Expansion of EPOR-negative macrophages besides erythroblasts by elevated EPOR signaling in erythrocytosis mouse models

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ABSTRACT

Activated erythropoietin (EPO) receptor (EPOR) signaling causes erythrocytosis. The important role of macrophages for the erythroid expansion and differentiation process has been reported, both in baseline and stress erythropoiesis. However, the significance of EPOR signaling for regulation of macrophages contributing to erythropoiesis has not been fully understood. Here we show that EPOR signaling activation quickly expands both erythrocytes and macrophages *in vivo* in mouse models of primary and secondary erythrocytosis. To mimic the chimeric condition and expansion of the disease clone in the polycythemia vera patients, we combined Cre-inducible *Jak2*$^{V617F/+}$ allele with *LysM*-Cre allele which expresses in mature myeloid cells and some of the HSC/Ps (*LysM*-Cre;*Jak2*$^{V617F/+}$ mice). We also generated inducible EPO-mediated secondary erythrocytosis models using *Alb-Cre, Rosa26-loxP-stop-loxP*-rtTA, and doxycycline inducible *EPAS1*-double point mutant (DPM) alleles (*Alb*-Cre;DPM mice). Both models developed a similar degree of erythrocytosis. Macrophages were also increased in both models without increase of major inflammatory cytokines and chemokines. EPO administration also quickly induced these macrophages in wild-type mice before observable erythrocytosis. These findings suggest that EPOR signaling activation could induce not only erythroid cell expansion, but also macrophages. Surprisingly, an *in vivo* genetic approach indicated that most of those macrophages do not express EPOR, but erythroid cells and macrophages contacted tightly with each other. Given the importance of the central macrophages as a niche for erythropoiesis, further elucidation of the EPOR signaling mediated-regulatory mechanisms underlying macrophage induction might reveal a potential therapeutic target for erythrocytosis.

Introduction

The glycoprotein hormone erythropoietin (EPO)[1,2] and EPO receptor (EPOR)[3,4] signaling tightly controls red cell mass (RCM). EPOR signaling activates several downstream pathways through Janus kinase 2 (JAK2).[5,6] Activation of EPOR signaling leads to erythrocytosis, which is defined as an absolute increase in RCM.[7] EPOR primarily expresses on colony forming unit-erythroid (CFU-E) to basophilic erythroblasts.[8,9] It has also been reported that EPOR is expressed in non-erythroid cells, such as macrophages;[2] and non-hematopoietic tissues, such as the brain, kidney, and heart.[14] However, the technical limitations for EPOR detection and the possibility of false-positive results in some cases have also been reported.[14,15]

Erythrocytosis is defined as primary when developed by an EPO-independent,

erythroid cell-intrinsic mechanism due to constitutively activated EPOR signaling by gain-of-function *EPOR* or *JAK2* mutations.[7,16,17] In contrast, secondary erythrocytosis is due to an erythroid cell-extrinsic, EPO-dependent mechanism.[7,16] EPAS1 (also known as HIF2A) is a master regulator of EPO gene expression.[18] In normoxia, EPAS1 protein is immediately degraded through a variety of posttranslational modifications. Hypoxia, or a defect in the oxygen-sensing signaling pathway due to tumors or mutations, leads to excess EPO production and secondary erythrocytosis.[16] Several mouse models mimicking primary or secondary erythrocytosis have been reported.[19-21] However, there is no head-to-head comparison of the pathobiology between primary and secondary erythrocytosis in these models. To understand the dynamics of EPOR signaling-mediated erythrocytosis and to establish new therapeutic strategies, animal models which present faithful clinically relevant phenotypes are needed.

It has been proposed over several decades that a subset of macrophages participate in erythroblastic island (EIs) formation and support erythropoiesis by providing iron, promoting proliferation of erythroblasts, and being involved in the enucleation process.[22-25] Recently, the critical role of macrophages, not only in baseline (homeostatic) erythropoiesis but also in stress erythropoiesis, has been demonstrated.[26,27] Specific depletion of macrophages in the *JAK2*V617F-mediated polycythemia vera (PV) mouse models was shown to rescue the PV erythrocytosis phenotype.[26,27] However, how EPOR signaling influences these macrophages in the erythrocytosis context is still not fully understood.

In this study, we generated novel erythrocytosis mouse models which faithfully recapitulate both primary and secondary erythrocytosis, and we utilized them to address the effect and significance of EPOR signaling activation on erythroid cells and other cell types *in vivo*. We found that EPOR signaling activation could induce not only erythroid cell expansion but also macrophages.

Methods

Mice

The mice with the genotype of Cre-inducible *Jak2*$^{V617F/+}$ and EPOR-Cre have been described previously.[28,29] *LysM-Cre, Alb*-Cre, and *Rosa26-LSL-rtTA-GFP* mice were purchased from Jackson Laboratory. C57BL/6 mice were obtained from the Cincinnati Children's Hospital Medical Center (CCHMC) / Cancer and Blood Diseases Institute mouse core. The *EPAS1* (also known as HIF2A) double point mutant (DPM) constructs (Pro531Ala and Asn847Ala) were generated by PCR mutagenesis using human *EPAS1* cDNA as a template. The IRES sequence was PCR amplified from the pIRES2-EGFP vector (Clontech, Mountain View, CA, USA) and cloned onto the multiple cloning site of the (tetO)7CMV-bGH-poly(A) vector. Next, a FLAG tagged human ARNT cDNA was cloned 3' of the IRES sequence and the EPAS1-DPM cDNA was cloned 5' of the IRES sequence. The constructs were linearized and microinjected individually into the pronucleus of fertilized eggs from FVB/N mice as previously described.[30] All mice were backcrossed to C57BL/6 strain mice at least eight times. All animals were housed in the animal barrier facility at CCHMC. All animal studies were conducted according to an approved Institutional Animal Care and Use Committee protocol and federal regulations.

Flow cytometric analysis

Flow cytometric analyses and cell sorting were performed with FACSCanto II or FACSAria II instruments (BD, San Jose, CA, USA). Peripheral blood (PB), bone marrow (BM), spleen (SP), and liver cells were immunostained using the following antibodies: CD45 (30-F11), CD71 (C2), CD44 (IM7) (BD), CD11b (M1/70), CD34 (RAM34), CD16/32 (93) (eBioscience, San Diego, CA, USA), CD115 (AFS98), Ly6G (RB6-8C5), Gr-1 (RB6-8C5), F4/80 (BM8), Ter119 (Ter-119), Sca-1 (D7) (Biolegend, San Diego, CA, USA), and c-Kit (2B8) (BD). For the staining of hematopoietic stem cells and progenitors, lineage marker cocktail containing anti-mouse CD3e (145-2C11), CD4 (RM4-5), CD8a (53-6.7), B220 (RA3-6B2), Ter119 (Ter-119), CD11b (M1/70), and Gr-1 (RB6-8C5) antibodies (all from BD) was used. Macrophage populations were defined as Gr-1^{low} CD115^{int} F4/80^{+} SSC^{low}.

