PLATELETS AND THROMBOPOIESIS

Genetic studies reveal an unexpected negative regulatory role for Jak2 in thrombopoiesis

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Key Points

- Jak2 deletion in PLTs and MKs leads to thrombocytosis due to dysregulated TPO turnover.
- Jak2 loss in PLTs/MKs induces non-autonomous expansion of stem/progenitors, and specifically of MK-primed hematopoietic stem cells (HSCs).

Introduction

Myeloproliferative neoplasms (MPN) are characterized by clonal proliferation of mature myeloid cells. Somatic mutations in the JAK2 pathway, including JAK2V617F, Jak2 exon 12 mutations, and in the thrombopoietin (TPO) receptor MPL are observed in MPN patients. JAK kinase inhibitors attenuate splenomegaly and constitutional symptoms in MPN, but cannot induce molecular responses. JAK inhibitor therapy results in the variable development of anemia and thrombocytopenia preventing dose intensification and maximal target inhibition. Cytopenias have been considered on-target effects of JAK2 inhibitors inseparable from therapeutic efficacy. However, the risk of thrombocytopenia varies among JAK2 inhibitors, suggesting thrombocytopenia may not be a direct consequence of JAK2 inhibition.

JAK2 mediates cytokine signaling from erythropoietin, TPO, and granulocyte-macrophage (GM)—colony-stimulating factor receptors. Germline deletion of Jak2 causes embryonic lethality by deficient definitive erythropoiesis, which was confirmed by a conditional Jak2 allele and germline Cre-recombinase. Conditional Jak2 loss in hematopoietic stem cells (HSCs)/progenitor cells induces anemia and thrombocytopenia, whereas deletion of Mpl in platelets (PLTs) leads to thrombocytosis. Although these studies highlight the significance of Jak-Stat signaling for steady state hematopoiesis, the specific requirement for Jak2 in different terminally differentiated myeloid lineages, including PLTs, has not been clarified. We, therefore, investigated the effects of PLT-specific Jak2 loss in vivo.

Study design

Mice

Jak2f/f mice were crossed with P4f-Cre mice. Animal care was performed according to institutional and international guidelines.

Signaling

PLTs were purified on a sepharose CL-2B column, and stimulated with TPO and lysates probed for Jak2, Stat3/5, Tyk2, β-actin (Cell Signaling), and Mpl (Wei Tong, Philadelphia).

Flow cytometry

Bone marrow was stained for lineage markers, Sca-1, c-Kit, CD41, CD150, CD48, CD16/32, and CD105 (eBioscience), and PLTs for CD42, CD61, and Mpl, and analyzed/sorted on FACSAria III (BD Biosciences). Megakaryocytes (MKs) were purified by CD41-phycocerythin-magnetic beads (Stemcell Technologies).

JAK inhibitor treatment is limited by the variable development of anemia and thrombocytopenia thought to be due to on-target JAK2 inhibition. We evaluated the impact of Jak2 deletion in platelets (PLTs) and megakaryocytes (MKs) on blood counts, stem/progenitor cells, and Jak-Stat signaling. P4f-Cre–mediated Jak2 deletion in PLTs and MKs did not compromise PLT formation but caused thrombocytosis, and resulted in expansion of MK progenitors and Lin−Sca1+Kit+ cells. Serum thrombopoietin (TPO) was maintained at normal levels in P4f-Cre–positive Jak2f/f mice, consistent with reduced internalization/turnover by Jak2-deficient PLTs. These data demonstrate that Jak2 in terminal megakaryopoiesis is not required for PLT production, and that Jak2 loss in PLTs and MKs results in non-autonomous expansion of stem/progenitors and of MKs and PLTs via dysregulated TPO turnover. This suggests that the thrombocytopenia frequently seen with JAK inhibitor treatment is not due to JAK2 inhibition in PLTs and MKs, but rather due to JAK2 inhibition in stem/progenitor cells. (Blood. 2014;124(14):2280-2284)

