) was quantified. Symbols indicate individual MKs. Representative images of 3 independent experiments are shown, scale bar represents 10 μm.

(legend continued on next page)
were all increased following MI compared to control mice (Figures S5E and S5F). Abrogation of neutrophil CXCR4 signaling corrected disproportionate platelet production (i.e. platelets: −35.6%; reticulated platelets: −49.1%; vs. sham treatment) and normalized distant thrombus formation in Mrp8-cre (+)/Cxcr4fl/fl mice with a previous MI compared to controls without MI (Figures 6D, 6E, S5G–S5I, and Video S2). Thus, neutrophils underly the increased supply of immature platelets in the course of MI, in turn generating a prothrombotic milieu favoring repeat arterial thrombotic events.

Patients with a recent MI carry an increased risk of VTE after the initial thrombotic event (Khan et al., 2021; Rinde et al., 2016). To determine whether the prothrombotic effect of neutrophil-driven thrombopoiesis is a general mechanism driving thrombotic endpoints, we defined its role for VTE. Induction of deep venous thrombosis (DVT) in a venous cava thrombosis model in mice (von Bruhl et al., 2012) resulted in increased reticulated platelet counts (i.e. platelets: +22.6%; reticulated platelets: +50.2%; vs. sham treatment; Figure S6A). In parallel, mouse VTE led to increased neutrophil counts, CXCR4 expression, and neutrophil tropism in BM resident MKs following adoptive transfer of neutrophils into DVT-treated animals (Figures S6B–S6D). When we exposed Mrp8-cre Cxcr4fl/fl mice to VTE, the number of prothrombogenic reticulated platelets was reduced (i.e. platelets: −28.2%; reticulated platelets: −44.3%; vs. sham treatment) (Figure S6E). At the same time, ablation of neutrophil CXCR4 resulted in reduced thrombus weights, while thrombus frequency (Figures 6F and S6G) and thrombus composition quantified by neutrophil, monocyte, and neutrophil-extracellular-trap (NET) densities (Figures S6F and S6G) were similar. Elevated reticulated platelet counts, arising from exacerbated neutrophil-driven thrombopoiesis, therefore foster thrombo-ischemic endpoints in two prototype thromboinflammatory cardiovascular diseases.

To test whether neutrophil plucking also controls platelet production in infection, we applied a model of lipopolysaccharide (LPS)-induced systemic inflammation. As expected, intraperitoneal LPS injection produced a rapid drop in platelet counts, followed by a prolonged platelet count recovery phase (Aslam et al., 2006). After 36 h, total platelet counts were still reduced in LPS-treated mice, while reticulated platelet counts were increased (Figure S7A) compared to control treatment. MK numbers remained unchanged (Figures S7B and S7C). We found increased neutrophil CXCR4 surface expression and ROS production after LPS treatment (Figures S7D and S7E), and in vivo imaging revealed increased MK-neutrophil interactions within the perisinusoidal space and accelerated PPL formation (Figures S7F–S7H). Ablation of neutrophil plucking in Mrp8-cre (+)/Cxcr4fl/fl mice decreased perisinusoidal MK-neutrophil interactions, attenuated PPL formation kinetics, and delayed platelet count recovery 36 h after LPS treatment (Figure S7I–S7L).

DISCUSSION

Our study has identified a so far unexplored function of neutrophils that links sterile inflammation to platelet homeostasis and thrombosis. We have shown that neutrophils control thrombopoiesis by plucking on forming PPLs, in turn accelerating their growth and release into the circulation.

Inflammatory diseases are known to impact thrombopoiesis, yet the mechanisms behind this observation remain unknown. Different mechanisms including cytokine gradients (Lane et al., 2000; Zhang et al., 2012), changes in local vasculature (Stegner et al., 2017) and extracellular matrix composition (Stegner et al., 2017), sympathetic innervation (Chen et al., 2016), and shear forces (Junt et al., 2007) are involved in the control of megakaryocyte and thrombopoiesis. Most of these pathways control platelet homeostasis in the long term but do not allow the rapid responses required for fine-tuning of platelet homeostasis and to provide immediate platelet supply in scenarios of increased platelet consumption. Here, we show that neutrophils are recruited to the PPL budding site of MKs via CXCR4-CXCL12 and control the last step of BM thrombopoiesis, i.e. release of PPLs by direct physical interaction. Plucking neutrophils accelerate PPL growth and release by induction of mechanosignaling in megakaryocytes through NOX2-dependent ROS (Figures 4A, 4C–4F, and S4). We propose that these mechanisms endow neutrophils with the ability to rapidly impact platelet homeostasis upon positioning around the PPL budding site. Thus, neutrophils outpace classical systemic feedback loops regulating platelet counts (i.e. cytokines) that partially depend on translational activity, posttranslational protein processing, and systemic distribution (Grozovskiy et al., 2015; Nishimura et al., 2015; Burzynski et al., 2019).

We have shown here that plucking neutrophils are a rheostat of platelet homeostasis that maintain platelet counts under steady state but also boost release of immature platelets during thrombopoiesis, thereby increasing the risk of arterial thrombosis and repeated ischemic events following MI. Previous data and our current findings suggest that immature platelets contribute to the increased risk of recurrent ischemia (Lakkis et al., 2004) and mortality (Cesari et al., 2013) in patients with MI. How immature platelets foster thrombotic processes remains to be defined, yet several mechanisms are likely to operate. Firstly, we demonstrated that immature platelets were preferentially recruited to sites of vascular damage after MI and are found more abundantly in arterial thrombi. Secondly, immature platelets showed a more activated phenotype,
Figure 5. Myocardial infarction triggers thrombopoiesis by neutrophil plucking

(A and B) Patients admitted because of acute ST-elevation myocardial infarction (STEMI) with symptom onset <12 h undergoing revascularization by percutaneous coronary intervention or control patients with stable coronary artery disease were recruited. (A) Platelet counts and reticulated platelet fraction (control patients: n = 10; STEMI patients: n = 10, symbols indicate individual patients) and (B) neutrophil-expressed CXCR4 was determined by flow cytometry (control patients: n = 5; STEMI patients: n = 7, symbols indicate individual patients).

(C) Adoptive transfer experiments of neutrophils into I/R or sham-treated C57BL/6 mice. Representative 2D pictures from 3 independent experiments of whole-mount-stained bones are shown (scale bar represent 10 μm). Minimal MK-neutrophil distance between MKs and neutrophils was quantified (n = 3 animals per group).

(D–G) In vivo multiphoton visualization of MK-neutrophil interactions 48 h after I/R or sham treatment in C57BL/6 mice. (D) Image series of PPL release in I/R and sham-treated mice is shown. Scale bars represent 20 μm (n = 3 animals per group), timeline (min) is indicated.

(E) Quantification of MK-neutrophil interaction frequencies within different compartments (n = 3 animals per group).

(F) Analysis of PPL growth speed and release time (n = 3 animals per group, each symbol indicates individual PPLs).

(G) Frequency of PPL-forming MKs (n = 3 animals per group, each symbol indicates individual ROI area).

(H) Platelet and reticulated platelet counts.

(I) CXCR4 surface expression on peripheral neutrophils was determined in I/R and sham-treated animals by flow cytometry (n = 4 animals per group), a representative histogram blot of 4 independent experiments is shown.