Phosphate buffered saline (PBS) with 2% fetal bovine serum (FBS) was used as a FACS buffer. For some experiments, 2-5 mM ethylenediaminetetraacetic acid (EDTA) was added to the FACS buffer. To obtain the hematopoietic cells, SP and liver were mashed through a 100 μm cell strainer and resuspended in the FACS buffer. Data were analyzed with the FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis

Statistical analyses were performed using Student *t*-test or one-/two-way ANOVA with multiple comparisons correction. Survival of mice was analyzed using the log rank test. $P<0.05$ was considered statistically significant.

Information concerning quantitative RT-PCR, multiplex immunoassay, ELISA, histology, Wright Giemsa staining, and colony forming assay is available in the *Online Supplementary Methods*.

Results

Pathophysiological modeling of primary and secondary erythrocytosis in novel mouse models

To explore in detail the role of EPOR signaling in erythrocytosis development *in vivo*, we generated two mouse models of primary and secondary erythrocytosis. PV is the most common disease characterized by primary erythrocytosis, arising from acquired somatic *JAK2* mutations in hematopoietic stem cells (HSCs).[31-34] Interestingly, not all of the HSCs and progenitors in the BM of PV patients have JAK2 mutations; normal or other clones besides *JAK2* mutant clones may also be present. The PV phenotype arises from HSC/Ps clones carrying JAK2 mutations over time. Although Vav1-Cre- or EIIA-Cre-mediated physiological PV models using this same conditional allele have been reported, the caveat is that, in these models, all of the hematopoietic cells have *Jak2* mutation before birth.[27,28] Mx1-Cre-mediated conditional allele has also been reported;[35] again, in these models, all of the hematopoietic cells have *Jak2* mutation. To faithfully recapitulate this chimeric condition and the natural clonal expansion process happening in PV patients with the *JAK2* mutation, we used LysM-Cre allele and combined it with Cre-inducible *JAK2*^{V617F} mutant knock-in heterozygous allele (*JAK2*$^{V617F/+}$),[28] then generated *LysM*-Cre;*Jak2*$^{V617F/+}$ mice (LysM-Cre;*Jak2*^{V617F} mice) (Figure 1A). LysM-Cre allele expresses not only in mature myelomonocytic cells, but also in some subsets of HSCs and progenitor cells.[36] To confirm the expression pattern of LysM-Cre, we used *Rosa26-loxP-Stop-loxP* (*Rosa26LSL*)-

