Deficiency of ATP-Binding Cassette Transporter B6 in Megakaryocyte Progenitors Accelerates Atherosclerosis in Mice

Andrew J. Murphy,* Vincent Sarrazy,* Nan Wang, Nora Bijl, Sandra Abramowicz, Marit Westerterp, Carrie B. Welch, John D. Schuetz, Laurent Yvan-Charvet

Objective—The ATP-binding cassette (ABC) transporter B6 (ABCB6) is highly expressed in megakaryocyte progenitors, but its role in platelet production and disease has not been elucidated.

Approach and Results—Among various ABC transporters, ABCB6 was highly expressed in megakaryocyte progenitors, exhibiting the same pattern of expression of genes involved in heme synthesis pathway. Transplantation of Abcb6 deficient (Abcb6−/−) bone marrow into low density lipoprotein receptor deficient recipient mice resulted in expansion and proliferation of megakaryocyte progenitors, attributable to increased reactive oxygen species production in response to porphyrin loading. The enhanced megakaryopoiesis in Abcb6−/− bone marrow–transplanted mice was further illustrated by increased platelet counts, mean platelet volume, and platelet activity. Platelets from Abcb6−/− bone marrow–transplanted mice had higher levels of chemokine (C-C motif) ligand 5, which was associated with increased plasma chemokine (C-C motif) ligand 5 levels. There were also increased platelet–leukocyte aggregates, which resulted in leukocyte activation. Abcb6−/− bone marrow–transplanted mice had accelerated atherosclerosis which was associated with deposition of the chemotactic agent, chemokine (C-C motif) ligand 5 in atherosclerotic plaques, resulting in increased macrophage accumulation.

Conclusions—Our findings identify a new role of ABCB6 in preventing atherosclerosis development by dampening platelet production, reactivity, and chemokine (C-C motif) ligand 5 deposition in atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2014;34:751-758.)

Key Words: ATP binding cassette transporter ■ atherosclerosis ■ platelet

Platelets facilitate the recruitment of inflammatory cells toward the atherosclerotic lesion and release a plethora of inflammatory mediators that contribute to both atherogenesis and atherothrombosis. Although the local role of platelets interacting with endothelial cells to enforce leukocyte activation, adhesion, and transmigration has been well documented in atherosclerosis,5,7 the mechanisms that control platelet production are not as clear. Nevertheless, increased mean platelet volume represents a major risk factor for cardiovascular disease (CVD) and is clinically used to denote increased platelet production.3,4

Recently, the molecular mechanisms that contribute to platelet production have begun to be elucidated; this is largely attributable to the characterization of megakaryocyte progenitors (MkPs), which reside within the bone marrow (BM) sinusoids.5,7 However, the relevance of these pathways to CVDs has not yet been fully explored. In myeloproliferative neoplasms such as myelofibrosis and essential thrombocyto- sis, mutations in myeloproliferative leukemia virus oncogene, the receptor for the platelet production factor thrombopoietin, or downstream signaling molecules (ie, janus kinase-2) have been discovered as the causative links to increased platelet production and thrombocytosis.8 More recently, we identified that ATP-binding cassette (ABC) transporter G4 expression was restricted to MkPs and modulation of the activity of this lipid transporter linked platelet production to thrombocytosis.9

ABC transporters are members of a large superfamily that transport a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids, and drugs. While screening for the expression of these ABC transporters in BM hematopoietic progenitors and myeloid cells, we identified that ABC transporter B6 (ABCB6) was highly expressed in...
MkPs and hypothesized that deficiency of this transporter may in vivo modulate megakaryopoiesis and atherosclerosis.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.9–12

**Results**

**Restricted Expression of ABCB6 and Genes Involved in Heme Synthesis in Megakaryocyte–Erythrocyte Progenitors**

While screening for the expression of various ABC transporters involved in lipid metabolism in BM hematopoietic progenitors and myeloid cells of wild-type (WT) mice, we observed that the expression of Abcb6 was restricted to megakaryocyte–erythrocyte progenitors (MEPs; Figure 1A). Because ABCB6 has been involved in porphyrin transport, we next investigated the expression of genes involved in heme homeostasis. Interestingly, genes involved in heme synthesis pathway, such as δ-aminolevulinic acid dehydratase (AlaD), hydroxymethylbilane synthase (HMBS), ferrochelatase (FECH), and the porphyrin transporter ABCG2,13 exhibited the same pattern of expression across the various BM and myeloid cells as ABCB6 (Figure 1B). In contrast, heme oxygenase-1, the rate-limiting enzyme in heme catabolism and the heme sensor Rev-erbα,14 was predominantly expressed in peritoneal macrophages (Figure 1B). These observations suggested a role of ABCB6 and heme synthesis in MEP.

