CD169-CD43 interaction is involved in erythroblastic island formation and erythroid differentiation

Jian Bai,[1]* Fan Fan,[1]* Chunchen Gao,[1]* Shaohua Li,[2]* Wei Li,[3] Tiaoxia Wei,[1] Shilin Cheng,[1] Jinmin Yu,[1] Chao Zheng,[1] Junlong Zhao,[1] Linru Zou,[1] Lei Feng,[1] Jing Yi[4] and Hongyan Qin[1]

[1]State Key Laboratory of Cancer Biology, Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Xi'an; [2]Department of Aerospace Physiology, Fourth Military Medical University, Xi'an; [3]Department of Hematology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou and [4]Department of Transfusion Medicine, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China

**JB, FF, CG and SL contributed equally as first authors.*

Correspondence: H. Qin
hyqin@fmmu.edu.cn

Received: September 28, 2022.
Accepted: February 23, 2023.
Early view: March 2, 2023.

https://doi.org/10.3324/haematol.2022.282192

Abstract

CD169, a specific marker for macrophages, is a member of the sialic acid-binding immunoglobulin-like lectin (Siglec) family which acts as an adhesion molecule implicated in cell–cell interaction via sialylated glycoconjugates. Although CD169$^+$ macrophages have been found to participate in erythroblastic island (EBI) formation and support erythropoiesis under homeostasis and stress, the exact role of CD169 and its counter receptor in EBI remains unknown. Herein, we generated CD169-CreERT knock-in mice and investigated the function of CD169 in EBI formation and erythropoiesis using CD169-null mice. EBI formation was impaired *in vitro* by both blockade of CD169 using anti-CD169 antibody and deletion of CD169 on macrophages. Furthermore, CD43 expressed by early erythroblasts (EB) was identified as the counter receptor for CD169 in mediating the EBI formation via surface plasmon resonance and imaging flow cytometry. Interestingly, CD43 was proven to be a novel indicator of erythroid differentiation due to the progressive decrease of CD43 expression as EB mature. Although CD169-null mice did not display defects in bone marrow (BM) EBI formation *in vivo*, CD169 deficiency impeded BM erythroid differentiation probably via CD43 under stress erythropoiesis, in concert with the role of CD169 recombinant protein in hemin-induced K562 erythroid differentiation. These findings have shed light on the role of CD169 in EBI under steady and stress erythropoiesis through binding with its counter receptor CD43, suggesting that CD169-CD43 interaction might be a promising therapeutic target for erythroid disorders.

Introduction

Erythropoiesis is a complicated progress wherein hematopoietic stem cells proliferate and differentiate into mature red blood cells (RBC) via multiple developmental stages regulated by various factors.[1] The last several decades have witnessed the emerging role of macrophages in supporting erythroid development. As the first described hematopoietic niche, the erythroblastic island (EBI) is composed of a central macrophage and surrounding developing erythroblasts (EB), seen in the fetal liver, bone marrow (BM), and spleen.[2,3] Central macrophages function as "nursing" cells in this niche, which anchor EB within EBI and provide cytokines and growth factors to promote the proliferation and differentiation of EB.[3–7] Moreover, these macrophages can transfer iron to attached EB for heme synthesis and phagocytose the nuclei extruded by EB at terminal differentiation.[3–7] Such functions are based on the adhesion of central macrophages and EB,[3–5] but the mechanisms mediating EBI formation have not been fully elucidated. Several adhesion molecule pairs participate in EBI formation, such as erythroblast macrophage protein (EMP)-EMP,[8–12] vascular cell adhesion molecule-1 (VCAM-1)-integrin $\alpha4\beta1$[13,14] and integrin αv-intercellular adhesion molecule-4 (ICAM-4).[15,16] However, recent studies found that not all of these molecules are essential for EBI formation *in vivo*.[12] EMP expressed by macrophages, not EB, is necessary for maintaining EBI.[12] Another adhesion molecule, VCAM-1, is dispensable for EBI formation *in vivo*.[12] In addition, CD163 expressed by macrophages might be involved in EBI formation *in vitro*.[17] Nevertheless, little is known regarding the interactions between other adhesion molecules mediating macrophages and EB.