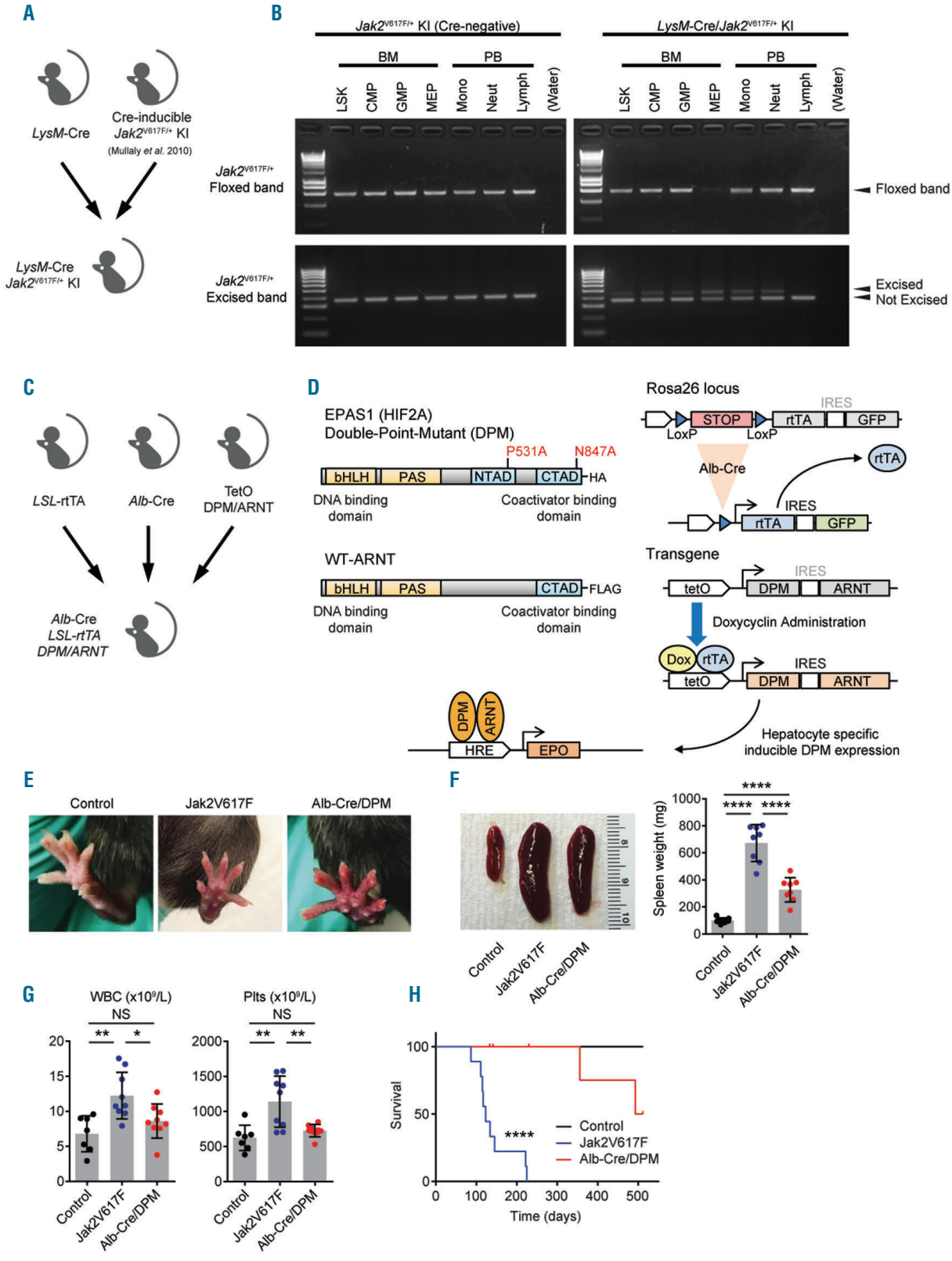

Figure 1. ***In vivo*** **pathophysiological modeling of primary and secondary erythrocytosis.** (A) Primary erythrocytosis model using *Lys*M-Cre allele, and Cre-inducible *Jak2*$^{V617F/+}$ knock-in allele. (B) PCR of DNA of sorted bone marrow (BM) or peripheral blood (PB) cells from *Jak2V617F*-mutant knock-in (KI) mice with or without *LysM*-Cre. (C and D) Inducible secondary erythrocytosis model using indicated alleles. Schematic for *EPAS1* double point mutant (DPM) and doxycycline-inducible hepatocyte-specific induction of the DPM protein is shown in (D). (E) Appearance of hind paw of the indicated mice. (F) Spleen size in the indicated mice (n=8 each). (G) White blood cell (WBC) counts and platelet (Plts) counts in PB from the indicated mice at 2-3 months of age (Control: n=7; *LysM*-Cre;*Jak2*V617F mice; n=9) or 2 months after HIF-2α induction (n=9). Data are mean±Standard Deviation (s.d.). (H) Survival of *LysM*-Cre; *Jak2*V617F mice (n=9) and doxycycline-administrated *Alb*-Cre;DPM mice (n=9). *P<0.05; **P<0.01; ****P<0.0001; NS: not significant [one-way ANOVA with multiple comparisons correction (G) or two-way ANOVA with multiple comparisons correction (F), and log rank test (H)].

eGFP reporter allele (*Online Supplementary Figure S1A*). GFP expression was observed in the subsets of lineage marker- Sca-1^{+} c-Kit^{+} cells (LSKs), common-myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythrocyte progenitors (MEPs), as well as in mature myeloid cells (granulocytes and monocytes) (*Online Supplementary Figure S1B*). We did not find GFP expression in the mature lymphoid lineage (*Online Supplementary Figure S1B*). These results are consistent with previous reports.[36] To confirm LysM-Cre mediated excision of wild-type exon and inversion of *Jak2*$^{V617F/+}$ mutant allele in *LysM*-Cre;*Jak2*^{V617F} mice, we sorted BM (LSKs, CMPs, GMPs, and MEPs) and PB (monocytes, neutrophils, and lymphocytes) cells from *Jak2*$^{V617F/+}$ mice with or without LysM-Cre. We then performed PCR using the primers previously described.[28] Consistent with the GFP expression pattern in the cells from LysM-Cre;GFP reporter mice (*Online Supplementary Figure S1B*), we found both bands for floxed allele and inversion of *Jak2*$^{V617F/+}$ mutant in LSKs, CMPs, GMPs, MEPs, monocytes, and neutrophils, but not in lymphocytes, from *LysM*-Cre;*Jak2*$^{V617F/+}$ mice (Figure 1B). Interestingly, in MEPs from *LysM*-Cre;*Jak2*^{V617F} mice, we found a weak band for the floxed allele and a strong band for Cre-mediated excision (Figure 1B), suggesting the expansion of the *Jak2*^{V617F} cells within the MEPs. Indeed, we found increased GFP^{+} cells in MEPs along with LSKs, CMPs, and GMPs in *LysM*-Cre;GFP/*Jak2*^{V617F} reporter PV mice (*Online Supplementary Figure S1C* and *D*). BFU-E and CFU-E were