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Figure 1. Jak2 loss in PLTs and MKs of Pf4-Cre–positive Jak2\(^{f/f}\) mice results in a thrombocytosis phenotype. (A) Jak2 mRNA expression is abolished in PLTs and MKs, and reduced in MKPs (mean reduction 33%, \(P = .029\)) of Pf4-Cre–positive (Pf4 Cre\(^{1}\)) Jak2\(^{f/f}\) mice, whereas it is preserved in Pre-MegE and LSK cells, consistent with Jak2 loss in late megakaryo-/thrombopoiesis. Mpl mRNA expression is unchanged in MKs of Pf4-Cre–positive Jak2\(^{f/f}\) mice (\(n = 3\) to 6 mice per group and population). (B) Jak2 protein is absent in PLTs of Pf4-Cre–positive Jak2\(^{f/f}\) mice and intact in Pf4-Cre–negative Jak2\(^{f/f}\) littermate controls, while the TPO receptor Mpl is expressed. (C) Mpl protein expression in PLTs, as assessed by densitometry of Western bands is slightly reduced (\(P = .05\)). (D) PLTs devoid of Jak2 in Pf4-Cre–positive Jak2\(^{f/f}\) mice are not reactive to stimulation with TPO (100 ng/ml) with inactive Stat3 and Stat5, whereas Jak signaling is activated in Pf4-Cre–negative Jak2\(^{f/f}\) littermate controls. Tyk2 remains inactive in the absence of Jak2. (E) Elevated PLTs with unaffected survival and volume in Pf4-Cre–positive Jak2\(^{f/f}\) mice, as compared with littermate controls. Tyk2 remains inactive in the absence of Jak2. (F) WBC count is slightly elevated in Pf4-Cre–positive Jak2\(^{f/f}\) mice. (G) Elevated WBCs are due to a proportionate expansion of all leukocyte subsets with a subtle relative disadvantage of lymphocytes in Pf4-Cre–positive Jak2\(^{f/f}\) mice. Results are representative of at least 2 independent experiments and shown as mean ± SEM. *\(P < .05\); **\(P < .01\); ****\(P < .0001\).
Figure 2. Cell non-autonomous expansion of stem/progenitor cells and LSK bias toward megakaryopoiesis in Pf4-Cre–positive Jak2<sup>ff</sup> mice. (A) MKs, MKPs, and Pre-MegE are expanded in Pf4-Cre–positive (Pf4 Cre<sup>1</sup> Jak2<sup>ff</sup>) mice as assessed by flow cytometry according to Pronk et al. 21 (n = 3 to 12 mice per group and population). (B) Megakaryocytic expansion is highlighted by hematoxylin and eosin stained bone marrow. (C) Formation of MK colonies from 0.5 x 10<sup>5</sup> whole bone marrow cells, 3000 sorted MKP is increased in Pf4-Cre–positive Jak2<sup>ff</sup> mice. (D) Flow cytometry gating scheme for megakaryocyte progenitors according to Pronk et al. 21 (E) LSK cells are expanded in Pf4-Cre–positive Jak2<sup>ff</sup> bone marrow analogous to Pre-MegE and MKP (n = 5 to 11 mice per group). (F) Formation of MK colonies from 3000 sorted LSK cells is increased in Pf4-Cre–positive Jak2<sup>ff</sup> mice. (G) Flow cytometry gating scheme for MK-primed LSK subsets based on c-Kit surface expression analogous to Shin et al. 26 (H) LSK CD150<sup>+</sup> CD48<sup>-</sup> HSCs are skewed toward MK-primed HSCs (c-Kit<sup>hi</sup>) in Pf4-Cre–positive Jak2<sup>ff</sup> mice. (I) Serum TPO is maintained at high steady state levels despite thrombocytosis in Pf4-Cre–positive Jak2<sup>ff</sup> mice, while serum TPO is reduced in thrombocytemic mice with the MPLW<sup>515L</sup> allele and intact Jak2<sup>ff</sup> (n = 5 to
Quantitative polymerase chain reaction

RNA was isolated in TRIzol (Life Technologies) and reverse transcribed by the Verso cDNA Kit (Thermo) for quantitative polymerase chain reaction with SYBR Green (Applied Biosystems) and β-actin for normalization.

Colony assays

Acetylcholinesterase-positive MK colonies were grown from 0.5 to 10^4 bone marrow cells or 3000 megakaryocyte-erythroid progenitors (Pre-MegE), MK progenitors (MKPs), or Lin^− Sca^1^ Kit^− ^ (LSK) cells in MegaCult-C (Stemcell Technologies) with TPO, IL-6, and IL-3 (R&D). Myeloid colonies from 4000 Pre-MegE, MKP, or 200 LSK cells were cultured in MethoCult (Stemcell Technologies) with stem cell factor, IL-3, IL-6, and erythropoietin.

Thrombopoietin measurement

TPO was determined by Quantikine enzyme-linked immunosorbent assay (R&D) in serum, or in media incubated with purified PLTs.

Results and discussion

To assess the requirement for Jak2 in PLTs, we deleted Jak2 in PLTs and MKs using Cre-recombinase expressed under the P4 promoter.14,19,20,23 P4-Cre–positive Jak2^f/f mice showed loss of Jak2 messenger RNA (mRNA) expression in PLTs and MKs demonstrating complete excision, and we observed a modest reduction in Jak2 mRNA expression in MKPs (P = .029) (Figure 1A). Jak2 mRNA expression was unaffected in Pre-MegE or LSK cells (n = 3 to 6 mice/group). Western blot analysis revealed loss of Jak2 protein expression in P4-Cre–positive Jak2^f/f PLTs, despite continued Mpl expression (Figure 1B-C). Jak2 deletion in PLTs abrogated Jak2, Stat3, Stat5, and Tyk2 phosphorylation upon TPO stimulation (Figure 1D). The absence of residual Tyk2 phosphorylation was consistent with cell line studies, suggesting that TPO-mediated activation of Tyk2 requires Jak2.24

We next investigated the impact of PLT-specific Jak2 deletion in vivo. P4-Cre–positive Jak2^f/f mice developed thrombocytosis with average PLTs of 4720 G/l (range 3436 to 6460 G/l, P < .0001) at 2 months and 5966 G/l (3204 to 7892 G/l, P < .0001) at 5 months of age (n = 5 to 17 mice/group; Figure 1E). PLT survival assessed by in vivo biotinylation and PLT volume were normal in P4-Cre–positive Jak2^f/f mice, as reflected by TPO depletion of media incubated with purified PLTs, is compromised as compared with littermate controls. TPO 884 pg/mL vs 1051 pg/mL, (P < .0001), (n = 2 to 3 mice/group; Figure 2F). TPO uptake by PLTs of P4-Cre–positive Jak2^f/f mice, as reflected by TPO depletion of media incubated with purified PLTs, is compromised as compared with littermate controls. Results are representative of at least 2 independent experiments and shown as mean ± SEM. *P < .05. **P < .01. ***P < .001. ****P < .0001.