CD169, also known as sialoadhesion (Sn) or sialic acid-binding immunoglobulin-like lectin 1 (Siglec1), is a specific marker for tissue resident macrophages.[18,19] As the largest member of the Siglec family, CD169 contains an extracel-

lular region made up of 16 C2-set immunoglobulin domains and 1 V-set immunoglobulin domain but lacks intracellular signaling motifs.[18,19] CD169 was originally named sheep erythrocyte receptor because of its ability to interact with sialylated structures on sheep erythrocytes, but later it was found to bind murine EB probably via sialylated glycoconjugates which is located at macrophages-EB contact zones.[20–22] Further *in vivo* investigations have shown that depletion of CD169$^+$ macrophages strikingly impairs EBI and markedly decreases the number of EB in BM, suggesting that CD169$^+$ macrophages are crucial for EBI formation *in vivo*.[23] In addition, the contribution of CD169$^+$ macrophages to stress and pathological erythropoiesis suggests that targeting CD169$^+$ macrophages has therapeutic implications in anemia, polycythemia vera, and β-thalassemia.[23,24] These studies emphasize the pivotal role of CD169$^+$ macrophages in supporting erythropoiesis under homeostasis and stress. A recent study has reported that EPOR$^+$ EBI central macrophages express a higher level of CD169 than EPOR$^-$ macrophages.[25] However, the mechanism of CD169 as an adhesion molecule for mediating macrophages and EB interaction remains unclear. Furthermore, the counter receptor on EB for CD169 has not been studied.

In this study, we found that CD43 is the counter receptor on EB for CD169 expressed by macrophages in EBI. In addition, similar to CD44, CD43 expression progressively decreased as EB mature, and thus it could be a novel marker to distinguish erythroid differentiation. Although blocking CD169 disrupted the adhesion between macrophages and EB *in vitro*, CD169 deletion did not impair EBI formation *in vivo*, suggesting that CD169 might play a dispensable role in the BM EBI niche. Moreover, in the model of high-altitude polycythemia (HAPC), CD169 might slightly promote BM erythroid differentiation through binding with CD43. Consistently, CD169 recombinant protein promoted hemin-induced K562 erythroid differentiation mildly with reduced CD43 expression. Collectively, our findings unravel the counter receptor on EB for CD169 and elucidate the role of CD169 expressed by macrophages in EBI formation and erythroid differentiation under homeostasis and stress conditions, replenishing the molecular mechanisms of adhesion molecules participating in EBI formation.

Methods

Mice

CD169-CreERT mice were generated on a C57BL/6 background by using CRISPR/Cas9 to knock CreERT into the exon 1 of CD169 (Biocytogen Biotechnology Company, China). Genotyping was performed by polymerase chain reaction (PCR) with two pairs of primers: WT-F: CATGCCACCAAGTGAGAGCATTTCC; WT-R: TGCACATTCTTGGGACTGGAGACACC; MUT-F: AGGAGACCAATTTCCGGTGCTTACG; MUT-R: GGCTTGCAGGTACAGGAGGTAG. The first pair of primers amplified a 539 bp wild-type (WT) band while the second pair amplified a 641 bp mutant (MUT) band from the CD169-CreERT allele. Rosa26tdTomato (Ai9) mice were kindly provided by Dr. Yuqiang Ding. In order to induce gene recombination in CD169-CreERT crossed with Ai9 mice, tamoxifen (100 mg/kg, Sigma) was administered intraperitoneally daily for 5 consecutive days, and analysis was performed 3 weeks after the last injection. In order to establish a high-altitude polycythemia (HAPC) model,[26] mice were exposed to hypoxia in a hypobaric chamber corresponding to an altitude of 5,000 m for 7 days. All mice with a C57BL/6 background were maintained in a specific-pathogen-free facility. All animal experiments were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University. Adult mice 2–3 months of age were used for the studies.

Cell isolation and culture

Murine BM-derived macrophages (BMDM) were induced and cultured as described previously.[27] The BM EB (CD45$^-$CD11b$^-$Ter119$^+$) were sorted by fluorescence activated cell sorting (FACS) using BD AriaIII or magnetic activated cell sorting (MACS) with the BD IMag™ cell separation system.

Erythroblastic island formation *in vitro*

EBI formation was performed as previously described.[25] Briefly, BMDM were pretreated with isotype or anti-CD169 antibody (5 μg/mL) for 1 hour (h), and were then washed with phosphate-buffered saline (PBS). Pretreated BMDM were mixed with sorted EB at a 1:20 ratio for 12 h in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS), 5 mM Mg^{2+}, and 5 mM Ca^{2+}. Afterwards, cells were collected for cytospin and May–Grunwald–Giemsa (MGG) staining to assess the ability of EBI formation *in vitro*. In some cases, EB were pretreated with isotype or anti-CD43 antibody for 1 h before co-culture or BM macrophages were sorted from CD169 WT and knockout (KO) mice using BD AriaIII or SONY MA900.