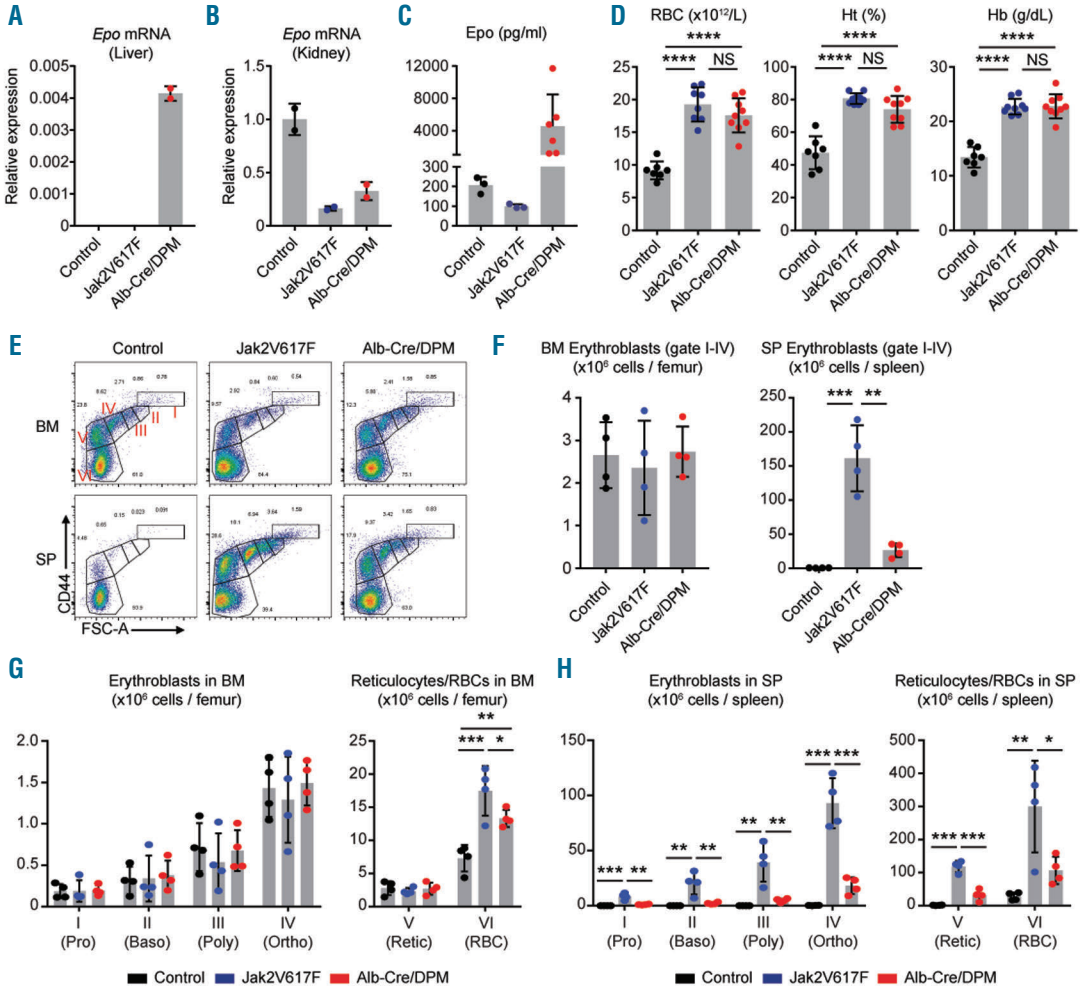

Figure 2. The novel mouse models *LysM*-Cre;*Jak2*V617F and *Alb*-Cre;DPM demonstrate a similar degree of erythrocytosis. (A and B) EPO mRNA expression in hepatocytes (A) and kidney (B) from indicated mice. Data are mean±Standard Deviation (s.d.) from duplicate samples. Results are representative of two independent experiments. (C) Serum erythropoietin (EPO) levels in the indicated mice (n=3-6 each). (D) Red blood cell (RBC) counts, hematocrit (Ht), and hemoglobin (Hb) counts in peripheral blood (PB) from the indicated mice at 2-3 months of age (Control, n=7; *Lys*M-Cre;*Jak2*V617F mice, n=9) or two months after HIF-2α induction (n=9). Data are mean±Standard Deviation (s.d.). (E-H) Flow cytometric analysis of erythroid lineage cells in bone marrow (BM) and spleen (SP) from the indicated mice. Representative plots of erythropoiesis profiles (CD44 and FSC) in BM and SP CD45-, Ter119$^{dim/+}$ population (E), and absolute number of erythroblasts (F) and each fraction of erythroblasts/mature erythroid cells in BM and SP (G and H) from indicated mice are shown (n=4 each). I (Pro): proerythroblasts; II (Baso): basophilic erythroblasts; III (Poly): polychromatic erythroblasts; IV (Ortho): orthochromatic erythroblasts; V (Retic): reticulocytes; VI (RBC): red blood cells. Data are mean±s.d. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS: not significant (one-way ANOVA with multiple comparisons correction (D) or two-way ANOVA with multiple comparisons correction (F-H)).

also increased in LysM-Cre;Jak2V617F mice (*Online Supplementary Figure S1E*).

Next, in order to model the EPO-induced secondary erythrocytosis, we generated a liver-specific inducible *EPAS1* double-point-mutant (DPM) (P531A and N847A) transgenic mouse (Figure 1C and D). Besides germline mutations, other acquired conditions, such as hypoxia or tumors, could also cause excessive EPO-mediated secondary erythrocytosis at various ages. Thus, we decided to generate a mouse model in which the excessive EPO condition can be induced. Liver is a major source of EPO during embryonic and early postnatal development in mice.[18] In adult erythropoiesis, although the kidneys become the major source of EPO, the hepatocytes still retain the capacity for EPO production.[37] Various gain-of-function *EPAS1* mutations, near or at the primary hydroxylation site proline-531 (P531), have been identified.[16,18,38] The EPAS1-DPM allele has mutations in P531 site, which is an important site for the normal EPAS1 protein degradation in normoxia, and in the asparagine-847 (N847) site, which is a critical site for transcriptional inactivation in normoxia (Figure 2B). Using hepatocyte-specific Alb-Cre allele and Rosa26LSL reverse-tetracycline-controlled transactivator (rtTA) driver (Rosa26LSL-rtTA), stable and constitutively active EPAS1 protein was induced in the adult hepatocytes in a doxycycline-dependent manner (Figure 1D). We compared the results from these mice with the results from wild-type control mice.

Both *LysM-Cre;Jak2*^{V617F} and Alb-Cre;DPM mice presented observable redness in the mouth and front and hind paws (Figure 1E). *LysM-Cre;Jak2*^{V617F} mice showed more severe splenomegaly than Alb-Cre;DPM mice (Figure 1F). We also determined red and white pulps of SP in these mice. The white pulp was diminished and the normal SP architecture was destroyed in both *LysM-Cre;Jak2*^{V617F} and *Alb*-Cre;DPM mice in comparison with control mice (*Online Supplementary Figure S2*). White blood cell (WBC) and platelet (Plts) counts in the PB from LysM-Cre;Jak2V617F mice were significantly higher than those in the PB from wild-type and *Alb*-Cre;DPM mice (Figure 1G). The median survival of the *LysM-Cre;Jak2*^{V617F} mice was 123 days, while the *Alb*-Cre;DPM mice survived longer (Figure 1H). Taken together, both our mouse models could quickly develop erythrocytosis, recapitulating the pathophysiological condition of human primary and secondary erythrocytosis.

Both primary and secondary erythrocytosis mouse models develop similar degree of erythrocytosis

Serum EPO level is a basic parameter used for differential diagnosis of erythrocytosis. Thus, we confirmed the EPO expression pattern in our erythrocytosis models. The expression levels of EPO mRNA in the hepatocytes from *Alb*-Cre;*Rosa26LSL*rtTA;*EPAS1*-DPM mice (Alb-Cre;DPM mice) were significantly up-regulated after doxycycline administration (Figure 2A) while the expression of EPO mRNA in the hepatocytes from *LysM-Cre;Jak2*V617F mice and wild-type control mice was undetectable (Figure 2A). The expression levels of EPO mRNA in the kidney cells from *LysM-Cre;Jak2*V617F and Alb-Cre;DPM mice were down-regulated compared to wild-type mice (Figure 2B). Serum EPO level in *Alb*-Cre;DPM mice was dramatically increased compared to that in wild-type mice (Figure 2C), while the EPO level in *LysM-Cre;Jak2*V617F mice was decreased (Figure 2C), consistent with observations in the patients with secondary or primary erythrocytosis, respectively.

Both models quickly developed a similar degree of erythrocytosis within three months after birth or doxycycline administration, respectively (Figure 2D). We next determined the erythroid expansion and differentiation in our erythrocytosis models according to the Ter119/CD44/cell-size-based gating strategy[39] (Figure 2E-H). Total erythroid population in BM and SP from both models was signifi-

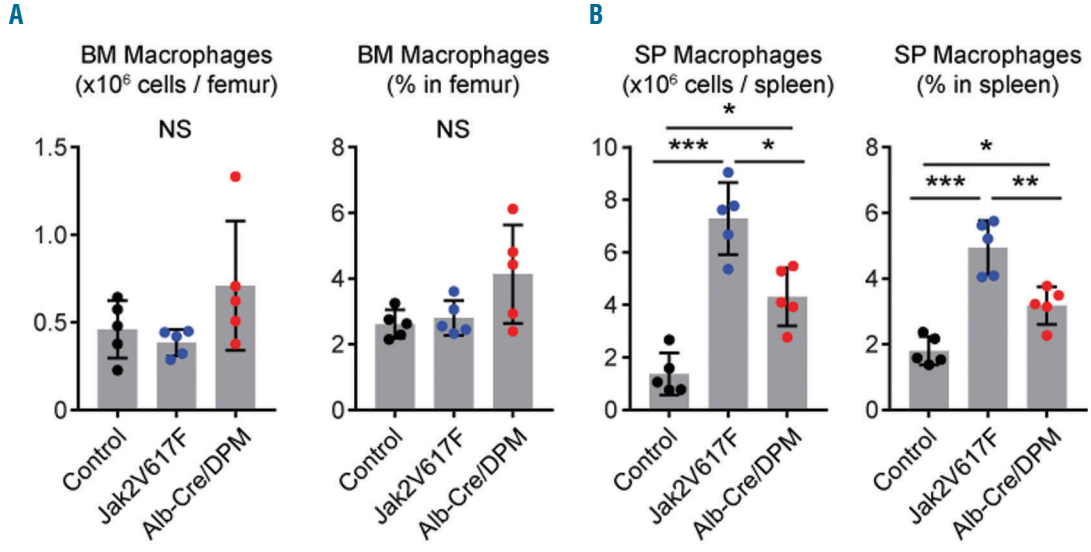

Figure 3. Alteration of macrophage population in the erythrocytosis mice. (A and B) Flow cytometric analysis of bone marrow (BM)/spleen (SP) macrophage population in the indicated mice (n=5 each). The absolute number and frequency of total macrophages from the indicated mice are shown. Data are mean±Standard Deviation (s.d.). *P<0.05; **P<0.01; ***P<0.001; NS: not significant (two-way ANOVA with multiple comparisons correction).