**ABCB6 Deficiency Promotes MkP Expansion**

Because we recently reported that lack of another ABC transporter, ABCG4 that is highly expressed in MEPs/MkPs suppressed megakaryocytosis, thrombocytosis, and atherosclerosis,9 we next investigated the relevance of ABCB6 in these pathways. We first transplanted WT and Abcb6 deficient (Abcb6−/−) BM into lethally irradiated low density lipoprotein receptor deficient (Ldlr−/−) recipient mice. Transplantation efficiency is depicted in Figure 2A. Interestingly, quantification of BM hematopoietic progenitor cells by flow cytometry revealed a selective 1.5-fold increase in both frequency and number of MEPs in Abcb6−/− BM-transplanted mice (Figure 2B, 2C, and 2D). Similar findings were observed when WT and

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>ABC</th>
<th>ATP-binding cassette</th>
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<tr>
<td>ABCB6</td>
<td>ABC transporter B6</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>CCL5</td>
<td>chemokine (C-C motif) ligand 5</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>MEP</td>
<td>megakaryocyte–erythrocyte progenitor</td>
</tr>
<tr>
<td>MkPs</td>
<td>megakaryocyte progenitors</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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AlaD indicates δ-aminolevulinic acid dehydratase; FECH, ferrochelatase; HMBS, hydroxymethylbilane synthase; and HO-1, heme oxygenase-1.
Abcb6−/− BM were transplanted into WT recipients (data not shown). Previous studies have shown that MEP population contains CD41+CD71− cells with MkP potential.5 Deficiency in ABCC6 also promoted a 1.5-fold increase in the percentage of MkPs in the BM (Figure 2E and 2F), consistent with the high expression of this transporter in these cells (Figure 2G).

**Lack of ABCB6 Increases MkP Proliferation and Platelet Expansion**

To get further insights into the mechanisms that promote Abcb6−/− MkP expansion, we first analyzed the scatter parameters of these cells as a crude viability assay because the forward-angle light scatter relates to the cell diameter and the side-angle light scatter reflects the conformation of inner cellular structure. Figure 3A revealed a shift in the size and granularity of Abcb6−/− MkPs. Thus, we next examined the in vivo proliferation of these cells by flow cytometry in the BM of WT and Abcb6−/− BM–transplanted mice. A significant increase in 5-ethynyl-2’-deoxyuridine incorporation into the DNA, reflecting enhanced proliferation, was observed in Abcb6−/− MkPs (Figure 3B). Although the switching of stem cells back and forth between quiescence and cycling are generally central to stem cell expansion, we also observed a decrease in annexin V staining, reflecting reduced apoptosis in these cells (data not shown). The in vivo relevance of these findings was revealed by increased circulating platelet counts in Abcb6−/− BM–transplanted mice throughout the study period (Figure 3C). Similar findings were observed in chow-fed Abcb6−/− mice especially as they aged (Figure 3D). Consistent with enhanced platelet production, reticulated platelets determined by thiazole orange staining were increased by 1.5-fold in these mice after analysis by flow cytometry (Figure 3E).