Preparation of native erythroblastic islands from bone marrow

Native murine BM EBI were prepared according to the described methods,[25,28] then stained with different antibodies listed in the *Online Supplementary Table S1*. Cells were washed and resuspended for flow cytometry or imaging flow cytometry analysis.

Flow cytometry and imaging flow cytometry

Isolated murine BM and spleen cells were stained with different antibodies listed in the *Online Supplementary*

Table S1. Flow cytometry analyses were performed with routine protocols using a BD CantoPlus flow cytometer. Dead cells were excluded by 7-AAD staining. Data was analyzed using the FlowJo 7.6.1 software. Imaging flow cytometry was done using a Luminex Amnis Imagestream Mk II instrument at 40× magnification with IDEAS 6.2 software for data analyses.

Surface plasmon resonance

BIAcore T200 (Cytiva) was used to determine the binding ability of CD169 to CD43. Based on the binding active unit of rmSiglec1-mFc (RD, 5610-SL) in N-terminal Ig-like V-set domain, mouse antibody capture kit (Cytiva, BR-1008-38) was utilized for rmSiglec1-mFc immobilization on a CM3 sensor chip via the C-terminal rmFc domain. Immobilization conditions and running conditions were set according to the mouse antibody capture kit instructions, and rmCD43-hFc (SinoBiological, 50735-M02H) was injected at a series of concentrations diluted with HBS-EP buffer. The association time was 60 seconds (sec), and the dissociation time was also 60 sec. The equilibrium dissociation constant (KD) value was obtained using a steady state affinity model on the BIAcore evalution software program.

Statistics

The data were quantitatively analyzed using the GraphPad Prism 7 software. All experiments were replicated at least three times and all data were reported as mean ± standard error of the mean (SEM). Statistical significance was assessed using the Student's *t*-test and one-way ANOVA with Tukey's multiple comparison tests, as appropriate. $P<0.05$ was considered statistically significant.

Results

Blockade of CD169 expression on bone marrow-derived marcrophages disrupts erythroblastic island formation *in vitro*

CD169$^+$ macrophages are reportedly involved in BM EBI formation.[23] In order to further assess the exact role of CD169 on EBI macrophages, we first examined CD169 expression during murine BM monocyte differentiation into macrophages at different time points. CD169 expression was found to increase at both the RNA and protein levels (Figure 1A–C) in the process of monocyte-to-macrophage differentiation, which is in line with the differentiation of healthy human peripheral blood monocytes to macrophages.[29] Meanwhile, the expression of CD169 on mature BMDM was confirmed by immunofluorescence staining (Figure 1D). Afterwards, we developed an *in vitro* EBI formation assay (*Online Supplementary Figure S1A*) to evaluate adhesion between CD169$^+$ BMDM and BM EB. BM EB were sorted (*Online Supplementary Figure S1B*) and then co-cultured with BMDM pretreated with isotype or anti-CD169 antibody. Representative images and quantitative analyses showed that the ability of EBI formation *in vitro* was impaired when the expression of CD169 on BMDM was blocked by anti-CD169 antibody (Figure 1E, F). These results demonstrated the crucial role of CD169 in EBI formation *in vitro*.

Regarding the lack of VCAM-1 on CD169$^+$ BMDM *in vitro* (*Online Supplementary Figure S1C*), it is reasonable to speculate whether overexpression of VCAM-1 on CD169$^+$ BMDM may rescue EBI formation upon blocking CD169. In order to prove this hypothesis, VCAM-1 was induced in cultured CD169$^+$ BMDM by lentivirus overexpression (*Online Supplementary Figure S1D*). Unexpectedly, the result showed that overexpression of VCAM-1 on CD169$^+$ BMDM might not rescue the impairment of EBI formation upon blocking CD169 *in vitro*, indicating that VCAM-1 may not compensate for the effect of CD169 deletion in EBI formation at least *in vitro* (*Online Supplementary Figure S1E, F*).