cantly increased compared to the control (Figure 2E-H). In BM from both models, mature RBCs, but not erythroblasts, were increased compared to the control (Figure 2F and G). We found that splenomegaly in *LysM-Cre;Jak2*V617F mice was more obvious than that in Alb-Cre;DPM mice (Figure 1F). Concordant with splenomegaly, expansion of erythroblasts and reticulocytes in SP from *LysM-Cre;Jak2*V617F mice was more prominent compared to *Alb*-Cre;DPM mice (Figure 2F and H). Extramedullary erythropoiesis could occur in liver under the stress conditions. Thus, we also determined erythropoiesis in liver (*Online Supplementary Figure S3A*). In control mice, we found only a small $CD71^{+}CD45^{-}$ population, which could contain erythroblasts in the liver. On

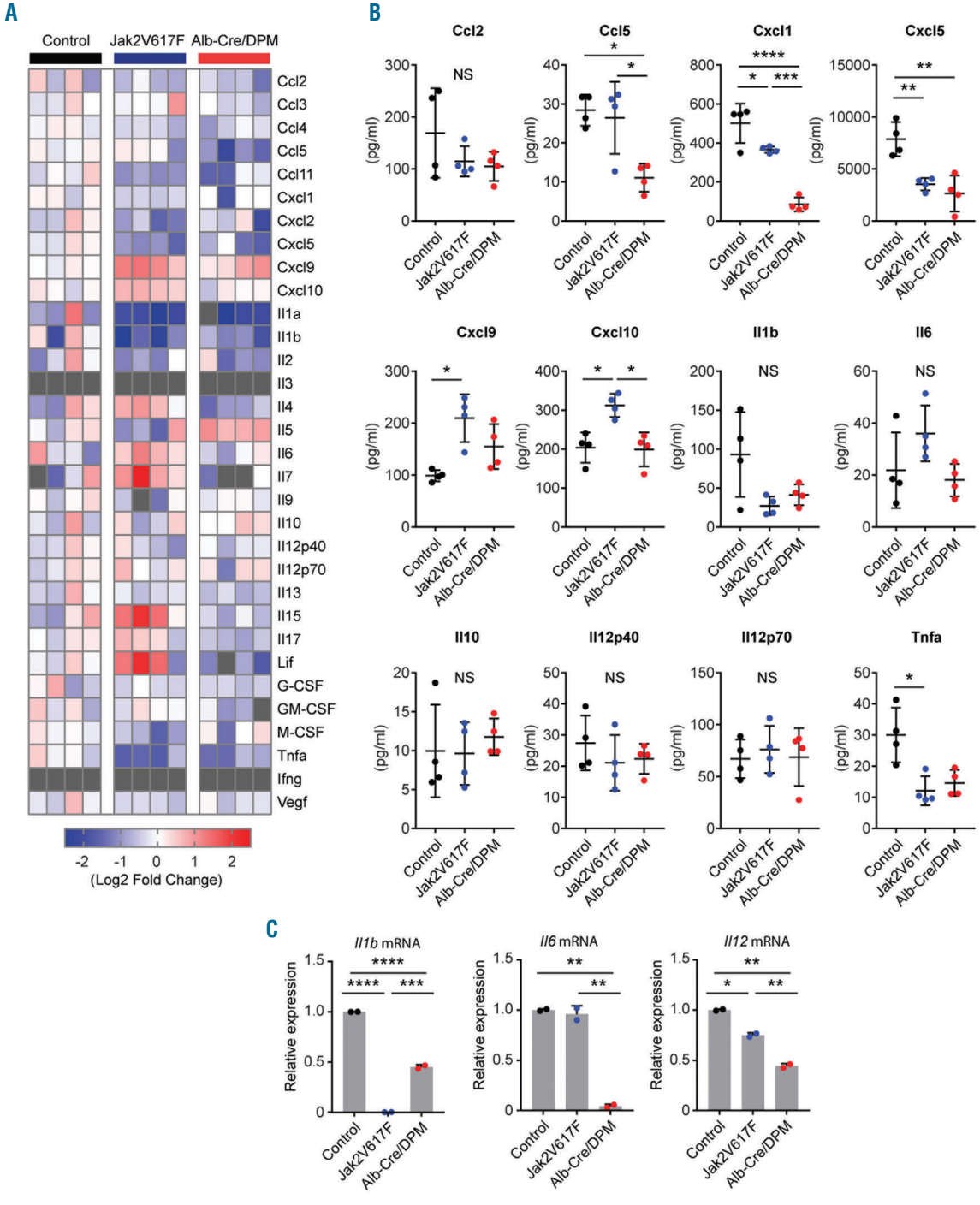

Figure 4. Inflammatory cytokine and chemokine profile in erythrocytosis mice. (A and B) Multiplex immunoassay using plasma samples from the indicated mice (n=4 each). Heat map showing $\log_2$-fold changes in concentration of 32 cytokines/chemokines normalized by mean value of control samples. Gray box indicates undetectable. Concentration of individual cytokines/chemokines are shown in (B). (C) The expression of *Il1b*, *Il6*, and *Il12* mRNA in sorted bone marrow (BM) macrophages from the indicated mice. Data are mean±Standard Deviation (s.d.). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS: not significant (two-way ANOVA with multiple comparisons correction).

the other hand, the CD71^{+}CD45^{-} population was significantly increased in Alb-Cre;DPM mice compared with control (*Online Supplementary Figure S3B* and *C*). This trend was also observed in *LysM-Cre;Jak2*V617F mice. However, there was no significant difference in the liver erythropoiesis between *LysM-Cre;Jak2*V617F and Alb-Cre;DPM mice (*Online Supplementary Figure S3B* and *C*).

Taken together, these results suggest that the sensitivity of, and response to the EPO-EPOR signaling pathway in erythroid cells and the niche environment could differ depending on whether activation is cell-intrinsic or extrinsic.

