SUPPLEMETAL INFORMATION

Stress-associated erythropoiesis initiation is regulated by type 1 conventional dendritic cells

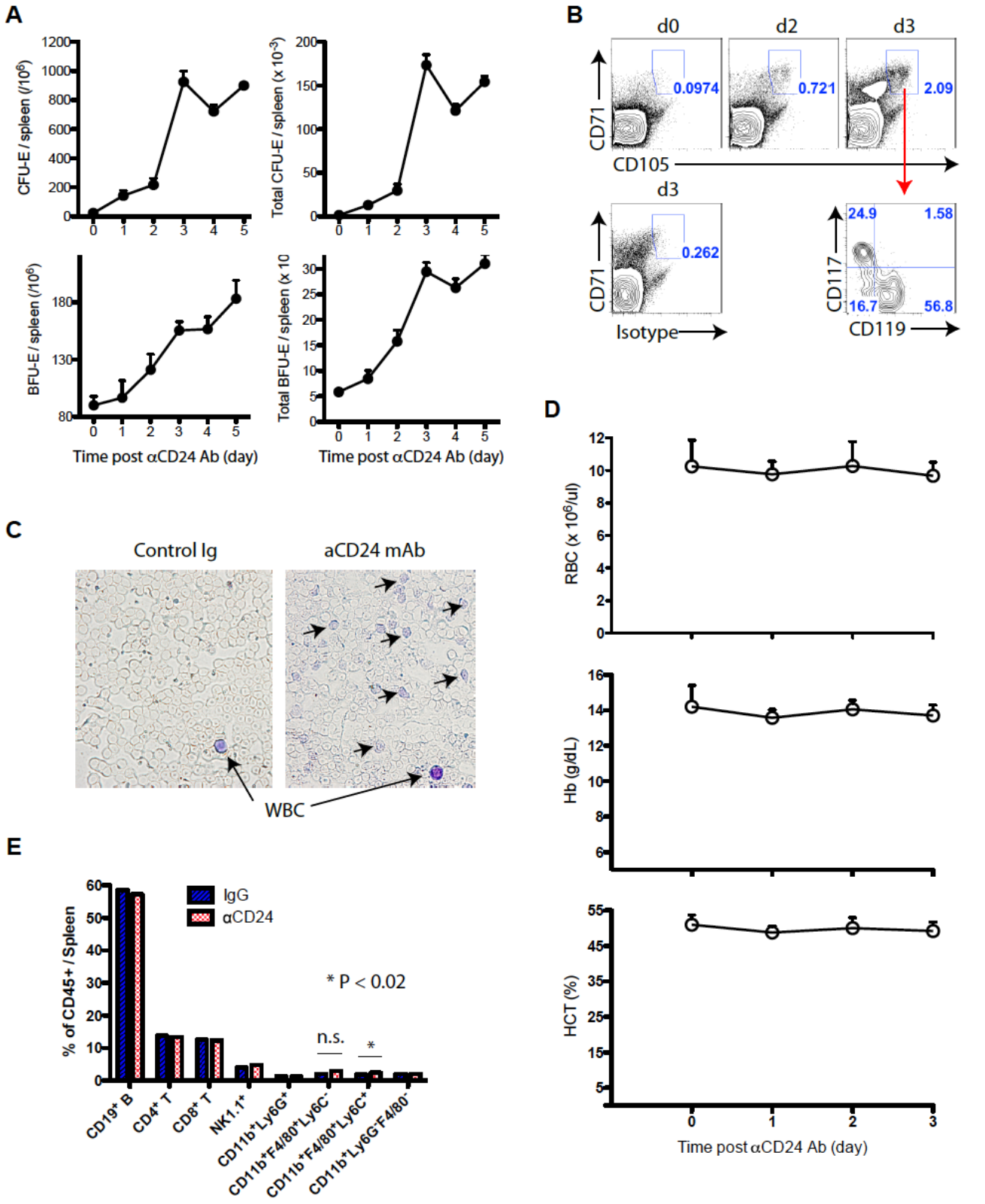
T.S. Kim *et al*.