(J) Reactive oxygen species in peripheral neutrophils in I/R and sham-treated animals (n = 4 animals). Bars represent mean ± SEM, symbols indicate individual animals or MKs or ROIs; p values are indicated, *p<0.05, **p<0.01, ****p<0.0001, n.s. not significant. P values were determined by unpaired Student’s t test (F, G, H, I, and J) or one-way (A and B) or two-way (C and E) ANOVA multigroup test. Please see also Table S2 and Figure S5.
facilitating platelet aggregation and driving coagulation at injury sites. Finally, immature platelets appeared to be less sensitive to current antiplatelet therapies (Bernlochner et al., 2015; Armstrong et al., 2017). Thus, targeting neutrophil-driven thrombopoiesis to attenuate supply of prothrombotic immature platelets may be an anthrombotic and antiischemic approach to prevent recurrent ischemia during the early phase of MI and other thromboinflammatory processes, including VTE. In summary, we have identified neutrophil-driven thrombopoiesis as a hallmark of common thrombotic cardiovascular diseases, highlighting its potential as a so far unexplored anthrombotic target.

Limitations of the study
Our study has some limitations arising from technical challenges in investigating cell-cell interactions within the bone marrow using life cell imaging. Mechanistically, we cannot dissect the contribution of neutrophil-transduced direct physical forces from neutrophil-induced mechanosignaling in MKs in vivo. While intracellular tension and force measurement using fluorescent probes are feasible, it is beyond the current state of the art to apply this approach on neutrophils within the bone marrow. Another limitation of our study is that we cannot directly prove the presence of neutrophil plucking in human bone marrow. However, our human data revealed similar changes within the peripheral platelet and immune cell populations as seen in mice with MK plucking neutrophils.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, T.P., Z.Z., I.B., A.H., S.M.; methodology, T.P., Z.Z., I.B., A.G., K.S., J.P., D.S., G.G., F.G., I.A., C.S., M.K., M.S.S.; investigation: I.B., A.P., I.S., M.T., L.L., Q.U.A., V.E., C.W., B.K., P.M., J.G., W.F., M.L., H.I.A., E.R., E.M., C.G., S.C., L.N.; visualization: Z.Z., L.L., Q.U.A., S.E.N.; supervision: T.P., A.P., M.K., A.H., S.M.; writing-original draft, T.P.; writing-review and editing, T.P., D.S., F.G., C.S., A.H., S.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                     | SOURCE                  | IDENTIFIER          |
|------------------------------------------|-------------------------|---------------------|
| **Antibodies**                           |                         |                     |
| Ultra-LEAF Purified anti-mouse Ly-6G/Ly-6C (clone RB6-8C5), neutrophil depletion | Biolegend               | Cat#108453; RRID:AB_2616681 |
| Ultra-LEAF™ Purified Rat IgG2b, κ Isotype Ctrl (clone RTK4530) | Biolegend               | Cat# 400671; RRID:AB_11147763 |
| Pacific Blue™ anti-mouse Ly-6G/Ly-6C (clone RB6-8C5) | Biolegend               | Cat#108430; RRID: AB_893556 |
| PE anti-mouse CD115 (clone AFS98)        | Biologend               | Cat#135506; RRID: AB_1937253 |
| APC anti-mouse/rat CD42d (clone 1C2)     | Biolegend               | Cat#148506; RRID: AB_2564602 |
| Pacific Blue™ anti-mouse Ly-6G Antibody  | Biolegend               | Cat#127612; RRID: AB_2251161 |
| PerCP/Cy5.5 anti-mouse CD154 Antibody   | Biolegend               | Cat#106514; RRID: AB_2563498 |
| APC/Cyanine7 anti-mouse CD63 Antibody    | Biolegend               | Cat#143908; RRID: AB_2565498 |
| Pacific Blue™ anti-mouse/rat CD29 Antibody | Biolegend             | Cat#102224; RRID: AB_2128079 |
| FITC anti-mouse CD3 complex (clone 17A2) | BD Bioscience           | Cat#555274; RRID: AB_395699 |
| FITC anti-mouse CD61 (clone 2C9.G2)     | BD Bioscience           | Cat#553346; RRID: AB_10895806 |
| BV711 Rat Anti-Mouse CD62P               | BD Bioscience           | Cat#740693; RRID: AB_2740377 |
| PE-Cyanine5 anti Human/Mouse CD45R (clone RA3-6B2) | ebioscience         | Cat#15-045282; RRID: AB_468755 |
| FITC anti-mouse CD41a (clone MWRreg30)   | ebioscience             | Cat#11-0411-85; RRID: AB_763483 |
| Biotin anti-mouse CD41a (clone MWRreg30) | ebioscience             | Cat#13-0411-82; RRID: AB_763484 |
| PE anti-mouse CXCR4 (clone 2B11)         | ebioscience             | Cat#12-9991-82; RRID: AB_891939 |
| Streptavidin eFlour 450                  | ebioscience             | Cat#48-4317-82;RRID:AB_10359737 |
| Streptavidin PE                          | ebioscience             | Cat#12-4317-87      |
| Biotin anti-mouse VE-cadherin (clone eBioBV13) | ebioscience          | Cat#13-1441-82; RRID: AB_1234997 |
| GFP recombinant rabbit monoclonal antibody | Invitrogen           | Cat#G10632; RRID: AB_2536526 |
| Goat anti-Rat IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 | Invitrogen           | Cat#A-21247; RRID: AB_141778 |
| 7F(ab')2-Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Invitrogen           | Cat#A-11070; RRID: AB_2534114 |
| DyLight 649 anti-mouse CD42b             | emfret                  | Cat# M040-          |
| Antibodies for In Vivo Mouse Platelet Labeling | emfret                | Cat#X488; RRID: AB_2890921 |
| Antibodies for In Vivo Mouse Platelet Labeling | emfret                | Cat#X649; RRID: AB_2861336 |
| Integrin alphabeta3 (GPIb/IIa, CD41/CD61) | emfret                | Cat#M023-2; RRID: AB_2833084 |
| Rabbit Anti-Myeloperoxidase Polyclonal Antibody | Abcam                | Cat# fab8535; RRID: AB_307322 |
| Alexa 488 anti-mouse GPIX                 | p0p6-derivative Stegner lab | N/A               |
| Mouse GPVI Alexa Fluor® 647-conjugated Antibody | R&D systems         | Cat# FAB6758R       |
| Human/Mouse CXCL12/SDF-1 Antibody        | R&D systems             | Cat#MAB350; RRID: AB_2088149 |
| Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody | Cell Signaling         | Cat#4370; RRID: AB_2315112 |
| p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Rabbit mAb antibody | Cell Signaling         | Cat#4695; RRID: AB_390779 |
| Phospho-Myosin Light Chain 2 (Ser19) Antibody | Cell Signaling         | Cat#3671; RRID: AB_330248 |
| Myosin Light Chain 2 Antibody            | Cell Signaling          | Cat#3672; RRID: AB_10692513 |
| Goat anti-Rabbit IgG (H + L) Secondary Antibody, HRP | Thermo scientific | Cat#31460; RRID: AB_228341 |
| MC-21(anti-CCR2) antibody                | Courtesy Matthias Mack | N/A               |

(Continued on next page)
**RESOURCE AVAILABILITY**

All data are available in the main text or the supplementary materials.

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tobias Petzold (Tobias.Petzold@med.uni-muenchen.de).