Macrophages are increased in both erythrocytosis models

Macrophages are an important erythroid cell-extrinsic component for baseline and stress erythropoiesis.[24,26] Thus, we next analyzed the macrophage population (Gr1^{low} CD115^{int} F4/80^{+} SSC^{low})[40] in our models. Although there was no change in the absolute number and frequency of total BM macrophages (Figure 3A), the absolute number and frequencies of total SP macrophages from both *LysM-Cre;Jak2*V617F mice and *Alb*-Cre;DPM mice were significantly increased compared to those in the wild-type mice (Figure 3B). These results suggest that macrophages are increased in the pathogenesis of erythrocytosis.

Inflammatory cytokines and chemokines are not elevated in both erythrocytosis models

Cytokines and chemokines play an important role in regulating dynamics of macrophages. To characterize the profile of cytokines and chemokines, we performed multiplex immunoassay using plasma samples from our erythrocytosis models (Figure 4A). Surprisingly, although the levels of some inflammatory cytokines/chemokines, such as Cxcl9 and Cxcl10, were increased in the plasma from *LysM-Cre;Jak2*V617F mice, the levels of most inflammatory cytokines/chemokines, such as Ccl2, Ccl5, Cxcl1, Cxcl5, Ccl2, Il1b, Il6, Il12, Tnf were not increased in the plasma from *LysM-Cre;Jak2*V617F mice and *Alb*-Cre;DPM mice compared to the control (Figure 4A and B). We also measured the gene expression levels of several inflammatory cytokines in sorted BM macrophages obtained from our erythrocytosis mice. The expression levels of *Il1b, Il6,* and *Il12* mRNA were significantly decreased in the erythrocytosis mice (Figure 4C). These results indicate that it is unlikely that an increase in macrophages in our erythrocytosis models is the result of inflammatory signaling activation.

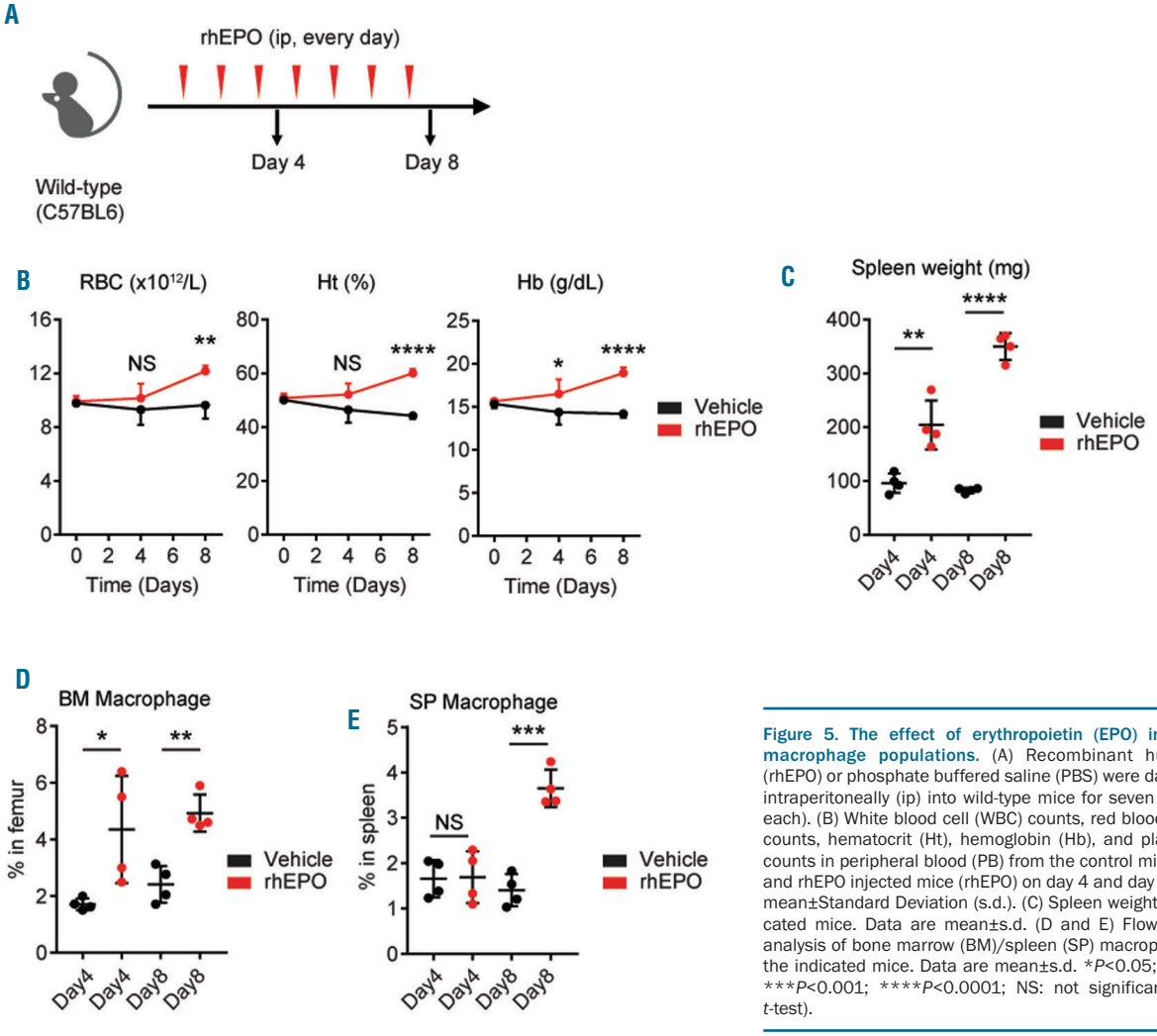

Figure 5. The effect of erythropoietin (EPO) injection on macrophage populations. (A) Recombinant human EPO (rhEPO) or phosphate buffered saline (PBS) were daily injected intraperitoneally (ip) into wild-type mice for seven days (n=4 each). (B) White blood cell (WBC) counts, red blood cell (RBC) counts, hematocrit (Ht), hemoglobin (Hb), and platelet (Plts) counts in peripheral blood (PB) from the control mice (Vehicle) and rhEPO injected mice (rhEPO) on day 4 and day 8. Data are mean±Standard Deviation (s.d.). (C) Spleen weight in the indicated mice. Data are mean±s.d. (D and E) Flow cytometric analysis of bone marrow (BM)/spleen (SP) macrophages from the indicated mice. Data are mean±s.d. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS: not significant (Student t-test).