Supplemental Figures 1-10

Supplemental Table 1

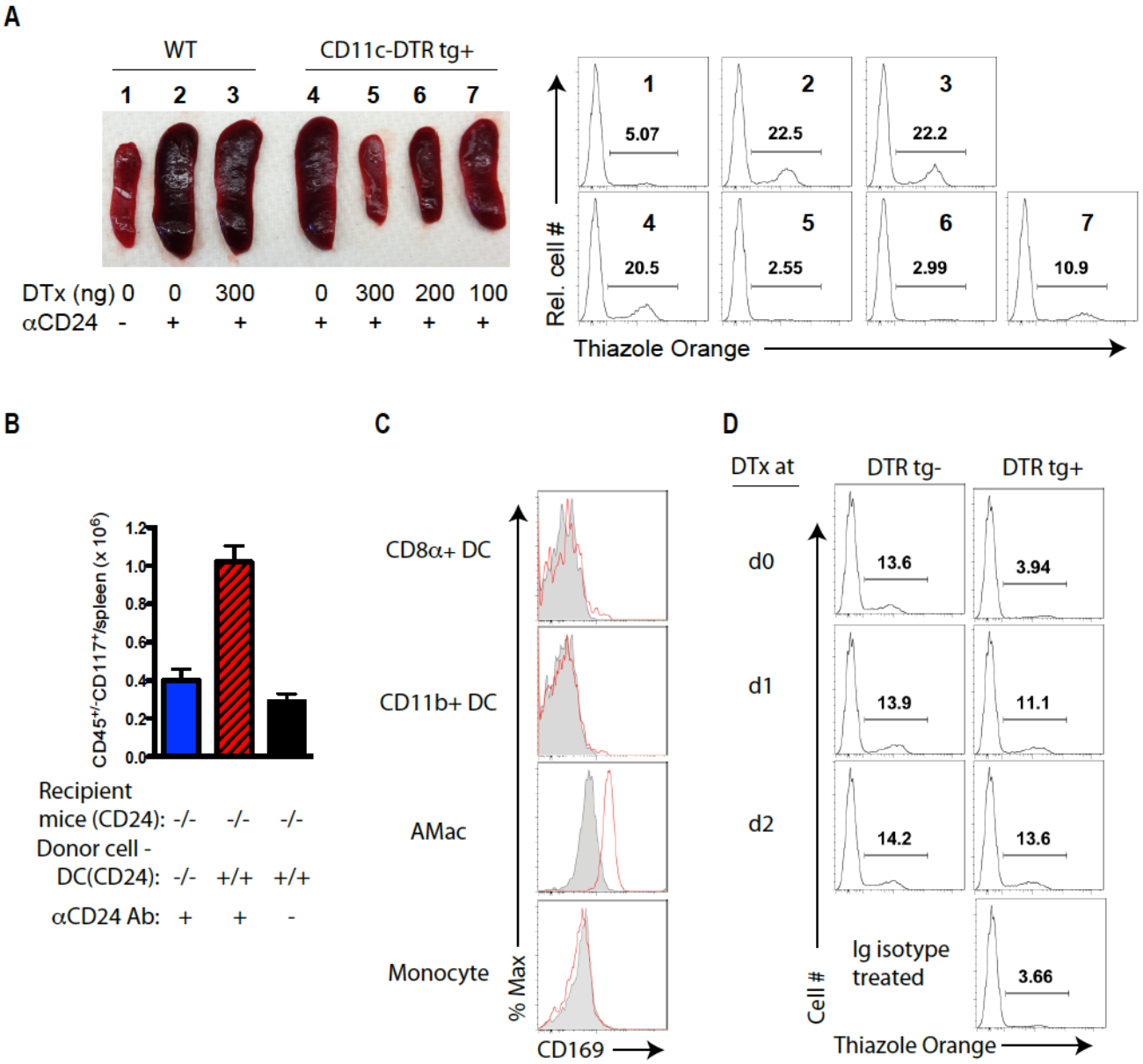
References for supplemental data

Supplemental Figure 1 **Kim et al.**



Supplemental Figure 1. Analyses on erythroid progenitors, reticulocytes, hematologic values, and leukocyte subpopulations ($Ter119^{-}CD45^{+}$ cells) in the spleen following administration of monoclonal αCD24 antibody. WT B6 mice were infused intraperitoneally (i.p.) with 100 μg control rat IgG2b or αCD24 mAb (clone M1/69). (**A** and **B**) Analyses on the erythroid progenitors in the spleens of Ab treated mice. Erythroid progenitors were identified by i) colony forming assays and ii) surface marker expression. (**A**) For colony forming assays, single cell suspensions were prepared from spleens at indicated days post Ab treatment. Selective CFU-E and BFU-E colony forming activities were measured using methylcellulose-based media (MethoCult™ SF M3436, Stem Cell Technologies). CFU-E and BFU-E colonies were identified at d2 and d7 (and d10 – data not shown), respectively, in culture by morphologic examination under light microscope (4x), and frequency (per 10^6) (left panels) and total (right panels) CFU-E (top panels) and BFU-E (bottom panels) colonies were enumerated over time (**A**). Data represent triplicates of 2 - 4 independent experiments at each time point. (**B**) Flow-cytometric analyses on the identification of erythroid progenitors. Splenocytes prepared from mice treated with αCD24 Ab were examined for surface expression of Kit, CD105, and CD71 at indicated days post treatment. At 5 days post Ab treatment, (**C**) peripheral blood smear was prepared and subjected for Wright Giemsa staining for reticulocytosis (small arrows) under light microscope (10x). (**D**) Hematological values (RBC counts, Hb and HCT levels) were analyzed on the Hemavet 850 FS automated CBC analyzer (n = 5 - 10). (**E**) Single cell suspensions prepared from spleens were analyzed for cellular types by flow cytometric-based phenotyping (n ≥ 5).