**Role of Reactive Oxygen Species Generation in Abcb6−/− MkP Proliferation and Platelet Expansion**

Because ABCB6 has been previously shown to prevent heme-dependent mitochondrial stress responses,10,15 we next incubated WT and Abcb6−/− BM cells for 30 minutes with or
without the heme precursor, protoporphyrin IX, and quantified the generation of reactive oxygen species (ROS) in MkP cells by flow cytometry. Lack of ABCB6 led to increased ROS generation in MkP cells, which was further enhanced when treated with protoporphyrin IX (Figure 3F). This was associated with an increase in tetramethylrhodamine ethyl ester staining, reflecting increased mitochondrial membrane potential (data not shown). Because ROS can induce a variety of responses in...
hematopoietic progenitors including cellular proliferation, and porphyrin accumulation is a key feature of highly proliferating tumor cells, we next performed a megakaryocyte-colony-forming unit assay to assess megakaryopoiesis in presence or absence of the antioxidant glutathione. The 1.5-fold increase in the number of megakaryocyte colonies in Abcb6−/− BM was partially reversed by the addition of glutathione (Figure 3G), providing evidence that ROS contributes to the enhanced proliferation. Finally, platelet counts were determined over time after platelet depletion with monoclonal antibody to CD41 (Figure 3H). At 6 hours after injection, very low platelet counts were observed in both WT and Abcb6−/− mice, whereas platelet counts were higher in Abcb6−/− mice compared with WT controls from 30 hours post injection to the end of the study (Figure 3H). Inhibition of ROS by N-acetylcysteine treatment had no effect on platelet counts in WT mice but clearly blunted the platelet recovery in Abcb6−/− mice, confirming the central role of ROS in the thrombocytosis of these mice.

**Lack of ABCB6 Promotes Platelet Expansion and Reactivity**

Consistent with the clinical use of mean platelet volume to denote increased platelet production, we found a higher mean platelet volume in Abcb6−/− BM–transplanted mice (Figure 4A). In addition, a significant increase in platelet-derived microparticles, previously shown to promote atherosclerosis by facilitating chemokine deposition to the arterial endothelium, was also observed in these mice (Figure 4B). This was associated with increased platelet reactivity as shown by the increased numbers of P-selectin (CD62P)+ platelets in Abcb6−/− BM–transplanted mice (Figure 4C and 4D). An increase in CD62P+ platelets has been associated with high platelet turnover.

**Figure 4.** Lack of ATP-binding cassette (ABC) transporter B6 (ABCB6) promotes increased platelet counts and platelet reactivity. Mean platelet volume (MPV; A) and plasma platelet-derived microparticle levels (B) in 12 weeks high-fat diet–fed low density lipoprotein receptor deficient (Ldlr−/−) recipient mice transplanted with wild-type (WT) or Abcb6−/− bone marrow (BM; n=16 animals per genotype). Representative dot plots and histograms (C) and quantification of activated CD62P+ platelets and intracellular platelet chemokine (C-C motif) ligand 5 (CCLS) content (D). Representative dot plots (E) and quantification of platelet/Ly6C+ and platelet/neutrophil interaction (F). Data are means±SEM and are representative of an experiment performed with 8 animals per group. *P<0.05 between genotypes.
Enhanced platelet chemokine (C-C motif) ligand 5 (CCL5) content was also observed in the Abcb6−/− mice (Figure 4C and 4D) and likely explained the increase in plasma CCL5 levels (Table 1). Finally, we determined the ability of platelets to interact with inflammatory leukocytes. Interestingly, although ABCB6 was barely detectable in platelets or leukocytes (data not shown), lack of ABCB6 led to an increase in platelet–leukocyte interactions, both Ly6hi monocytes and neutrophils (Figure 4E and 4F). This occurred without any significant changes in peripheral leukocyte counts (Table 2).