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# REAGENT or RESOURCE SOURCE IDENTIFIER

**Experimental models: Organisms/strains**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| C57BL/6J           | Jackson Laboratory | N/A |
| Mrp8-cre           | Passegué et al., 2004 | B6.Cg-Tg(S100A8-cre,-EGFP)1Iiw |
| Rosa26-Confetti     | Livet et al., 2007  | Gt(Rosa26Sor1Cre1)1bra | B6.129P-Lyz2tm1(EGFP)1.1graf |
| Rosa26-iDTR         | Buch et al., 2005  | C57BL/6-Gt(Rosa26Sor1Cre1)1AWai | Gt(ROSA)26Sortm1(MTm1)Cle/J |
| Lyz2-eGFP          | Faust et al., 2000 | B6.129P-Lyz2tm1(EGFP)1.1graf |
| Cxcr1/2             | Greenbaum et al., 2013 | B6(FVB)-Cxc1/2tm1.1Lin/J |
| P4h-cre            | Tiedt et al., 2007 | C57BL/6-Gt(P4h-cre)3Rsko/J |
| Cxcr4              | Nie et al., 2004   | B6.129P2-Cxcr4tm1Yzo/J |
| Selpig/kg             | Yang et al., 1999 | B6.Cg-Selpig1Caa/J |
| Catchup             | Hasenberg et al., 2015 | B6.Ly6g1Tm1.1Kunz |
| Cyba/+/+             | Nakano et al., 2008 | B6.Tyr + -Cyba19m333/J |
| Cd19-cre            | Demircik et al., 2013 | B6.129P2(Cd19-cre)1jag/J |
| Cd3e/f−/−            | Sommers et al., 2000 | B6.129-Cd3etm1Lov/J |
| Rag1+/−              | Mombaerts et al., 1992 | B6.129S7-Rag11tm1Mon/J |

**Chemicals and recombinant proteins**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Thiazole Orange     | Sigma-Aldrich | Cat# 390062 |
| 2',7' -Dichlorodihydrofluorescein diacetate | Sigma-Aldrich | Cat# D6883 |
| EZ-LinkTM Sulfo-NHS-Biotin | Thermo Fisher | Cat# 27217 |
| Phorbol 12-myristate 13-acetate | Sigma-Aldrich | Cat# P8139 |
| Mouse recombinant thrombopoietin | Immunotools | Cat# 12343615 |
| Mouse recombinant SDF-1 alpha | Immunotools | Cat# 12343656 |
| Apocynin            | Sigma-Aldrich | Cat# 10809 |
| Qtracker705 Vascular labels | Thermo Fisher | Cat# Q21061MP |
| AMD3100             | Tocris | Cat# 3299 |
| U46619              | Cayman | Cat# 16450 |
| Thrombin            | Chrono-Log | Cat# P/N386 |
| Proteome Profiler Mouse XL Cytokine Array | R&D systems | Cat# ARY028 |
| ML-7 hydrochloride  | MedChemExpress | Cat# HY-15417 |
| FR180204            | MedChemExpress | Cat# HY-12275 |

**Critical commercial assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Neutrophil Isolation Kit, mouse | Miltenyi Biotec | Cat# 130-097-658 |
| μ-Slide 4 Well      | Ibidi | Cat# 80426 |
| Pan T Cell Isolation Kit II, mouse | Miltenyi Biotec | Cat# 130-095-130 |
| Monocyte Isolation Kit (BM), mouse | Miltenyi Biotec | Cat# 130-100-629 |
| Pan B Cell Isolation Kit II, mouse | Miltenyi Biotec | Cat# 130-095-813 |

**Software and algorithms**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Imaris              | Bitplane | RRID:SCR_007370 |
| Flowjo vX           | Treestar | RRID:SCR_008520 |
| Prism               | Graphpad | RRID:SCR_002798 |
| ImageJ             | NIH | RRID:SCR_003070 |

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Materials availability
This study did not generate new unique reagents. All reagents generated or used in this study are available on request from the lead contact with a completed Materials Transfer Agreement. Information on reagents used in this study is available in the key resources table.

Data and code availability
All the data supporting the findings of the article are available within the main text or supplementary materials.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse strains
C57BL/6J, Pf4-cre (C57BL/6-Tg(Pf4-cre)Q3Rsko/J), Rosa26-Confetti (Gt(Rosa)26Sortm1(CAG-Brainbow2.1)Cle/J), Lyz2-eGFP (B6.129P-Lyz2tm1(EGFP)1.1Graf), Cxcl12 e3 Immunity Pf4-cre mice were kindly provided by Dr. Markus Moser. CD19-Cre/Rosa26iDTR mice were kindly provided by Dr. Wenyan He. To generate C57BL/6J, Pf4-cre Mouse strains

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EXPERIMENTAL MODEL AND SUBJECT DETAILS

METHODS

Primary-fetal-liver-cell-derived megakaryocyte culture
Murine fetal liver cells were harvested on embryonic day 13.5–14.5 as described before (Zhang et al., 2012). Suspended cells were cultured in DMEM medium (10% fetal bovine serum+1% penicillin/streptomycin) containing 70 ng/ml thrombopoietin (TPO, ImmunoTools) for 5 days at 37°C and 5%CO2. On day five, megakaryocytes were enriched using a bovine serum albumin (BSA) gradient method.

BM-derived MK isolation
Femur and humerus bones were isolated and BM was flushed with 2% FCS/PBS. Cells were resuspended as single cell population to pass a 70µm cell strainer. Cells were centrifuged, resuspended in DMEM medium and supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were cultured in 6-well plates in DMEM medium with 10% FBS and 1% penicillin/streptomycin, 70 ng TPO and 15 U/ml heparin for 5 days at 37°C (5% CO2). MKs were enriched using a BSA gradient (3%–1.5%–0% BSA in PBS) over 30 min twice.

Leukocyte isolation
Murine neutrophils were isolated from large bones by flushing the BM with PBS supplemented with 2% FCS. BM cells were resuspended with a 20-gauge syringe needle and filtered by 40µm strainer, followed by centrifugation at 300 x g for 5 min at room temperature (RT). Erythrocyte lysis was performed by addition of lysis buffer (155 mM NH4Cl2, 10 mM KHCO3, 0.1 mM EDTA, pH 7.3). Lysis reaction was stopped after 5 min by addition of PBS containing 2 mM EDTA. Neutrophils were enriched using a murine neutrophil enrichment kit (Miltenyi Biotech) according to the manufacturer’s instruction. B-cells, T-cells and monocytes were harvested from spleen (B-cells and T-cells) or BM. Murine spleen was cut and grinded into small pieces by scalpel and syringe. Then cells were resuspended into single cells suspension by 20-gauge syringe needle with 2% FCS/PBS. Afterward, B-cells and T-cells were isolated and enriched by isolation kit (Miltenyi Biotech) following the manufacturer’s instructions. Monocytes were isolated in analogy to neutrophils. For monocyte isolation from the BM an murine monocyte isolation kit (Miltenyi Biotech) was used.