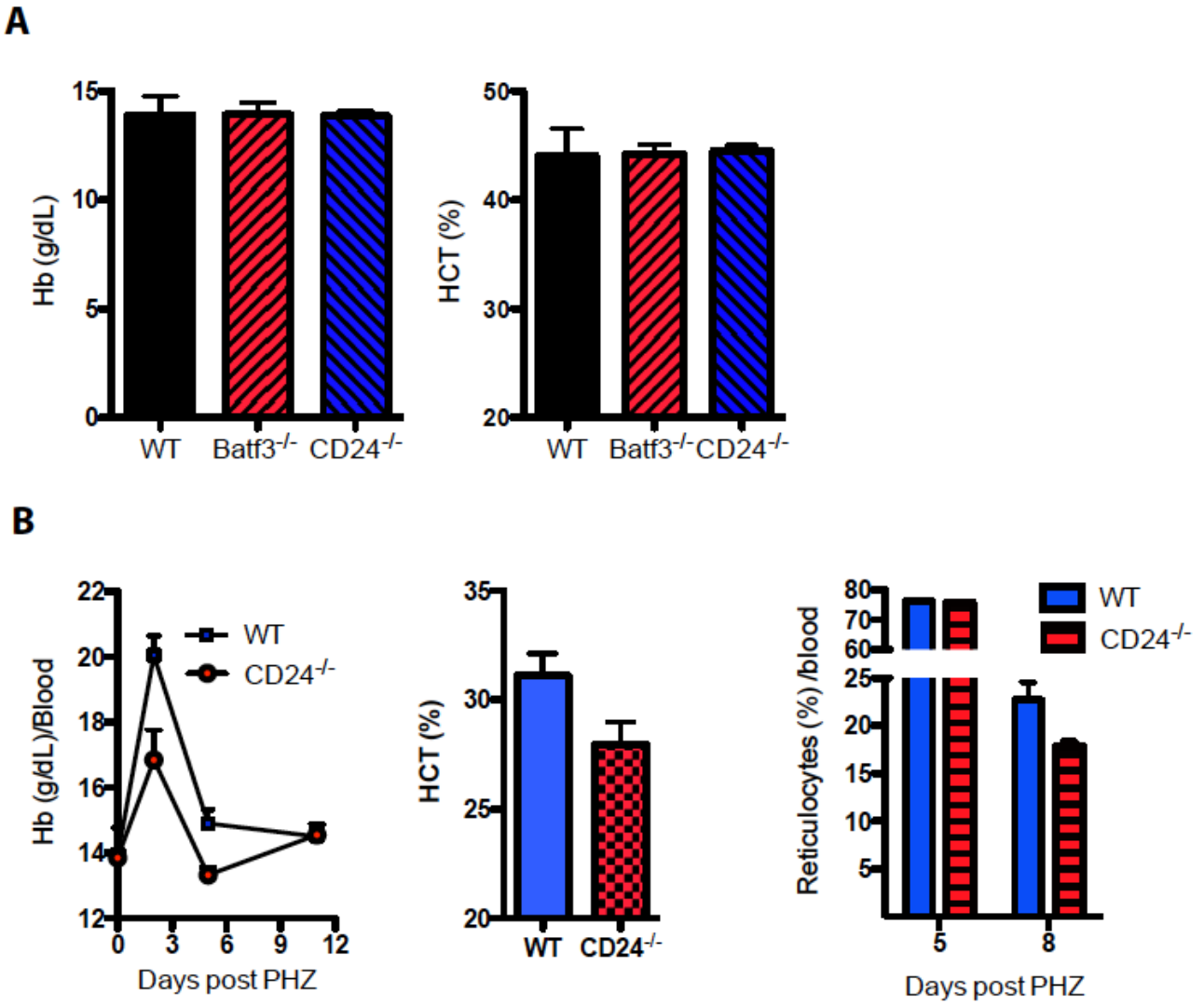
Supplemental Figure 2 **Kim et al.**



Supplemental Figure 2. Dendritic cells are critical during the inductive phase of stress erythropoiesis after CD24 engagement. (**A**) CD11c-DTR mice were administrated i.p. graded doses of diphtheria toxin (DTx). At 4 hrs post DTx administration, DC-ablated mice were infused i.p. with Ig or M1/69, and were necropsied at d5 for gross appearance of the spleens (left panels), circulating reticulocytes (right) and $CD45^{-}Ter119^{+}$ erythroid progenitors in the spleen (data not shown). Data represent at least two independent experiments. (**B**) $CD24^{-/-}$ mice were received intravenously (i.v.) bone marrow-derived DC (BMDC) prepared from either wt or $CD24^{-/-}$ mice prior to αCD24 mAb (M1/69) treatment. At d5 post treatment, erythroid progenitors ($CD45^{+/-}CD117^{+}$) in the spleens were enumerated (n = 4-5/group). (**C**) Surface expression of CD169 in naïve mice. $CD8\alpha^{+}$ DC, $CD11b^{+}$ DC and $F4/80^{+}Ly6C^{+}$ monocytes