BM/SP macrophages increase fast in number in response to EPO in an EPO-injection model of erythrocytosis

The critical role of subset of macrophages has been reported in erythrocytosis.[27] We found that macrophages are increased in our primary and secondary erythrocytosis mouse models. To confirm whether EPOR signaling could induce macrophages, we injected intraperitoneally recombinant human EPO (rhEPO) into wild-type mice daily for seven days (50 U/day) (Figure 5A). On day 4, erythrocytosis was not yet prominent (Figure 5B), while splenomegaly was significant (Figure 5C). On day 8, the rhEPO injected mice had developed observable erythrocytosis (Figure 5B). On day 4, after three days of rhEPO injections, BM macrophages in rhEPO injected mice were also significantly more increased than those from vehicle-injected control mice (Figure 5D and E). The increase in SP macrophages in rhEPO injected mice was prominent on day 8 but not on day 4 (Figure 5F and G). These results suggest that BM/SP macrophages are immediately induced in response to EPO-EPOR signaling.

Macrophages may not express EPOR but tightly interact with erythroblasts

We found that EPO injection quickly increased macrophages as well as erythroid cells. EPOR expression

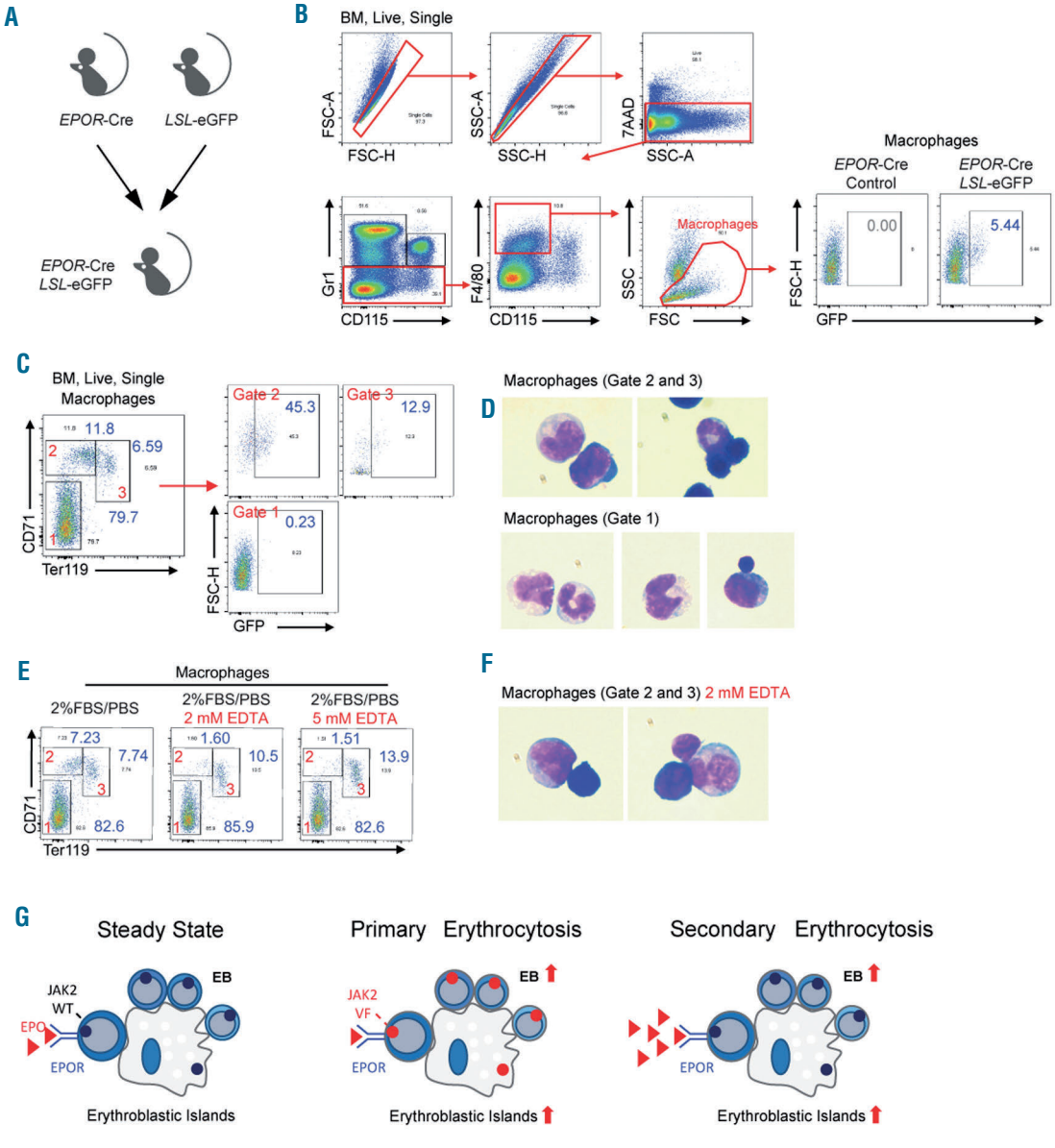

Figure 6. Most of the macrophages do not express EPOR. (A) GFP-reporter mice using *EPOR*-Cre knock-in allele and *Rosa26/loxP-Stop-loxP* (*LSL*)*/eGFP* allele. (B) Expression pattern of *EPOR*-Cre (GFP) in bone marrow (BM) single-cell-gated macrophage fraction. (C) CD71 and Ter119 expression pattern in the single-cell-gated BM macrophage population in (B). Expression pattern of *EPOR*-Cre (GFP) in the individual fractions are shown. (D) Wright Giemsa staining of single-cell-sorted macrophages in the indicated fraction defined in (C). Samples were prepared with normal FACS buffer. (E) Flow cytometric analysis of the same BM sample with different preparation (normal FACS buffer with or without EDTA). Representative plots are shown. (F) Wright Giemsa staining of single-cell-sorted macrophages in the indicated fraction defined in (E) with the FACS buffer containing 2 mM EDTA. (G) Summary of this study. EPOR signaling activation expands erythroid and macrophage populations.

is required for direct response to EPO. It has been reported that EPOR expresses not only on erythroid progenitors/precursors but also on macrophages.[10-13] To confirm the distribution of EPOR expression in hematopoietic cells using a genetic approach, we used *EPOR*-Cre knock-in allele and *Rosa26LSL*-GFP reporter allele (*EPOR*-Cre;GFP reporter mice) (Figure 6A). As expected, in erythroid lineage we found GFP expression on the surface of $CD71^+$ $Ter119^{-/dim}$ cells (pro-erythroblasts) and $CD71^+$ $Ter119^+$ FSC^{high} cells (basophilic erythroblasts) (*Online Supplementary Figure S3*). As previously suggested, we found GFP expression in a single-cell-gated macrophage fraction in *EPOR*-Cre;GFP reporter mice (Figure 6B). However, surprisingly, we found $CD71^+$ and/or $Ter119^+$ populations in this single-cell-gated macrophage fraction (Figure 6C), and a majority of GFP^+ cells belong to this $CD71^+$ and/or $Ter119^+$ subset (Figure 6C). FACS sorting revealed that those GFP^+ cells are not just macrophages, but macrophages with erythroblasts adhered to them (Figure 6D). On the other hand, most of the $CD71^-$ $Ter119^-$ macrophages were single macrophages, while some macrophages bind with a nucleus which is likely to be extruded nucleus ('pyrenocyte') (Figure 6D). We confirmed these findings by analyzing the same samples with a buffer containing EDTA. By adding 2-5 mM EDTA, we found reduced $CD71^+$ $Ter119^{-/+}$ population in the macrophage fraction while some macrophages still retained bound $CD71^+$ $Ter119^+$ erythroblasts (Figure 6E and F). These results suggest that macrophages may not present EPOR on the cell surface, but that they tightly connect with erythroblasts that express EPOR on their surface.