Co-culture
For co-culture assays 2.5 x 10⁶ BM-derived megakaryocytes or fetal liver cells (FLC) (for stainings) were seeded in 6-wells dishes coated with 100 µg/ml fibrinogen and cultured in medium [40% (DMEM+10% fetal bovine serum+1% Penicillin/Streptomycin) with 60% Leibovitz L15 medium] for 6 hours at 37°C and 5%CO2. After 30 min 2.5 x 10⁶ neutrophils were added to the MK culture. MK-leukocyte ratios were adapted according to their neutrophil count ratio (100% neutrophils, 75% B-cells, 24% monocytes, 12% T-cells). If indicated, blocking antibody treatment was started immediately before co-culturing. If indicated, neutrophils were pre-incubated with cytochalasin D (100 nM) or blebbistatin (50 nM) for 30 min at 37°C before being added into the co-culture with MKs. After 6 h supernatants were collected and fixed with 1% paraformaldehyde (PFA) for 10 min at RT. Released platelet particles
(PP) were centrifuged and stained with directly fluorescently labelled antibodies for CD42d-APC, Ly6G-PE and CD61-FITC before being analyzed by flow cytometry.

**Co-culture (immunoblot)**

Co-culture experiments were performed as described above. MKs were incubated with 2.5 x 10⁶ neutrophils in the presence of 30 nM apocynin or 30 nM H₂O₂ for 6 hours. To isolate a clean MK population, neutrophils were flushed away by repetitive washing steps with 1x cold Tris-buffered saline. Successful removal was confirmed by microscopy. Next, adherent megakaryocytes were scraped off before homogenized in cold RIPA buffer containing 50 nM protease and phosphatase inhibitors (Thermo Fischer). Samples (10 μg) were prepared and subjected to immunoblotting. Following the blot step, the PVDF membrane was incubated with the following antibodies: p-ERK, t-ERK, p-MLC and t-MLC. Thereafter, membranes were incubated with HRP coupled secondary antibody (1:5000 or 1:30000 respectively). Chemiluminescence signals were detected on X-ray film (Fuji) after addition of chemiluminescent detection reagent to the membrane (ECL, Merck). Relative signal strengths were determined by densitometry using ImageJ software.

**Blood count analysis**

Murine blood was drawn by intracardial puncture from anesthetized [isoflurane (DeltaSelect), fentanyl i.p. (0.05 mg/kg body weight; CuraMed Pharma)] mice and collected in a tube containing acid citrate dextrose (39 mM citric acid, 75 mM sodium citrate, 135 mM dextrose; ACD) buffer. Blood counts were analyzed on a hematology system blood counter (IDEXX ProCyte DX) or Gallios flow cytometer (Beckman) / BD FACSCantoII flow cytometer (BD) after addition of specific labelling antibodies (i.e. CD41) and fluorescent bead calibration. Therefore, 2 μL murine blood was stained with the respective antibody (dilution 1:100) for 20 min at RT in the dark before 4 μL multifluorescent counting beads were added and flow cytometry analysis was performed.

**Flow cytometry**

Erythrocyte-lysed whole blood or bone marrow cells were surface-stained in PBS at 4°C. Multiparametric flow cytometric analyses were performed on Gallios flow cytometer (Beckman) or BD FACSCantoll flow cytometer (BD). Dead cells were excluded by FSC, SSC and 49,6-diamino-2-phenylindole (DAPI) stain. Neutrophils were gated as Gr-1⁺ (RB6-8C5)/CD115⁻ (AFS98) events; monocytes, B-cells or T-cells were gated as Gr-1⁺/CD115⁺, CD45R⁺ (RA3-6B2) or CD3⁺ (17A2) events respectively. Mature MKs were gated by as CD41⁺/CD42d⁺ double positive population. MK ploidy was quantified following propidium iodide staining in MKs.

**Quantification of reticulated platelets**

Reticulated platelet counts were determined by analyzing fixed (1% PFA for 10 min) citrate blood. Human reticulated platelets were stained with CD41-PE (1:100) and thiazole orange (250 ng/ml) for 20 min, before being analyzed by flow cytometry. Murine reticulated platelets were detected following a double staining with CD42d-PE antibody and thiazole orange (1 μg/ml).

**Neutrophil depletion**

For neutrophil depletion 50 μg Gr-1 antibody (RB6-8C5 clone) was injected by tail vein or 3 times every second day. For diphtheria toxin (DT)-induced neutrophil depletion, Mrp8-cre/Rosa26-iDTR mice received 10 ng/g body weight DT over 5 consecutive days by i.p. injection.

**Monocyte and B cell depletion**

To deplete monocytes in vivo, 10–12 weeks old, C57BL/6 male mice received (i.p.) 5 consecutive days anti-CCR2 (MC-21) or isotype control antibody, 20 μg/per mouse daily. For B-lymphocyte depletion, Cd19-cre/iDTR mice received (i.p.) 3 consecutive days diphtheria toxin, 10 ng/g body weight per day.

**N-acetylcysteine (NAC) treatment in vivo**

10–12 weeks old mice were assigned to receive 200 mg/kg body weight N-acetylcysteine or 0.9%NaCl (vehicle) i.p. over 5 days. At treatment day five mice were euthanized and blood and bones were harvested for further analysis.

**Platelet lifespan measurement**

To quantify platelet lifespan a biotin pulse labelling assay was performed. Therefore 30 mg/kg body weight Sulfo-NHS-biotin was injected i.v. At the indicated time points 20 μL blood was collected in ACD buffer after tail vein puncture. Whole blood was stained with 1:100 streptavidin-PE (SA-PE) to stain biotin labelled cells in addition to a CD42d-APC labelling to identify the total platelet fraction.

**Cell labeling for in vivo visualization**

To visualize neutrophils and MKs simultaneously in vivo, 5 μg Ly6G-PE antibody (Biolegend) or 15 μg anti-GPIX-Alexa488 derivative antibody was administered i.v.
Intravital multiphoton imaging
Preparation of the calvarium was performed as described before (Zhang et al., 2012). Briefly 10–18 weeks old mice received anesthesia with 5.0 Vol. % isoflurane and 2% oxygen, followed by an i.p. injection of MMF solution (90 μL Midazolam (0.5 mg/ kg), 15 μL Meditomidin (0.05 mg/kg) and 90 μL Fentanyl (5 mg/kg)) with repetitive injections every 45 min. A PE-10 polyethylene catheter was placed into the murine tail vein for fluids and reagents administration. Mice were immobilized using custom made stage on a heating pad to maintain body temperature. A LaVision Biotech intravital multiphoton microscope system, based on Ti:sapphire system and to acquire images. For visualizing of P44-cre(+)/Confetti/ Lyz2-eGFP mice following laser settings were used: Ti:sapphire laser (wavelength 800 nm) and an OPO system (wavelength 1050 nm). For clarity MKs are shown single colored in red representing the brightest color in this imaging setup. The vasculature was visualized after i.v. injection of 15 μL Qtracker705 following excitation by Ti:sapphire laser (wavelength 800 nm). For 4-dimensional image acquisition, image stacks were acquired by capturing a region of interest of 405 × 405 μm² with 2 μm thick imaging layers over a total depth of 40–50 μm every 60 seconds over 1 h. Original image data was processed by IMARIS software (Bitplane).

Time lapse imaging of MK-neutrophil interactions in vitro
Neutrophil plucking in co-culture was visualized over a period of at least 3 hours by Brightfield and Phase Contrast Microscopy (Zeiss, Axiovert 200) with time-lapse images acquisition every 60 seconds. ImageJ software was used for movie generation.

Adoptive neutrophil transfer
Neutrophils were isolated from the BM as described before. Next, 2.5 × 10⁶ neutrophils were injected i.v. into the recipient mice. After one hour, recipient mice were anaesthetized, sacrificed and perfused with PBS and 4% PFA. Bones were embedded in tissue-tek before stored at −20 °C.