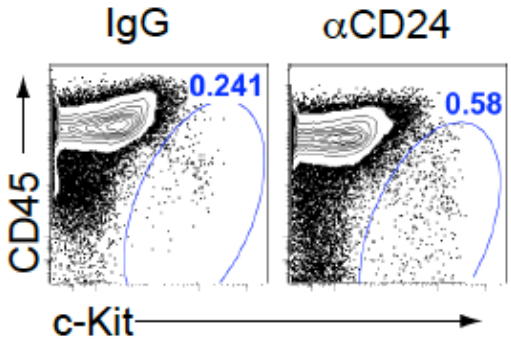
were isolated from spleens and stained with anti-CD169 mAb. Alveolar macrophages were used as a positive control for CD169 staining. (**D**) CD11c-DTR mice were administrated i.p. with 100 μg of DTx at the indicated days post M1/69 treatment. DC-ablated Ab-treated mice were necropsied at d5 for measuring circulating reticulocytes. A representative of circulating reticulocytes in the blood (%) is shown. Data represent at least three independent experiments.

Supplemental Figure 3 **Kim et al.**

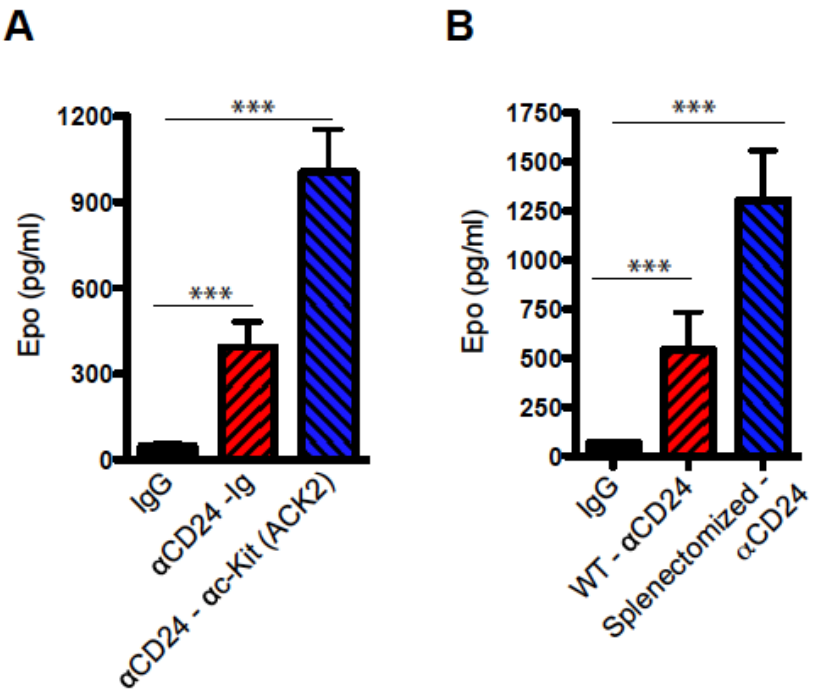


Supplemental Figure 3. Levels of Hb and HCT in mice deficient in *Batf3* or *CD24* under normoxic condition and after phenylhydrazine-induced hemolytic anemia. Phenylhydrazine (PHZ; Sigma-Aldrich) diluted in saline-buffered solution (40 mg/kg body weight, daily) was injected intraperitoneally on three consecutive days. Control mice were similarly injected with an equivalent volume of sterile saline solution (0.15 M NaCl). Mice were sacrificed the indicated days. HCT was measured at d5. Data represent mean ± SEM (n > 5 in **A** and n = 4-5 in **B**).