Accelerated Atherosclerosis in Abcb6−/− BM Recipients

To test the relevance of increased platelet production and reactivity to disease, we next determined the atherosclerosis development in Ldlr−/− recipient mice transplanted with WT or Abcb6−/− BM that were fed a high-fat diet for 12 weeks. Quantification of the atherosclerotic plaques by hematoxylin and eosin staining revealed a significant 20% increase in lesion development in mice that received Abcb6−/− BM compared with controls (Figure 5A). Histological analysis of lesions revealed a 2-fold increase in F4/80+ macrophage foam cells in atherosclerotic lesions, which was associated with enhanced CCL5 staining in the Abcb6−/− BM recipients (Figure 5B and 5C). Consistent with platelet production and reactivity being independent risk factors for CVDs beyond traditional risk factors, we observed similar body weight, plasma triglycerides, low-density lipoprotein cholesterol; plasma aspartate aminotransferase, and blood urea nitrogen levels (Table 1). However, there was a significant 2-fold increase in the adhesion molecule CD11b on the cell surface of platelet-interacting Ly6-Chi monocytes indicating they were more activated compared with platelet-interacting monocytes in the WT BM recipients (Figure 5D). We attempted to deplete platelets in Abcb6−/− BM–transplanted mice by repeated injection of anti-CD41 antibodies. However, preliminary studies suggest that repeated injections even with isotype control antibodies confounded the results. Nevertheless, our findings provide clear evidences for a role of ABCB6 in atherosclerosis by modulating megakaryopoiesis, circulating platelet levels and reactivity, CCL5 deposition, and platelet-induced activation of Ly6-C+ monocytes, ultimately manifesting as increased macrophage-rich atherosclerotic lesions.

Table 1. Metabolic Characteristics of Ldlr−/− Recipient Mice Transplanted With WT or Abcb6−/− BM After 12 Weeks of HFD

<table>
<thead>
<tr>
<th></th>
<th>WT BM→Ldlr−/−</th>
<th>Abcb6−/− BM→Ldlr−/−</th>
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<tbody>
<tr>
<td></td>
<td>12-Week HFD</td>
<td>12-Week HFD</td>
</tr>
<tr>
<td>BW, g</td>
<td>27.5±0.9</td>
<td>28.9±1.0</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>524±38</td>
<td>485±33</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>993±84</td>
<td>1044±71</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>84±3</td>
<td>88±2</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>196±18</td>
<td>239±17</td>
</tr>
<tr>
<td>BUN, mg/dL</td>
<td>18.6±0.7</td>
<td>17.1±0.7</td>
</tr>
<tr>
<td>Rantes, ng/mL</td>
<td>27.4±2.4</td>
<td>35.3±0.7*</td>
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</table>

Data are means±SEM (n=10 per group). AST indicates aspartate aminotransferase; BM, bone marrow; BUN, blood urea nitrogen; BW, body weight; HDL-C, high-density lipoprotein cholesterol; HFD, high-fat diet; LDL-C, low-density lipoprotein cholesterol; Ldlr−/−, low density lipoprotein receptor deficient; and WT, wild type.

*P<0.05 between genotypes.

Table 2. Leukocyte Counts in Ldlr−/− Recipient Mice Transplanted With WT or Abcb6−/− BM After 12 Weeks of HFD

<table>
<thead>
<tr>
<th></th>
<th>WT BM→Ldlr−/−</th>
<th>Abcb6−/− BM→Ldlr−/−</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>12-Week HFD</td>
<td>12-Week HFD</td>
</tr>
<tr>
<td>WBC, ×10^9/mL</td>
<td>7.4±0.7</td>
<td>7.5±0.5</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>54.7±2.2</td>
<td>52.6±3.2</td>
</tr>
<tr>
<td>Ly6hi monocytes, %</td>
<td>11.8±1.0</td>
<td>10.3±0.6</td>
</tr>
<tr>
<td>Ly6lo monocytes, %</td>
<td>3.7±0.5</td>
<td>4.0±0.5</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>5.4±0.3</td>
<td>6.1±0.5</td>
</tr>
<tr>
<td>B cells, %</td>
<td>19.0±2.3</td>
<td>19.7±1.5</td>
</tr>
<tr>
<td>T cells, %</td>
<td>2.5±0.2</td>
<td>3.0±0.7</td>
</tr>
</tbody>
</table>

Data are means±SEM (n=10 per group). BM indicates bone marrow; HFD, high-fat diet; Ldlr−/−, low density lipoprotein receptor deficient; WBC, white blood count; and WT, wild type.