Adoptive neutrophil transfer in Mrp8-cre(+)IDTR mice
Neutrophilia was induced in Mrp8-cre (+) IDTR mice as described before. Neutrophil plucking was visualized by multiphoton intravital microscopy over one hour before and after transfer of 2.5 × 10⁶ neutrophils labeled with Ly6G-PE (1: 50, clone 1A8).

Bone whole-mount staining
Frozen, tissue-tec embedded bones (i.e. tibia, humeri) were cut horizontally using a CryoStar NX70 cryotome (Leica). Bones were immerssed in a small tube with blocking buffer (10% goat serum, 3%BSA, 0.5% Triton-X100) at 4 °C overnight. Bones were then incubated in the staining solution containing an anti-CD44-biotin and an anti-GFP antibody for 6–8 hours at 4 °C. For the secondary antibody staining, bones were washed and incubated in a secondary antibody solution (i.e. streptavidin eFluor450, goat anti-rabbit Alexa488 and CD41-PE) at 4 °C overnight. For confocal 3D imaging, bones were immobilized in a plastic sample holder. 3D images were acquired using an inverted Zeiss LSM 880 Airyscan confocal microscope with a plan-Apochromat 100x/1.46 oil-immersion objective and auto-imaging system (Zeiss). Picture analysis was done by using IMARIS software (Bitplane).

Immunofluorescent staining
For MK staining, fetal liver-derived MKs were seeded on coverslips coated with 100 ng/mL fibrinogen for 6 hours in DMEM medium (10% FCS and 1% penicillin/streptomycin) at 37 °C. Cells then were fixed with 4% PFA for 10 min. After a three-times PBS washing step, MKs were blocked and permeabilized with a blocking solution containing 10% goat serum and 0.5% Triton-X100 for 1 hour at RT. DCF-1 was stained with anti-mouse DCF-1 alpha purified antibody (R&D systems). MKs and nuclei were labeled by an anti-mouse CD41-FITC antibody and Hoechst 33342, respectively.

ROS quantification
ROS amounts were determined in neutrophils isolated from murine BM. Neutrophils were stimulated with 50 nM phorbol 12-myristate 13-acetate (PMA) or 200 ng/mL SDF-1 + 50 nM PMA for 1 h at RT. Cells were subsequently stained with 10 nM 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) for 30 min before flow cytometric analysis. For negative control, equal number of neutrophils received same DCFDA staining procedure without prior stimulation.

ROS visualization in vitro
Co-cultured cells were stained with 10 nM 7'-Dichlorodihydrofluorescein diacetate (DCFDA) for 30 min before being fixed (4% PFA), three times washed with PBST(0.5% tween 20), and stained with Ly6G-PE (1A8) for 30 min. Nuclei were counterstained with DAPI for 5 min at RT. ROS fluorescence was imaged using a Zeiss Axio Observer fluorescent microscopy equipped with 20X objective. Images were analyzed by IMARIS software.

Murine BM cytokine profile analysis
After induction of neutropenia by Gr-1 or control antibody treatment, two tibia and femur bones were isolated. Bones were centrifuged and flushed with 400 μL PBS as described before to wash out BM interstitial fluid. Cytokine amounts were analyzed using a murine proteome cytokine array kit (R&D system, cat#ARY028) according to the manufacture’s instruction. Briefly, 200 μL BM...
samples were mixed with 15 µL detection cocktail antibody and incubated on a membrane for 1 hour at RT. Next, membranes were incubated with streptavidin-HRP for 30 min at RT. For signal detection, ECL chemiluminescence substrate was added and specific chemiluminescence signals on the membrane were visualized by X-ray film exposure. Relative signal strengths (mean gray intensity compared to reference signal) of each dot were determined by densitometry using ImageJ software.

**Acute myocardial infarction mouse model**
Male 12- to 20-week old mice were subjected to 1 hour occlusive ligation of left anterior descending (LAD) coronary artery or a sham operation without ligation. Briefly, anesthetized mice were placed on the heat pad to maintain body temperature. Afterward trachea was exposed, a tube connected with ventilator was placed into the trachea to keep mouse respiration rate (RR) at 150/min. Chest cavity was opened at the site between 3rd and 4th ribs and the LAD artery was exhibited by carefully removing pericardium. Thereafter an 8-0 silk suture was slowly passed under the LAD 2–3 mm below left atria avoiding to tear heart tissue. The ligature was closed for one hour. For sham-operated mice, the suture was slowly pulled under the LAD without ligation. After one hour, the ligature was carefully released from the LAD. The chest cavity, muscle and skin layer were closed with a 5-0 suture afterward. All operated mice were kept in a clean cage with heat pad for 48 hours and thereafter used for further investigations.

**Ferric(III) chloride-induced thrombus formation in the murine carotid artery model**
In anesthetized mice the right common carotid artery was surgically prepared and vessel injury was induced by application of FeCl₃ (1 µL of 10% FeCl₃ soaked in 1 mm² Whatman paper) for 2 min. For intravitral microscopy, platelets were labelled by injection of a fluorescently labelled non-blocking platelet antibody (anti-mouse-GP Ib-X488, Emfret, 0.5 µg/g body weight) before application of FeCl₃. Vessel injury-induced thrombus formation was monitored and imaged for 60 min by in vivo video fluorescence microscopy (Axio Zoom.V16 Zeiss microscope equipped with a Zeiss AxioCam). Vessel occlusion was defined as complete cessation of blood flow for at least 10 seconds. Thrombus size was calculated from single images using ImageJ software.

**Platelet recruitment into Fe(III) chloride-induced mesenteric artery thrombosis**
Single cell imaging of platelet recruitment into thrombi in vivo was investigated in murine mesenteric arterioles after vessel injury using FeCl₃. In anesthetized mice the mesentery was surgically prepared and mounted on a custom-made stage for intravitral microscopy. After the surgical preparation of the mouse mesentery, 3–5 arterioles were chosen and 1% FeCl₃ was applied for 10 seconds. To selectively identify freshly released platelets a fluorescently labelled non-blocking platelet antibody (anti-mouse-GP Ib-X649, Emfret, 0.5 µg/g body weight) was applied 12 hours before the experiment and the same antibody but with different fluorochrome (anti-mouse-GP Ib-X488, Emfret, 0.5 µg/g body weight) was applied shortly before the experiment. Accordingly, newly produced platelets within the last 12 hours were single positive while all other platelets were positively stained for both fluorochromes. Intravitral fluorescence microscopy was performed using a confocal microscope Zeiss LSM 880 with airyscan microscope. Images were recorded digitally with a Zeiss AxioCam) and analyzed with ImageJ software.

**Venous thrombosis model by flow restriction in the vena cava inferior**
After a median laparotomy the vena cava inferior (IVC) was exposed and a space holder was positioned followed by a narrowing ligature. Subsequently, the wire was removed to avoid complete vessel occlusion. Side branches were not ligated or manipulated. All groups were age, sex, and weight matched. Mice with bleedings or any injury of the IVC during surgery were excluded from further analysis. For thrombus weight measurement after 48 hours, the IVC was excised just below the renal veins and proximal to the confluence of the common iliac veins.

**LPS model**
Inflammation was induced by a single intraperitoneal injection of 0.1 mg/kg (bodyweight) LPS as done before (Aslam et al., 2006).