Supplemental Figure 4 **Kim et al.**

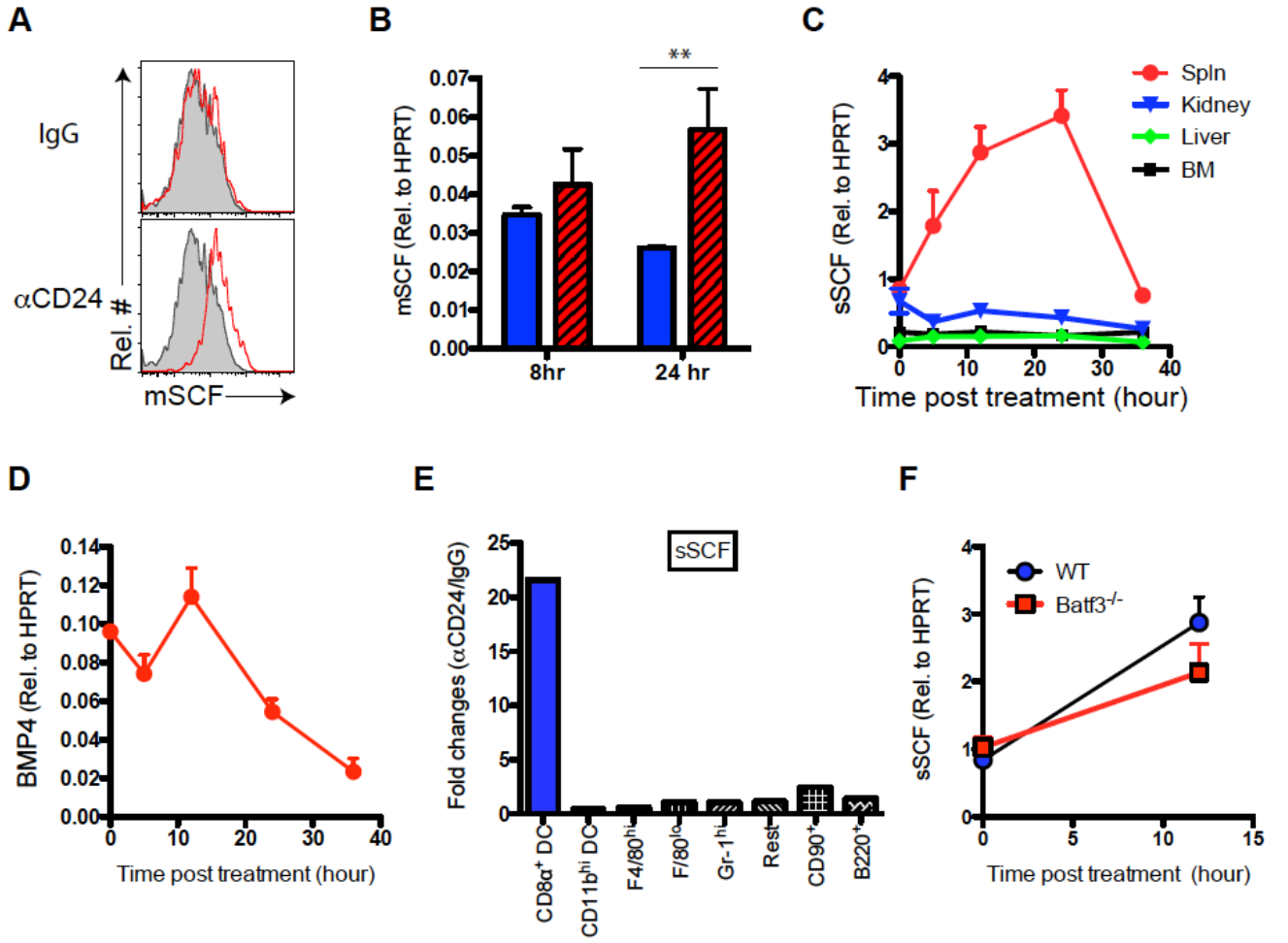


Supplemental Figure 4. Representative flow cytometric analyses of $CD45^{-}Kit^{+}$ proerythroblastic cells in the spleen of M1/69-treated WT B6 mice at 24 hrs post treatment (> 20).



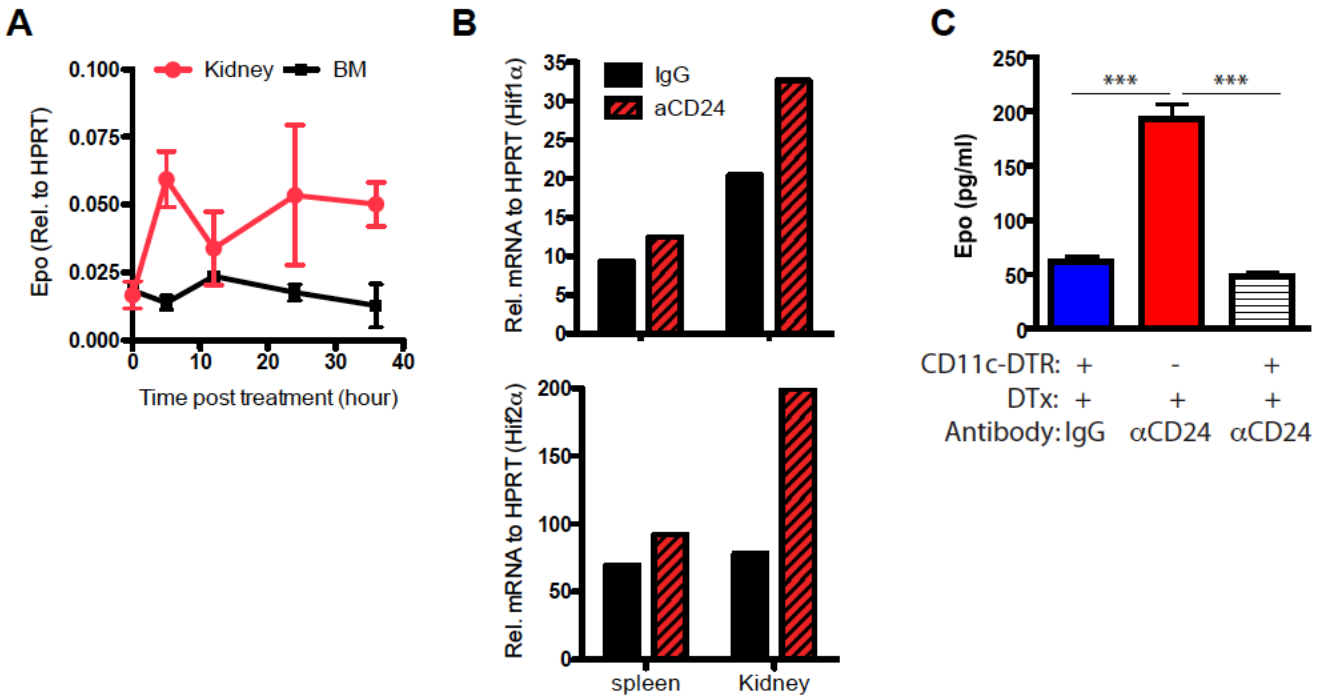
Supplemental Figure 5. Serum Epo production in the presence of anti-Kit blocking mAb (ACK2) and splenectomized mice after aCD24 engagement. (**A**) WT B6 mice were treated i.p. with Kit signaling blocking antibody (clone ACK2) at days 0 and 1 (200 μg/each injection) post M1/69 treatment. (**B**) Splenectomized mice were treated i.p. with 100 μg of M1/69. Sera were collected on day 3 post Ab treatment and subject to ELISA for EPO detection. Data represent mean ± SEM (n= 3 - 7); ***P < 0.001.