Discussion

ABC transporters represent the largest family of transmembrane proteins involved in the transport of a wide variety of substrates across biological membranes. Several ABC transporters have been involved in the process of atherosclerosis through their pivotal role in lipid trafficking.26,27 Recent evidences suggest that some of these transporters may play a key function in hematopoietic stem and progenitor cells. We recently discovered a role for the cholesterol efflux transporters ABCA1 and ABCG1 in preventing the development of atherosclerosis through their antiproliferative effects on hematopoietic stem cells,12 whereas ABCG4 prevented atherosclerosis by suppressing the proliferation of Mkp4.9 Similarly, ABCB6 was highly expressed in Mkp cells and prevented platelet production by modulating the survival of these cells. Deficiency of ABCB6 ultimately led to increased circulating platelet levels, interaction of platelets with inflammatory leukocytes, and accelerated atherosclerosis.

Although the role of ABCB6 is still a subject of debate,10,28–30 this transporter was originally identified as transporter facilitating porphyrin import into the mitochondria and contributed to understanding the movement of porphyrins across intracellular membrane during porphyrin synthesis.31 Although the enzymatic process that produces heme is initiated by aminolevulinic acid synthase in the mitochondria and ends in the mitochondria, 4 intermediate steps are present in the cytoplasm. Consistent with this proposed function of ABCB6, we now show that the expression of Abcb6 follows the expression pattern of other key enzymes of heme synthesis in MEP cells and Abcb6-deficient cells exhibit an increased oxidative stress response that was further amplified after protoporphyrin overload. This suggested a new and unexpected function of heme synthesis in megakaryopoiesis and atherosclerosis distinct from the antioxidant and vasoprotector role of heme catabolism mediated by heme oxygenase-1 in macrophages and vasculature, respectively.31,32

By contributing to heme synthesis, ABCB6 may protect cells against a variety of mitochondrial stresses in part by
regulating hemoproteins, mitochondrial electron transport, free radical detoxification, and metabolism. Consistent with porphyrin accumulation being a key feature of highly proliferating tumor cells, and the observations that mitochondrial ROS production in BM hematopoietic progenitors may have a key role in promoting cellular proliferation, we observed an increased megakaryopoiesis and mean platelet volume in Abcb6−/− BM–transplanted mice, a major risk factor for CVDs, in part through production of young reactive platelets. This, indeed, resulted in more activated platelets in Abcb6−/− BM–transplanted mice that housed higher levels of the potent chemokine, CCL5. We also observed increased plasma CCL5 levels and deposition in the atherosclerotic lesions of these mice, which could facilitate the recruitment of leukocytes, especially Ly6hi monocytes, that were preactivated in platelet–leukocyte aggregates. These key processes likely explain the increased macrophage accumulation and atherosclerosis observed in Abcb6−/− mice.

In conclusion, our data indicate the possibility that activation of the heme synthesis pathway through upregulation of ABCB6 in MKPs may provide a novel thrombolytic approach for the treatment of CVDs in part by limiting production of young reactive platelets and their associated downstream contributions to atherogenesis.

Acknowledgments
We thank Dr Kristie Gordon for assistance with flow cytometry. The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

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Disclosures
None.

References

Significance
The porphyrin transporter ATP-binding cassette transporter B6 is highly expressed in megakaryocyte progenitor and can prevent megakaryocyte progenitor proliferation and reactive oxygen species-dependent megakaryopoiesis. Thus, by dampening platelet production and reactivity, ATP-binding cassette transporter B6 prevents chemokine (C-C motif) ligand 5 deposition in atherosclerotic lesions and macrophage accumulation. This may translate into therapeutic strategies for atherosclerosis.
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Deficiency of ATP binding cassette transporter B6 in megakaryocyte progenitors accelerates atherosclerosis in mice.

Material and Methods

**Mice.** Abcb6<sup>-/-</sup> mice on the C57BL/6 background were provided by Dr. Schuetz and were as previously described.<sup>1</sup> All mice were housed at Columbia University Medical Center according to animal welfare guidelines and the study was approved by the Columbia University animal ethics committee. Animals had ad libitum access to both food and water.

**Bone marrow transplantation.** Bone marrow (BM) transplantation was performed as previously described.<sup>2</sup> The atherosclerosis studies were conducted in female C57BL/6 Ldlr<sup>−/−</sup> mice fed a Western diet (TD 88137, Harlan Teklad) for 12 weeks.<sup>3,4</sup> Mice were allowed to recover for 5 weeks after irradiation and BM transplantation before diet studies were initiated.