Generation of CD169-CreERT mice to investigate the role of CD169 in erythroblastic island formation

In order to enunciate the role of CD169 in EBI, we constructed CD169-CreERT knock-in mice on a C57BL/6 background using CRISPR/Cas9 technology. As shown in the schematic model, CreERT was knocked into exon 1 to allow the independent expression of CreERT under the control of the CD169 promoter (Figure 2A), and the genotype was confirmed by PCR (*Online Supplementary Figure S2A*). Meanwhile, the different genotype of CD169-CreERT mice was determined by FACS using BM cell suspension. As shown in Figure 2B and C, CD169 expression was reduced by about half in heterozygotes, and CD169 was knocked out successfully in CD169-CreERT double knock-in mutant mice (CD169 KO mice). Further analysis revealed that CD169 deletion did not affect BM macrophage percentage and numbers (*Online Supplementary Figure S2B*). Because CD169$^+$ macrophages maintain the hematopoietic stem cells niche,[30,31] we then examined the hematopoietic stem cell development by FACS. The percentage and cell numbers of hematopoietic stem and progenitor cells showed negligible changes in CD169 KO mice (*Online Supplementary Figure S2C, D*).

Moreover, we observed CD169 distribution in immune cells by crossing CD169-CreERT mice and Rosa26tdTomato mice with tdTomato followed by the loxp-flanked STOP sequence inserted into the Rosa26 locus. After tamoxifen administration, tdTomato expression was observed in the EBI multiplets (Figure 2D), but not in T cells, B cells, or NK cells (*Online Supplementary Figure S2E*), suggesting that CD169 is a macrophage-restricted marker and that CD169-CreERT could be utilized for tracing EBI central macrophages. However, the percentage of tdTomato in EBI central macrophages was not as high as we