We observed an increase in colony-forming unit (CFU)-GEMM and CFU-GM colonies but not BFU-E colonies from P4-Cre–positive Jak2^f/f mice (see supplemental Figure 1A-B, available on the Blood Web site). These data demonstrate that PLT/MK-specific Jak2 loss induces expansion of megakaryopoesis in vivo.

We next assessed the impact of PLT-specific Jak2 loss on HSC/progenitor cells in vivo. We observed a significant expansion in LSK number (567% of controls, P < .0001, (n = 5 to 11 mice/group; Figure 2E). LSK cells from P4-Cre–positive Jak2^f/f mice showed enhanced CFU-GEMM and CFU-GM colony formation (supplemental Figure 1C) and marked expansion in megakaryocytic colony formation on a per-cell basis (Figure 2F). This suggested the possibility that P4-Cre–positive Jak2^f/f mice had selective expansion of MK–^primed^ stem cells.25 Consistent with recent studies showing MK bias of c-Kithi HSC,26 P4-Cre–positive Jak2^f/f mice had significant expansion of c-Kithi Sca^1^ Kit^− ^ CD150^− ^ CD48^− ^ HSC (Figure 2G–H). These data demonstrate PLT-specific Jak2 loss induces expansion of MK-biased stem/progenitors.

Previous studies have shown that PLTs and MKs regulate serum TPO through internalization attenuating TPO levels in thrombocytosis.27,28 We hypothesized that Jak2 loss in PLTs and MKs would compromise TPO internalization and maintain high steady state TPO despite thrombocytosis. We assessed TPO levels in P4-Cre–positive Jak2^f/f mice, controls, and mice expressing MPLW515L,29 which develop a similar extent of thrombocytosis and MK expansion as P4-Cre–positive Jak2^f/f mice. Serum TPO was markedly reduced in MPLW515L mice (TPO 230 ± 37 pg/mL in MPLW515L vs 2787 ± 204 pg/mL in controls, P < .0001), but not in P4-Cre–positive Jak2^f/f mice (TPO 2524 ± 230 pg/mL, P < .0001) (n = 8 to 13 mice/group; Figure 2F). Jak2-deficient PLTs internalized less TPO than PLTs from P4-Cre–negative Jak2^f/f controls (ATP 884 pg/mL vs 1051 pg/mL, P < .01; Figure 2K). Mpl mRNA expression was unchanged in MKs of P4-Cre–positive Jak2^f/f mice (Figure 1A), while Mpl total and surface protein was modestly reduced in Jak2-deficient PLTs (Figures 1C and 2J). These data suggest that TPO levels are maintained at an inappropriately high steadystate in P4-Cre–positive Jak2^f/f mice due to reduced TPO internalization by Jak2-deficient PLTs.

In conclusion, our data demonstrate that Jak2 is dispensable for the production of MKs and PLTs. This finding suggests that thrombocytopenia observed with certain JAK2 inhibitors is due to JAK2 inhibition in more proximal stem/progenitor cells consistent with conditional Jak2 knockout studies in hematopoiesis.15,16 or potentially due to inhibition of other targets. Analogous to genetic Jak2 loss, Jak2 inhibition in PLTs/MKs may actually enhance PLT production,30 but may be overruled by the impact of Jak2 inhibition on stem/progenitors. Complementary to previous studies on the role of Mpl in PLTs,17–19 our results demonstrate an important role for PLT-specific Jak2 signaling in regulating TPO turnover, and show that PLT Jak2 signaling negatively regulates stem/progenitor cells, including MK–primed HSCs. Notably, HSCs biased toward megakaryopoesis have been shown to express higher levels of MK-specific genes, including Mpl.25,26 Increased Mpl in MK–primed HSCs might enhance susceptibility to TPO in P4-Cre–positive Jak2^f/f mice and contribute to thrombocytosis. Recent studies further suggest a role for MKs in regulating HSCs and have shown that PLT secretory factors can impact HSC numbers.31 Taken together, these data underscore the dynamic interaction between PLTs, MKPs, and stem/progenitors.
and suggest that signaling in mature myeloid lineages can alter stem/progenitor function in vivo.

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Authorship

Contribution: S.C.M., M.D.K., B.A.W., L.M.L., L.B., M.K., N.B., and S.M. performed experiments; and S.C.M. and R.L.L. designed research, analyzed data, and wrote and edited the manuscript.

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