**Complete blood counts.** Leukocytes, differential blood counts, platelets and mean platelet volume (MPV) were quantified from whole blood using a hematology cell counter (Ac-Tdiff, Beckman Coulter).

**Histological analysis of proximal aortas.** Proximal aortas were serially paraffin-sectioned from eight to ten animals per group and stained with hematoxvlin and eosin as previously described.<sup>2</sup> Aortic lesion size of each animals was obtained by average lesion areas in five sections from the same mouse. Platelet and macrophage immunostainings were performed on xylene-deparaffinized slides. After rehydratation and antigen retrieval with citrate buffer, stainings were performed overnight with AlexaFluor 488 F4/80 (BM8, Biolegend) and AlexaFluor 647 CCL5 antibodies (VL1, Ozyme) according to the manufacturer's instructions. Sections were treated with autofluorescence eliminator reagent (Millipore) before visualization under fluorescence microscopy. The number of positive cells per section was quantified by automated processing of the images by ImageJ software.

**Quantification of intracellular reactive oxygen species.** The generation of intracellular ROS in macrophages was estimated by incubating carboxy-H2 DCFDA reagent (Molecular Probes) according to the manufacturer’s instructions. Macrophages were visualized using a fluorescent microscope (Olympus, 20x) equipped with CCD amera (RS Photometrics). At least three separate fields from triplicate wells for each treatment condition were randomly selected and counted for cells positively stained with carboxy-H2 DCFDA reagent. Total cell
within the field were also counted using DAPI staining to give the percentage of cells generating ROS.

**Platelet preparation.**
Whole blood was collected from the inferior vena cava in ACD (10%, vol/vol) from WT or Abcb6−/− mice under anesthesia. Platelet-rich plasma (PRP) from each individual mouse was obtained from whole blood by centrifugation at 300g for 7 minutes at room temperature. Washed platelets were prepared from PRP by centrifuged at 1,000g for 10 min. After two washing steps, platelets were resuspended in modified Tyrodes-HEPES buffer (137 mM NaCl, 0.3 mM Na₂HPO₄, 2 mM KCl, 12 mM NaHCO₃, 5mM HEPES, 5 mM glucose, 1 mM CaCl₂, pH 7.3) containing 0.35% bovine serum albumin. P-selectin (CD62P, BD Pharmigen) positive platelets were detected by flow cytometry as previously described. Platelet intracellular Rantes (CCL5, Ozyme) content was determined after platelet fixation using BD Perm/Wash buffer (BD Pharmigen) according to the manufacturer's instructions. For measurement of 'reticulated platelets', platelets were resuspended in a thiazole orange solution ("Retic-Count", BD Biosciences) 30min at room temperature and then, analyzed by flow cytometry.

**Platelet depletion.**
Mice were injected i.v with 0.2mg/kg of anti-CD41 to deplete platelet and follow platelet recovery over time as previously described. Baseline platelet counts were obtained from mice before treatment and platelets were counted over time using a hematology cell counter. In one group of each genotype, mice were treated with 1mg/mL N-acetylcysteine in their drinking water. N-acetylcysteine treatment was renewed every 6 hours during daytime.

**Flow cytometry analysis.** Bone marrow cells were collected from the femas and tibias, lysed to remove RBCs and filtered before use as previously described. Freshly isolated bone marrow cells were stained with the appropriate antibodies for 30 min on ice. For haematopoietic subsets, the following lineage antibodies were used: c-Kit, Sca-1, CD135 (also known as Flt3), CD150 (Slamf1), CD34, FcgRII/III, CD41, CD71 and IL7Rα as previously described. The fluorescent carboxy-H2 DCFDA reagent was used to quantify ROS production after incubation of BM cells for 30min with 1μM protoporphyrin IX (Sigma). Proliferation assay was performed as previously described. Briefly, mice were injected with Edu 18 hours prior BM harvest and proliferation was quantified as percentage of Edu⁺ cells according to the manufacturer's instructions (eBioscences). Apoptosis was determined by Annexin V staining (Molecular Probes). Viable cells, gated by light scatter or exclusion of CD45− cells, were analyzed on a four-laser BD LSRII cell analyzer or sorted on a BD
FACSAria Cell Sorter both running with DiVa software (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.)