Supplemental Figure 6 **Kim et al.**



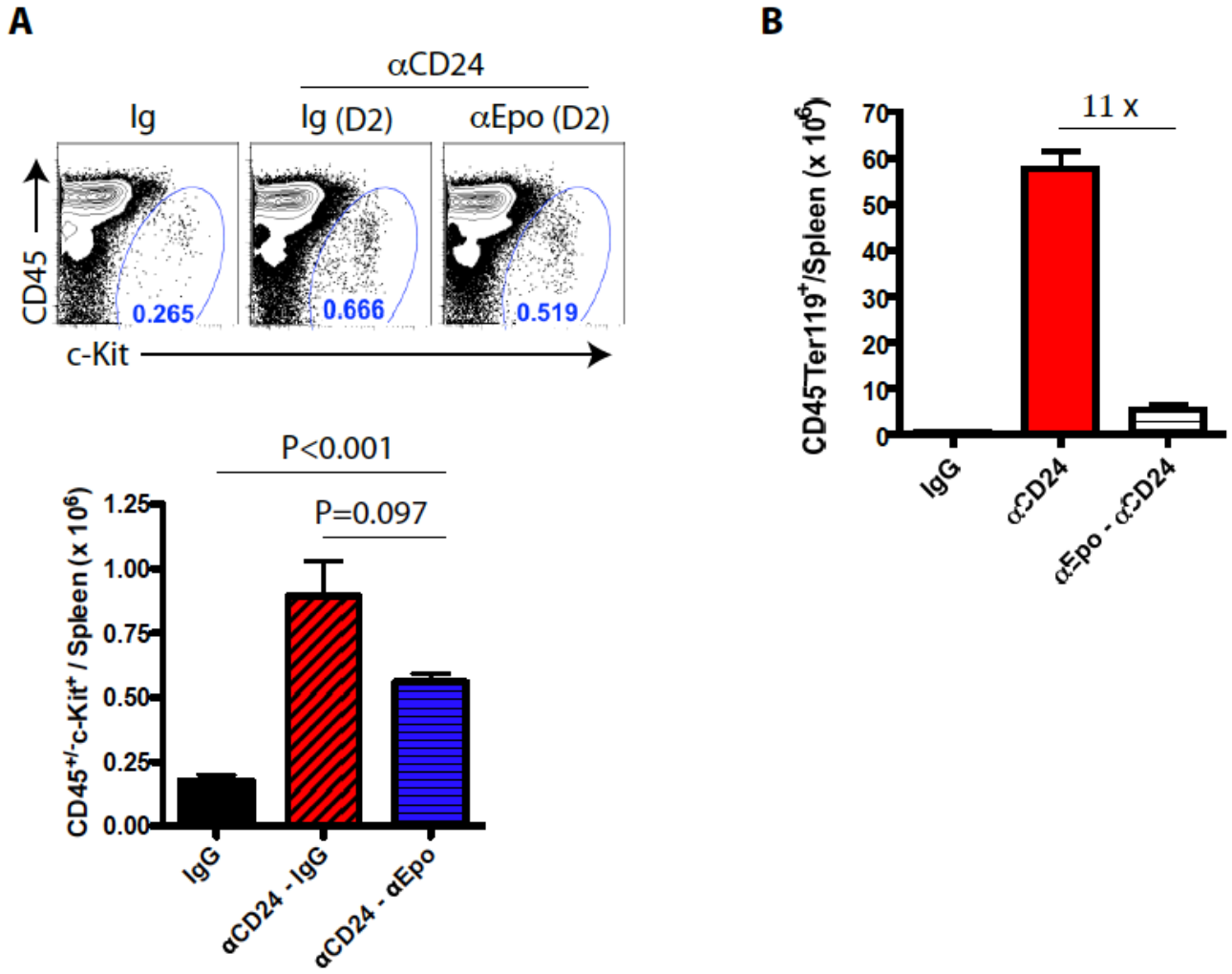
Supplemental Figure 6. DC production of SCF upon CD24 ligation. (a and **b)** Murine BMDCs were stimulated *in vitro* with M1/69 for 18 hrs and stained for surface (membrane) SCF expression for analysis by flow cytometry (**A**) or mRNA by qRT-PCR at 8 and 24 hrs post M1/69 incubation (**B**). Data in **B** represent mean ± SEM of triplicates of two independent experiments; **P < 0.01. (**C**) Kinetic analyses of soluble SCF mRNA in various organs after M1/69 treatment. (**D**) BMP4 mRNA expression in the spleens of the M1/69-treated mice at the indicated times. (**E**) Analyses of soluble SCF mRNA expression among cell types sorted from spleens of M1/69-treated mice at 15 hrs (n = 4 mice pooled/sorting). Data represent two independent experiments. (**F**) Examination of sSCF mRNA expression in the spleens of M1/69-treated WT or Batf$^{-/-}$ mice at the indicated time points. Data in **C**, **D**, and **F** represent mean ± SEM (= 3 – 5).