**Generation of bone marrow chimera**
Bone marrow chimeras were generated by injecting 6x10⁶ bone marrow cells isolated from C0/C0 deficient mice (Pollock et al., 1995) into lethally irradiated 8 weeks old CD45.1 recipient mice as done before (Petzold et al., 2013).

**Human samples**
Acute ST-elevation myocardial infarction (symptom onset <12 h) patients were recruited after giving informed consent at the Heinrich-Heine University (HHU) in Düsseldorf, Germany. All STEMI patients underwent cardiac catheterization with percutaneous coronary intervention. Patients received standard of care medication at the discretion of the interventionalist. 24 h and 5 days after PCI patient blood was drained and analyzed using a blood counter and a BD FACSVersus flow cytometer to determine reticulated platelet fraction and CXCR4 expression on neutrophils. Reticulated platelets were stained with thiazole-orange as described above. Respective control patients with stable coronary artery disease were recruited in the department of cardiology at HHU after giving informed consent. All experiments were approved by the ethics committee of the HHU.
QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed using Prism8 (GraphPad). Normality testing was done using D’Agostino-Pearson omnibus test, where applicable, or Kolmogorov-Smirnov test. Depending on normality distribution, unpaired two-sided Student’s t test or Mann-Whitney test were used. In matched-sampled experiments, paired Student’s t test was performed. Tukey-HSD test for one-way ANOVA or two-way ANOVA was used for statistical analysis in multi-group comparison for normal distributed data (Kolmogorov-Smirnov test), otherwise Kruskal-Wallis- or Wilcox-rank-sum-tests were applied. Correlation analysis was performed using Pearson correlation coefficient test.

GWAS analysis

Vuckovic et al. (Vuckovic et al., 2020) reported a large GWAS on blood cell trait in European cohorts. In these analyses they identified common and rare variants associated with four platelet indices (PLT - Platelet count, MPV - Mean platelet volume, PDW - Platelet distribution width, PCT - Plateletcrit). A GWAS with the sample size N = 408,112 participants of European ancestry was carried out for the UK Biobank. Genome-wide genotyping was performed on all UK Biobank participants using the UK Biobank Axiom Array. More than 90 million variants were imputed using the Haplotype Reference Consortium and UK10K + 1000 Genomes reference panels. Association analyses were then conducted using a linear mixed effects model in order to account for known or cryptic relatedness with the additive genetic model. Details on quality control and statistical methods can be found in Vuckovic et al. (Vuckovic et al., 2020). Summary statistics for platelet traits are available to download from: ftp://ftp.sanger.ac.uk/pub/project/humgen/summary_statistics/UKBB_blood_cell_traits. We used this resource to look up our 20 candidate genes in four platelet indices. We identified all single nucleotide polymorphisms (SNP) and their results in the GWAS within the candidate gene and 50 kb upstream and downstream from the gene. Then we identified the SNP with minimal p-value. Finally, we visually checked local association plots and LD-blocks to see if the block covered the candidate gene. In some cases it was necessary to assign more than one gene to a gene cluster. Then we designated the SNP as sentinel SNP and reported it in Table 1 and Table S1. We used the software SNiPA v3.3 (June 2018, Genome assembly: GRCh37.p13, Ensembl version: 87, 1000 genomes: phase 3 version 5) and R version 3.5.2 to implement this strategy.
Supplemental information

Neutrophil “plucking” on megakaryocytes drives platelet production and boosts cardiovascular disease

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### Table S1: GWAS analysis of platelet traits (related to Figure 4)

| Gene Symbol(s) | Associated Alleles (rsID) | OR (95% CI) | BP (Gorisch) | REF (Gorisch) | ALT (Gorisch) | Minor Allele Frequency | Minor Allele Effect | Detailed Standard Error of Estimate | Genotype-wide significant (P > 0.10) |
|----------------|---------------------------|-------------|-------------|--------------|--------------|------------------------|-------------------|-------------------------------------|----------------------|
| CECR4          | FLT rs1973228              | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |
| CECR4          | FLR rs1973228              | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |
| CECR4          | FLT rs1973228              | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |
| CECR4          | FLR rs1973228              | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |
| CYBA         | G02008.1            | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |
| CYBA         | G02008.1            | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |
| CYBA         | G02008.1            | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |
| CYBA         | G02008.1            | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |
| CYBA         | G02008.1            | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |
| CYBA         | G02008.1            | 1.000       | 0.994       | 0.992       | 0.992       | 0.992                  | 1.000             | 0.992                               | 0.992                 |

Supplemental Material

Supplemental Tables

Table S1
is always the rs-number of the SNP with the smallest p-value in the respective candidate gene region. Information about chromosomes, positions and reference as well as alternative alleles is with respect to Genome Reference Consortium Human Build 37 (GRCh37). GWAS summary statistics are calculated with UK biobank data (Vuckovic et al, Cell, Volume 182, Issue 5, 2020) and are downloaded from ftp://ftp.sanger.ac.uk/pub/project/humgen/summary_statistics/UKBB_blood_cell_traits. Genome wide significantly regulated traits are highlighted in bold.
Table S2

Clinical characteristics of patients for reticulated platelets analysis

|                      | Control patient | STEMI patient       |
|----------------------|-----------------|---------------------|
| Patients count       | 10              | 10                  |
| Age                  | 68.7 ± 11.56    | 67.2 ± 12.05        |
| Male, n [%]          | 8 [80]          | 6 [60]              |
| Family anamnesis, n [%] | 3 [30]     | 1 [10]              |
| Diabetes, n [%]      | 4 [40]          | 1 [10]              |
| Hypertension, n [%]  | 9 [90]          | 3 [30]              |
| CAD, n [%]           | 10 [100]        | 9 [90]              |
| Smoke, n [%]         | 6 [60]          | 4 [40]              |
|                      | day1            | day5                |
| Hemoglobin, g/dl     | 13.71 ± 1.62    | 12.98 ± 1.36        |
| Hematocrit, %        | 38.13 ± 12.46   | 39.41 ± 4.62        |
| Leukocyte, 10^9/L    | 7.95 ± 2.23     | 13.17 ± 6.51        |
| Erythrocyte, 10^12/L | 4.53 ± 0.45     | 4.22 ± 0.50         |

Clinical characteristics of patients for CXCR4 analysis

|                      | Control patient | STEMI patient       |
|----------------------|-----------------|---------------------|
| Patients count       | 5               | 7                   |
| Age                  | 75 ± 12.75      | 59 ± 11.15          |
| Male, n [%]          | 1 [20]          | 4 [57.1]            |
| Family anamnesis, n [%] | 0 [0]      | 1 [14.29]           |
| Diabetes, n [%]      | 1 [20]          | 2 [28.57]           |
| Hypertension, n [%]  | 5 [100]         | 4 [57.1]            |
| CAD, n [%]           | 4 [80]          | 7 [100]             |
| Smoke, n [%]         | 2 [40]          | 5 [83.3] *          |
|                      | day1            | day5                |
| Hemoglobin, g/dl     | 13.52 ± 3.36    | 12.24 ± 2.22        |
| Hematocrit, %        | 40.42 ± 9.81    | 37.24 ± 6.50        |
| Leukocyte, 10^9/L    | 7.8 ± 1.47      | 13.21 ± 4.66        |
| Erythrocyte, 10^12/L | 4.31 ± 1.14     | 4.21 ± 0.62         |