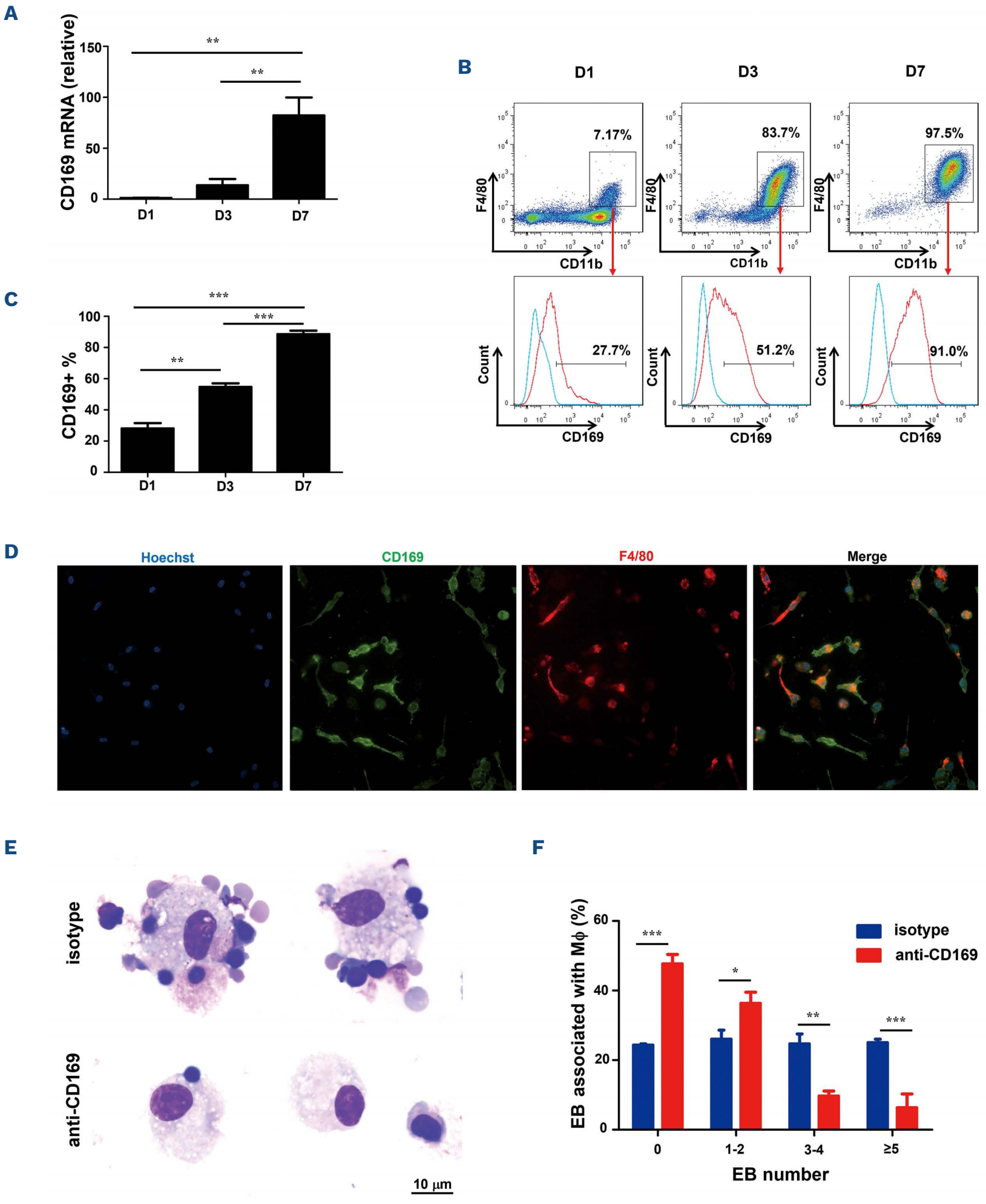


Figure 1. Blockade of CD169 expression on bone marrow-derived macrophages disrupts erythroblastic island formation *in vitro*. (A) The relative expression of *Cd169* was determined by quantitative real-time polymerase chain reaction (qRT-PCR) at different time points of bone marrow-derived macrophages (BMDM) differentiation *in vitro* (N=3). (B) The percentage of $CD11b^+F4/80^+$ macrophages (upper panel) and the CD169 expression of $CD11b^+F4/80^+$ macrophages (lower panel) were analyzed by fluorescence activated cell sorting (FACS) during BMDM differentiation. The representative FACS plot is shown (N=3). (C) The percentage of $CD169^+$ macrophages was quantitatively analyzed as shown in (B) (N=3). (D) BMDM on day 7 (D7) were stained with CD169 (green), F4/80 (red), and Hoechst (blue) using immunofluorescence staining (N=3). (E) Sorted BM erythroblasts (EB) were cultured with BMDM pretreated with immunoglobulin G (IgG) isotype or anti-CD169 antibody, then *in vitro* EBI formation was examined by May–Grunwald–Giemsa (MGG) staining (N=3). (F) Quantitative analyses of *in vitro* erythroblastic island (EBI) formation by counting the number of surrounding EB associated with BMDM in (E) (N= 3). Data are shown as mean ± standard error of the mean; *P<0.05; **P<0.01; ***P<0.001.

expected, probably due to the efficiency of tamoxifen induction.

We then sorted BM macrophages from WT and CD169 KO mice as well as BM EB from WT mice, and performed EBI formation *in vitro*. EBI formation was undermined *in vitro* in the CD169 KO group (Figure 2E, F), which resembled the impairment of EBI by blocking CD169 using anti-CD169 antibody (Figure 1E, F). Based on the abovementioned results, CD169-CreERT double knock-in mice could be utilized as CD169 KO mice for studying CD169 function in EBI formation *in vivo*.

CD43 is the counter receptor on erythroblasts for CD169

As the counter receptor on EB for CD169 in EBI has not been determined, we attempted to determine its potential counter receptor from some reported sialylated molecules interacting with CD169, such as MUC1 and CD43.[32,33] Since the counter receptor candidate is likely to share a similar proportion with that of recombinant CD169-Fc protein binding to BM EB, we first measured the binding of recombinant mouse CD169-Fc protein to sorted BM EB. In fact, recombinant CD169-Fc bound only to about 10% of BM EB (Figure 3A; *Online Supplementary Figure S1G*). Next, we examined the expression of MUC1 and CD43 on EB by western blotting. We found that CD43, but not MUC1, was expressed on sorted BM EB (*Online Supplementary Figure S1H*). Accordingly, we also observed approximately 16% CD43$^+$ BM EB by FACS (Figure 3B), indicating that CD43 might be the counter receptor for CD169 on EB.

For further insight regarding the interaction between CD169 and CD43, surface plasmon resonance was per-