Supplemental Figure 7 **Kim et al.**



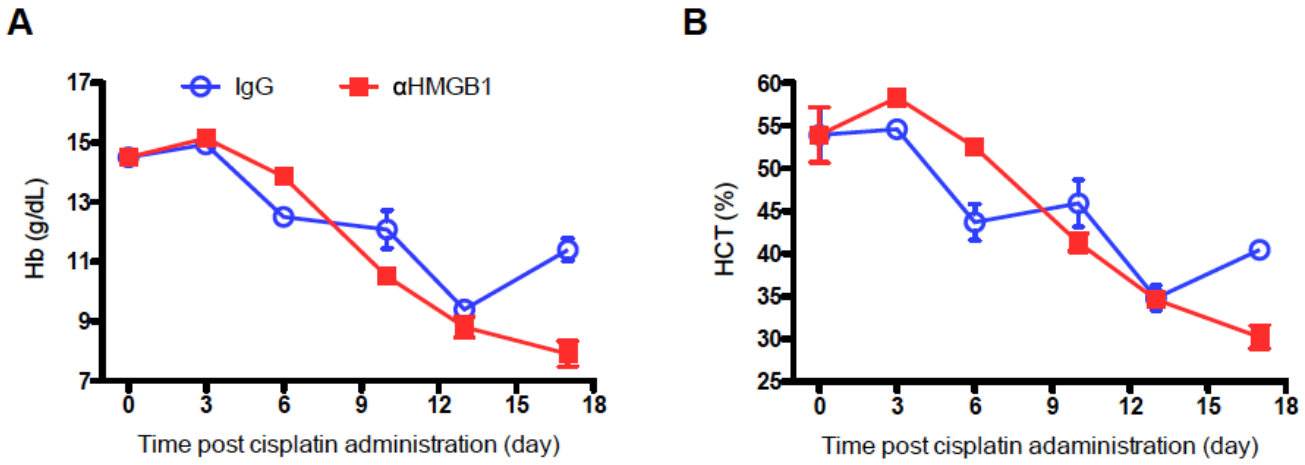
Supplemental Figure 7. **cDCs are required for renal EPO production upon CD24 engagement.** (**A**) Kinetic analyses of EPO mRNA expression in the Kidney and BM of M1/69-treated mice over time. (**B**) Analyses of Hif-1/2α mRNA expression in the Kidney and spleen of M1/69-treated mice at 1 day post Ab treatment. (**C**) DC requirement for serum EPO production after CD24 engagement. CD11c-DTR mice were administrated i.p. with DTx (100 ng) at 4 hr prior to M1/69 treatment. Sera were collected and measured at day 3 post Ab treatment by ELISA. Data represent mean ± SEM (= 3 – 5); ***P < 0.001.

Supplemental Figure 8 **Kim et al.**



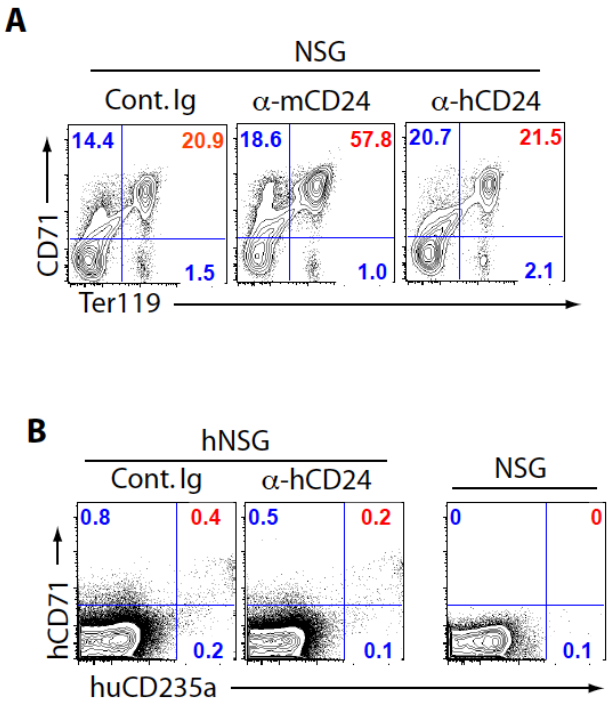
Supplemental Figure 8. Impact of Epo signaling blockade on the activation and expansion of early and late erythroid progenitors. WT B6 mice were treated i.p. with anti-Epo antibody (MAB959, R&D systems) at days 0 and 1 (200 μg/each injection) post M1/69 treatment. (**A**) Spleens were collected on day 3 post Ab treatment and evaluated for early erythroid progenitors ($CD45^{+/-}CD117^{+}$) by flow cytometry. Data represent mean ± SEM (n = 4 - 6). (**B**) Late erythroid progenitors ($CD45^{-}Ter119^{+}$) in the spleen were examined at d5 post M1/69 treatment. Data shown represent mean ± SEM of pooled mice of at least two independent experiments (n = 4 – 6).