Table S2: Clinical characterization of STEMI patients (related to Figure 5)
Figure S1: Characterization of MK-neutrophil interactions in vivo (related to Figure 1 and 2)

(A) Frequency distribution of MK-neutrophil interactions over 1h is shown (n=4 animals per group, 7 movies per group, 29 MKs were observed and analyzed). (B) Frequency distribution of interaction times over 1h is shown (n=4 animals per group, 7 movies per group). (C) Ratio of PPL forming MKs to non-PPL forming MKs grouped by sphericity indices (n=4 animals, 7 movies). (D) Correlation of MK surface area to occurring MK-neutrophil interactions (p=0.22), (n=4 animals, 7 movies). (E-F) Gr-1 induced neutrophil depletion model. (E) Treatment scheme and quantification of (F) leukocyte subpopulation in the peripheral blood and BM by flow cytometer (n=4 animals per group) and whole mount BM staining.
Scale bar represents 50μm. (G) Gr-1 staining of platelet and MKs, a representative histogram is shown (n=3 independent experiments). (H-J) Analysis of MK-neutrophil interaction in Gr-1 treated neutropenic and control antibody treated dual reporter mice (Pf4-cre/Confetti/Lyz2-eGFP) by video analysis. (H) Quantification of interaction frequencies within different compartments by distance to the PPL budding site (control: n=4 animals, ND: n=3 animals). (I) Frequency of PPL forming MKs in each region of interest (ROI), each symbol indicates one ROI area, multiple ROI per mice were compared (Gr-1 treatment n=4 animals; control AB treatment n=4 animals) (J) Analysis of PPL release from individual MKs per hour and maximal PPL lengths (Gr-1 treatment n=3 animals; control AB treatment n=4 animals). (K-L) Diphtheria toxin induced neutropenia in Mrp8-cre(+)/iDTR and littermate control Mrp8-cre(-)/iDTR mice. (K) Treatment scheme and (L) leukocyte subpopulations in the peripheral blood and BM were quantified by flow cytometer (n=4 animals per group). Whole mount BM staining (bar represents 50μm). Bars represent mean±SEM; symbols indicate individual animals; p-values are indicated, **<0.01, ***<0.001, ****<0.0001, n.s. not significant. P-values were determined using unpaired (I, J) Student’s t-test, two-way (F, H, L) ANOVA multigroup test and (D) Pearson’s correlation coefficient.
Figure S2: Thrombopoiesis under neutropenia and in vitro (related to Figure 2)

(A-D) Characterization of platelets and MKs in GR-1 or isotype control antibody treated mice are shown. (A) Platelet life span following biotin pulse labelling, (B) MK numbers (each symbol indicates individual MK from n=7 animals per group), (C) MK diameters (each symbol indicates individual MK) and MK ploidy (n=4 animals per group). (D) Platelet count recovery following antibody induced double depletion of platelets and neutrophils in GR-1 induced neutropenic mice. (n=4 animals per group). (E-F) Gr-1 depletion in Ly6G deficient homozygous (homo, n=4 animals) and littermate control heterozygous (hetero, n=3 animals) catchup mice are shown. (E) Leukocyte, neutrophil and (F) platelet counts are shown. (G-J) Platelet and MK
characterization in Mrp8-cre/iDTR mice with diphtheria toxin treatment. (G) Platelet life span following biotin pulse labelling. (H) MK numbers. (I) MK diameters (n=4 animals per group, each symbol indicates individual MKs analyzed in total) and MK ploidy (n=4 animals per group) are shown. (J) Platelet count recovery following double depletion of platelets and neutrophils in diphtheria toxin treated Mrp8-cre/iDTR mice (n=4 animals per group). (K) Cytokine levels of BM interstitial fluid were determined by a cytokine profile assay in Gr-1 treated neutropenic or control antibody treated mice. Cytokines related to thrombopoiesis are shown on the right (n=3 animals per group). (L) Flow cytometry gating strategy of platelet particles (PP) from co-culture supernatants. (M) In vitro analysis of PP production in co-culture supernatant after indicated treatments (n=3 independent experiments). Bars represent mean±SEM; symbols indicate individual animals; p-values are indicated, **<0.01, ****<0.0001, n.s. not significant. P-values were determined using unpaired (E, F (left panel), G) Student’s t-test and two-way (C, D, F (right panel)) ANOVA multigroup test.
Figure S3: CXCR4-CXCL12 axis regulates thrombopoiesis (related to Figure 3)