Colony-Forming assay. Primary Lin⁻ bone marrow cells (5x10⁴) were plated in methylcellulose-based media containing a cocktail of recombinant cytokines including TPO (50ng/mL), IL-6 (20ng/mL), and IL-3 (10 ng/mL) and incubated for 8 days according to manufacturer’s protocol (Megacult-C, Stemcell Technologies). The number of CFU-Mk per dish was scored after acetylcholinesterase staining as previously described.⁵

Leukocyte – platelet reactivity
Leukocyte-platelet interaction assay was performed as previously described.⁵ Briefly, red blood cells were lysed and the washed cells were then stained with CD45, CD115, Gr1 (Ly6-C/G), CD11b and CD41 for 30mins on ice. The cells were carefully washed, resuspended in FACS buffer and run on the LSRII to detect leukocyte platelet interactions and leukocyte activation. Viable cells were selected based on forward and side scatter characteristics and then CD45⁺ leukocytes were selected. Ly6-C⁻⁴ monocyte platelet aggregates were identified as CD115⁺Gr1⁻⁴ (Ly6-C⁻⁴) CD41⁺. Neutrophils platelet aggregates were identified as CD115⁻ Gr1⁺ (Ly6-G⁺) CD41⁺. Platelet dependent activation of Ly6-C⁻⁴ monocytes was measured as CD11b MFI after subtracting the expression of CD11b on Ly6-C⁻⁴, which were not interacting with platelets.

Platelet derived microparticles
Blood was collected via the tail vein into EDTA lined tubes on ice and plasma was obtained via centrifugation. Equal amounts of plasma (20 μL) from each sample was diluted with HEPES binding buffer (80 μL) and then incubated with annexin V and anti-CD41. Equal amounts of 1 μm beads (Invitrogen) were added to the sample, which was then run on an LSR-II. Platelet derived microparticles were detected as particles less than 1 μm in size that stained positive for CD41 and annexin V. A stopping gate was placed over the beads, to ensure accurate counting in each sample. Data was converted to number of microparticles per 1 μL of whole blood.

Antibodies. CD2 (RM2-5), CD3e (145-2C11), CD4 (GK1.5), CD8b (53-6.7), CD19 (eBio1D3), CD45R (B220, RA3-6B2), Gr-1 (Ly6G, RB6-8C5), Cd11b (Mac1, M1/70), Ter119 (Ly76) and NK1.1 (Ly53, PK136)-FITC were all from eBioscience and used for lineage determination. c-Kit (CD117, ACK2)-APCeFluor780 from eBioscience, Sca-1-Pacific blue from Biolegend, FcγRII/III-PE (CD16/32, 2.4G2), CD34 (RAM34)-AlexaFluor 647, CD135
(Flt3, A2F10)-PE, IL7Rα (87R34)-PECy7, CD41 (MWReg30)-PECy7 were from Biolegend and used to quantify progenitor subsets. FcyRII/III-Pacific blue (CD16/32, 2.4G2), CD34 (RAM34)-AlexaFluor 647, CD71-PE and CD41-PECy7 were from eBioscience and used to quantify erythrocyte-megakaryocyte progenitors. For BM monocytes, neutrophils, oesinophils, T and B-cells analysis, CD115 (AFS98)-APC, B220 (RA3-6B2)-FITC, CD45 (30-F11)-APCCy7, Gr-1 (RB6-8C5)-PercPCy5.5, CD3 (17A2)-PE, CD8a (53-6.7)-PECy7 were from eBioscience and BD Biosciences, respectively.

**RNA analysis.** Total RNA extraction, cDNA synthesis and real-time PCR was performed as described previously. Ribosomal m36B4 RNA expression was used to account for variability in the initial quantities of mRNA.

**Statistical analysis.** Statistical significance was performed by two-tailed parametric student’s t test, non-parametric Mann-Whitney U test (2-group lesion area), or by one-way analysis of variance (ANOVA, 4-group comparisons) with a Bonferroni multiple comparison post-test (GraphPad software, San Diego, CA). Differences were considered significant at P < 0.05.

**References**