Supplemental Figure 9 **Kim et al.**



Supplemental Figure 9. HMGB1/CD24 sensor system is required for efficient stress erythropoiesis. Mice were treated i.v. with cisplatin daily at days 0, 1, and 2. The cisplatin-treated mice also received αHMGB1 mAb (clone 3E8, 200 μg) i.p. at days 1, 2 and 3 post cisplatin treatment. Retro-orbital blood samples (30 – 50 μl) were collected at the indicated days and immediately analyzed on the Hemavet 850 FS automated CBC analyzer for Hb (**A**) and HCT (**B**). Data shown represent mean ± SEM of pooled mice of at least two independent experiments (n = 3 – 5).

Supplemental Figure 10 **Kim et al.**



Supplemental Figure 10. Detection of murine, but not human, erythroid progenitors in the spleens of humanized mice after CD24 engagement. NOD/SCID/IL-2Rγ-null (NSG) mice and humanized mice (hNSG) were treated i.p. with control Igs, or Abs against mouse (M1/69) or human (eBioSN3 or ML5) CD24, respectively. Ab-treated mice were necropsied at d5 for the measurement of erythroid progenitors in the spleens after staining with erythroid-lineage markers specific for mouse ($CD71^+Ter119^+$) (**A**) or human ($hCD71^+CD235a^+$) (**B**). Data represent at least two independent experiments.

Supplemental Table 1. Primer sequences used for qRT-PCR

Gene Name	Sequence	Reference
Mouse		
EPO	Forward: 5′-GGGTGCCCGAACGTCCCAC-3′ Reverse; 5′-AGATGAGGCGTGGGGGAGCA-3′	Sakoda *et al*.
Hif1a	Forward: 5′-TCTGGATGCCGGTGGTCTAG-3′ Reverse; 5′-TGCAGTGAAGCACCTTCCAC-3′	Sakoda *et al*.
Hif2a	Forward: 5′-TCAGTGCGAACATGGCCCCCGA-3′ Reverse; 5′-AGCACTGTGGCGGGAACCCA-3′	Sakoda *et al*.
BMP4	Forward: 5′-TGCTGGCGAGCCCGCTTCTG-3′ Reverse; 5′-TGCGTCGCTCCGAATGGCACTAC-3′	Millot *et al.*
mSCF	Forward: 5′-TCCCGAGAAAGGGAAAGC-3′ Reverse; 5′-CTGCCCTTGTAAGACTTGACTG-3′	Gu *et al.*
sSCF	Forward: 5-′TTATGTTACCCCCTGTTGCAG-3′ Reverse; 5′-CTGCCCTTGTAAGACTTGACTG-3′	Gu *et al.*
Human		
mSCF	Forward: 5′-GATGTTTTGCCAAGTCATTGTTGG-3′ Reverse; 5′-ACTGACTCTGGAATCTTTCTCAGG-3′	Hachiya *et al.*
sSCF	Forward: 5′-GGACTTTGTAGTGGCATCTGAA-3′ Reverse; 5′-CTAAGGGAGCTGGCTGCAA-3′	Wygrecka *et al.*
b-actin	Forward: 5′-ATTGCCGACAGGATGCAGGAA-3′ Reverse; 5′-GCTGATCCACATCTGCTGGAA-3′	Wygrecka *et al.*

Reference:

Sakoda, Y. *et al.* Herpesvirus entry mediator regulates hypoxia-inducible factor–1α and erythropoiesis in mice. *J Clin Invest.* 2011;121(12):4810-4819.

Chow, A. *et al*. $CD169^{+}$ macrophages provide a niche promoting erythropoiesis under homeostasis and stress. Nat. Med. 19, 429–436 (2013).

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Millot S. *et al*. Erythropoietin stimulates spleen BMP4-dependent stress erythropoiesis and partially corrects anemia in a mouse model of generalized inflammation. Blood 2010;116(26):6072-6081.

Gu Y. *et al*. (2009) Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. Development 136: 1295–1303.