(A) Representative confocal images of in vitro cultured, fetal liver cell derived megakaryocytes, scale bars represent 30μm (n=3 independent experiments). (B) Whole mount staining for CXCL12 in P4-cre+/cxcl12−/− deficient mice or littermate controls (P4-cre−/−/cxcl12fl/fl). Representative confocal
images of three independent experiments are shown. Scale bars represent 10μm. (C-E) Two-Photon-Imaging video analysis of Mrp8-cre/Cxcr4 mice. (C) Quantification of MK-neutrophil interaction frequencies within different compartments by distance to the PPL budding site (multiple movies per mouse were included in analysis, symbols indicate individual MKs; Mrp8-cre(+)/Cxcr4ΔΔ n=3 animals; Mrp8-cre(-)/Cxcr4ΔΔ n=4 animals). (D) Flow cytometry based analysis of leukocyte subpopulation in the blood and BM (Mrp8-cre(-): n=5 animals; Mrp8-cre(+): n=4 animals). (E) Analysis of PPL lengths (symbols indicate individual MKs; Mrp8-cre(+)/Cxcr4ΔΔ n=3 animals; Mrp8-cre(-)/Cxcr4ΔΔ n=4 animals). (F) MK numbers and MK ploidy (n=3 animals per group). (G) Analysis of platelet-neutrophil aggregates in the peripheral blood of Mrp8-cre/Cxcr4 mice (n=4 animals per group). (H) Scheme of adoptive neutrophil transfer experiment is shown. (I) Distribution analysis of adoptively transferred neutrophils was done following whole mount BM staining. Representative 2D pictures of stained bones are shown (scale bar represents 100μm). Minimal MK-neutrophil distance analysis between MKs and neutrophil (n=3 animals per group). (J) Analysis of platelet and reticulated platelet counts are shown. (K) Platelet life span after biotin pulse labeling (n=3 animals per group). (L) Platelet count recovery following antibody induced immune-thrombocytopenia in Mrp8-cre(+)/Cxcr4ΔΔ and littermate control mice (n=4 animals per group). Bars represent mean±SEM; symbols indicate individual animals; MKs or proplatelets; p-values are indicated, *<0.05, **<0.01, ****<0.0001, n.s. not significant. P values were determined using unpaired (E, F (left panel), G, J) Student’s t-test and two-way (C, D, F (right panel), I, K, L) ANOVA multigroup test.
Figure S4: Neutrophil derived ROS augments platelet biogenesis in vivo and in vitro (related to Figure 4)
(A) Gating strategy to verify the efficiency of chimerism (in accordance with the ratio of CD45.2 positive cells) of Cybb⁻/⁻ Chimera is shown. (B) Analysis of platelet and reticulated platelet counts in Cybb⁺/⁺ Chimera (n=4 animals). (C) Platelet Lifespan as measured by clearance of biotinylated platelets. (D-G) In-vivo multiphoton visualization of MK-neutrophil interactions in Cybb⁺/⁺ Chimera. (D) Image sequence showing PPL formation in Cybb⁻/⁻ Chimera. Scale bar represents 20μm, Timeline (min) is indicated. (E) Analysis of proplatelet growth speeds, release time and PPL length (n=3 animals per group). (F) Quantification of MK-neutrophil interaction frequencies within different compartments and (G) within different distance categories. (H) Megakaryocyte numbers and (I) characterization of the leukocyte subpopulation ratio in blood and bone marrow by flow cytometry (n=4 animals). (J-N) N-Acetylcysteine or vehicle treatment in C57BL/6 mice over 5 days (n=5 animals per group), (J) Treatment scheme is shown (left). Quantification of platelet and reticulated platelet counts is shown. (K) Platelet life span following biotin pulse labelling. Analysis of (L) MK numbers and (M) MK ploidy. (N) Leukocyte subpopulations in the BM and peripheral blood are shown (n=3 animals per group). (O) Quantification of PP release in vitro co-culture assay with MKs isolated from Cyba⁻/⁻ or littermate control mice (n=3 independent experiments). (P) Mean fluorescence intensity of DCFDA in MKs co-cultured with or without neutrophils (individual MKs from three independent experiments are shown). (Q) ROS production in neutrophils following stimulation with indicated agonists (i.e. 200ng/ml SDF-1; 50nM PMA) was determined by 2′,7′- Dichlorfluorescin-Diacetat (DCFDA). (R) In vitro quantification of PP release following ERK, MLC inhibition or vehicle treatment (n=3 independent experiments). Bars represent mean±SEM; symbols indicate individual animals or MKs; p-values are indicated, *<0.05, **<0.01, ***<0.001, ****<0.0001, n.s. not significant. P-values were determined with unpaired (B, E, H, J, L, P) Student’s t-test, one-way (O, Q, R) or with two-way ANOVA (C, F, G, I, K, M, N) multigroup test.
Figure S5: Increased reticulated platelet counts drive risk of thrombosis in MI (related to Figure 5 and 6)
C57BL/6 mice underwent MI I/R or sham treatment, analysis was performed after 48h (n=4 animals per group). (A) Analysis of P-selectin and GPIIb/IIIa expression on immature (reticulated platelets) vs. mature platelets. Gating strategy and representative histogram plots following thrombin (0.1U/ml) stimulation are shown, respectively. (B) Quantification of platelet surface and activation marker expression on immature or mature platelets under unstimulated condition and following stimulation with U46619 (2μM) or thrombin (0.1U/ml) (n=3 animals). (C, D) Characterization of blood and BM following MI. Analysis of (C) MK numbers, MK maturation and (D) leukocyte subpopulations within the blood and BM. (E-F) Fe-(III) chloride carotid artery thrombosis was induced 48h after myocardial ischemia reperfusion (I/R) injury and sham treatment in C57BL/6 mice. (E) Thrombus size and (F) total thrombus burden over 60min was quantified by video analysis in C57BL/6 mice. (Sham: n=8 animals, MI: 9 animals). (G) Platelet and reticulated platelet counts were determined after 48h I/R and sham treatment (Mrp8-cre(-)/Cxcr4(fl/fl): n=4 mice; Mrp8-cre(+)/Cxcr4Δ/Δ: n=3 mice). (H-I) Fe-(III) chloride carotid artery thrombosis was induced 48h after MI I/R and sham treatment in Mrp8-cre/Cxcr4fl/fl mice. (H) Thrombus size and (I) total thrombus burden (over 60min) was analyzed by video analysis. (Mrp8-cre(-)/Cxcr4fl/fl: n=6 animals; Mrp8-cre(+)/Cxcr4Δ/Δ: n=5 animals). Bars represent mean±SEM, symbols indicate individual animals; p-values are indicated, *<0.05, **<0.01, ***<0.001, ****<0.0001, n.s. not significant. P-values were determined using unpaired (C (left panel), F, G, I) Student's t-test or two-way (B, C (right panel), D, E, H) ANOVA multigroup test.
Figure S6: Thrombopoiesis during venous thrombosis (related to Figure 6)

(A) Quantification of platelet, reticulated platelets, (B) white blood cells (WBC) and neutrophil counts 48 hours after induction of venous thrombosis (DVT). (C) CXCR4 surface expression was determined on peripheral neutrophils of DVT and sham treated mice by flow cytometer (n=3 animals per group), a representative histogram blot is shown. (D) Adoptive transfer experiments of neutrophils in DVT or sham treated C57BL/6 mice. Representative 2D pictures of whole-mount-stained bones are shown (scale bar represents 100μm). Minimal MK-neutrophil distance between MKs and neutrophils (n=3 animals per groups). (E) Platelet and reticulated platelet counts in peripheral blood were shown (Mrp8-cre(-)/Cxcr4fl/fl).
n=6 animals; Mrp8-cre(+)/Cxcr4Δ/Δ n=6 animals). Leukocyte count and thrombus composition analysis 48h after induction of thrombosis. (F) Immunohistological staining of vena cava thrombi harvested from Mrp8-cre(+)/Cxcr4Δ/Δ or Mrp8-cre(-)/Cxcr4fl/fl control mice (n=3 animals per group). Representative images of stained thrombi are shown, scale bar indicates 100μm. Quantification of neutrophil (MPO+/Ly6G+) and monocytes (MPO+/Ly6G-) densities by IMARIS software. (G) NET formation in thrombi was quantified by immunofluorescence microscopy. Representative images are shown, scale bar indicates 200μm. Symbols indicate individual animals; p-values are indicated, *<0.05, **<0.01, ****<0.0001, n.s. not significant. P-values were determined using unpaired (A, B, C, E, F, G) Student's t-test or two-way (D) ANOVA multigroup test.
Figure S7: Neutrophil plucking in LPS induced inflammation (related to Figure 6)

(A-E) LPS model 36h after intraperitoneal LPS application (0.1 mg/kg Bodyweight). (n=4). (A) Platelet and reticulated platelet counts. (B) Megakaryocyte number (C) Characterization of the leukocyte subpopulation ratio in blood and BM by flow cytometry. (D) Neutrophil expressed CXCR4 and (E) ROS (measured by DCFDA staining) was determined by flow cytometry. (F-H) In vivo multiphoton
visualization of MK-neutrophil interactions 36 hours after LPS treatment. (F) Image sequence showing PPL formation under LPS induced inflammation. Scale bar represents 20μm, Timeline (min) is indicated. (G) Analysis of proplatelet growth speeds, release time and PPL length (n=3 animals per group. (H) Quantification of MK-PMN interaction frequencies within different compartments (n=3 animals per group). (I) Platelet and reticulated platelet counts in Mrp8-cre/Cxcr4 mice 36h after intraperitoneal LPS injection (0.1mg/kg Bodyweight) (n=4). (J) Image sequence showing PPL formation under LPS induced inflammation. Scale bar represents 20μm, Timeline (min) is indicated. (K) Analysis of proplatelet growth speeds, release time and PPL length (n=3 animals per group). (L) Quantification of MK-neutrophil interaction frequencies within different compartments (n=3 animals per group). Bars represent mean±SEM; symbols indicate individual animals or MKs; p-values are indicated, *<0.05, **<0.01, ***<0.001, ****<0.0001, n.s. not significant. P-values were determined with unpaired (A, B, D, E, G, I, K) Student’s t-test or with two-way ANOVA (C, H, L) multigroup test.