Discussion

In this study, we generated novel mouse models of primary and secondary erythrocytosis. Using these, we demonstrated that activation of EPOR signaling could alter not only erythrocytes but also macrophages (Figure 6H). *LysM*-Cre;*Jak2*V617F mice can recapitulate the chimeric condition in BM and a clonal expansion of mutant clone with *JAK2*V617F. Since conventional stabilization of EPAS1 has been reported to result in short-life phenotype (only 6-8 weeks),[21] our inducible EPO expression model (*Alb*-Cre;DPM mice) has an obvious advantage in that an excessive level of EPO can be induced in adult mice of various ages. Thus, these are by far the most physiologically relevant primary and secondary erythrocytosis models.

Both erythrocytes and macrophages co-operate to achieve an absolute increase in RCM. Interestingly, *Alb*-Cre;DPM adult mice had longer survival, while *LysM*-Cre;*Jak2*V617F mice quickly developed a lethal phenotype of PV. High WBC and/or Plt counts in the PV patients have been considered risk factors for life-threatening thrombosis.[41] Indeed, WBC and Plt counts in *Lys*M-Cre;*Jak2*V617F mice were higher than those in *Alb*-Cre;DPM mice. It has also been suggested that the *JAK2*V617F mutation promotes adhesion of eythrocytes through an EPOR-independent pathway.[42] This EPOR-independent pre-thrombotic effect could also contribute to shorter survival in *LysM*-Cre;*Jak2*V617F mice.

Major inflammatory cytokines and chemokines were not increased in *Alb*-Cre;DPM mice. Consistent with previous reports,[11,13] this indicates that EPOR signaling activation by itself may not induce systemic inflammation. A *JAK2*V617F mutation may activate not only EPOR signaling in erythrocytes but also a variety of cytokine receptor signaling which also uses JAK2 for signaling in other hematopoietic cells.[43,44] It has been reported that retroviral overexpression of JAK2-mutant in the bone marrow transplantation mouse models results in elevated Tnfa expression in the serum.[45] TNFα has also been reported to be increased and to promote the expansion of JAK2-mutant clones in the myeloproliferative neoplasm.[46] However, the expression levels of Tnfa in the plasma from *LysM*-Cre;*Jak2*V617F mice were decreased. The pathophysiological expression level of JAK2-mutant (but not retroviral overexpression) by itself might not induce a systemic inflammatory cytokinemia. Given that a variety of mutations, such as mutations in *TET2, ASXL1, DNMT3A*, and *EZH2*, have recently been identified in PV patients as well as *JAK2* mutation.[47] The combined effect of *JAK2* mutation and those other mutations may create an inflammatory environment, and further accelerate the expansion of the disease clone and full blown disease progression in PV patients.

Despite the fact that a similar degree of erythrocytosis was observed in both *LysM*-Cre;*Jak2*V617F and *Alb*-Cre;DPM mice, the number of erythroblasts in the SP of *LysM*-Cre;*Jak2*V617F was significantly higher than that in *Alb*-Cre;DPM mice. However, there was no significant difference in the BM and liver erythropoiesis between those two models. Since *JAK2* mutation could activate a variety of cytokine receptor signaling, JAK2 activation-mediated non-EPOR signaling in hematopoietic cells may intrinsically or extrinsically affect the dynamics of mature erythroid cell production, maintenance, or life-span. Indeed, some inflammatory chemokines, such as Cxcl9 and Cxcl10, were actually increased in the serum from *LysM*-Cre;*Jak2*V617F mice.

By a genetic approach, using EPOR-Cre knock-in and GFP reporter allele, we found restricted EPOR expression on limited stages of erythroid cells (from CFU-E to asophilic-erythroblasts), which is consistent with previous findings.[8,9] On the other hand, we did not identify EPOR expression on most of the macrophages, despite the fact that, in our study, the macrophage population was quickly expanded in response to EPO injection. Instead, we found a tight interaction between macrophages and EPOR-expressing erythroid cells. This may give the mistaken impression that macrophages also express EPOR. However, we still cannot exclude the possibility that a subpopulation of macrophages, which are the central macrophages binding to erythroblasts, express EPOR on their cell surface. In rhEPO injected wild-type mice, expansion of macrophages first occurred in BM. Although several macrophage populations reside in SP, the expansion of SP macrophages in response to EPO was delayed. Since BM is the main site of erythropoiesis in steady state, most of the early erythroblasts which express EPOR are to be found in BM but not in SP. Thus, this delay of SP macrophage expansion after EPO injection may also suggest: 1) a lack of proper macrophages in SP at steady state; and/or 2) that the expansion of macrophages in SP in response to EPO needs EPOR expressing erythroblasts which first expand in BM and then migrate from BM to SP.

EPOR signaling may alter the macrophages into erythropoiesis-supportive macrophages *via* inter-cellular signaling by erythroid cells. Interestingly, erythroblasts are known

to secrete erythroid factors, including GDF15, GDF11, TWSG1, and ERFE, which suppress hepcidin and increase iron availability for hemoglobin synthesis.[48] Studies so far have focused on their effect on hepcidin production in hepatocytes. Central macrophages in the erythroblastic islands are where the cells provide the erythroblasts with iron. Thus, these potential erythroid factors may influence macrophages *via* a paracrine mechanism. Future study is needed to understand the details of growth factors, cytokines, or chemokine signaling that may play an important role in both steady stage and stress erythropoiesis. Blocking this erythroblast-macrophage communication through direct cell-cell contact or through indirect interactions could be an alternative therapeutic target for both primary and secondary erythrocytosis.

Our current study sheds light on the significance of EPOR signaling in erythroid cells and macrophages during erythrocytosis. Without expressing EPOR on their cell surface, macrophages expanded in response to EPO. Further elucidation of the mechanism by which EPO/EPOR signaling could expand macrophages and affect their function will: 1) provide us with a clue for controlling those macrophages which may form erythroblastic islands *in vivo*; and 2) lead to identification of novel therapeutic targets, beside JAK2 inhibition, for erythrocytosis. Our new mouse models are also useful *in vivo* tools to test the efficacy of new therapeutic options on cellular and molecular components involved in the pathogenesis of erythrocytosis.

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