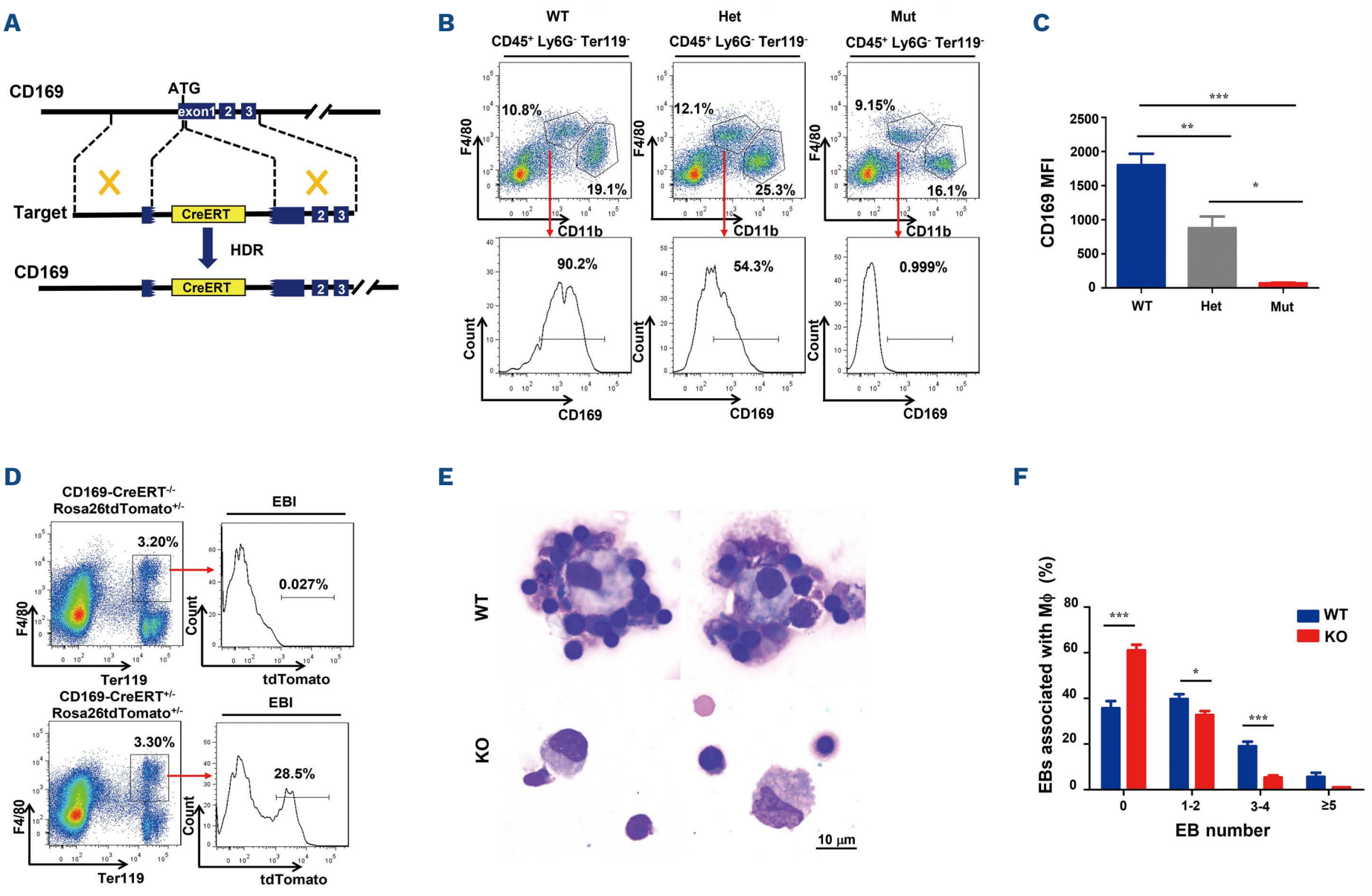


Figure 2. Generation of CD169-CreERT mice for investigating the function of CD169 in erythroblastic island formation. (A) Schematic model for the generation of CD169-CreERT knock-in mice. (B) The expression of CD169 in bone marrow (BM) macrophages from CD169 wild-type (WT), heterozygous (Het), and mutant (Mut) mice was analyzed by fluorescence activated cell sorting (FACS) (N=3). (C) The deletion efficiency of CD169 on BM macrophages was determined by mean fluorescence intensity (MFI) as shown in (B) (N=3). (D) Representative flow cytometry plots of BM erythroblasts (EB) gated as Ter119$^+$F4/80$^+$ live multiplets and representative histograms showing tdTomato expression of erythroblastic island (EBI) central macrophages in CD169-CreERT$^{-/-}$ Rosa26tdTomato$^{+/-}$ and CD169-CreERT$^{+/-}$ Rosa26tdTomato$^{+/-}$ mice (N=3). (E) Sorted BM EB were cultured with BM macrophages sorted from CD169 WT and KO mice, followed by May–Grunwald–Giemsa (MGG) staining for evaluating *in vitro* EBI formation (N=3). (F) Quantitative analyses of *in vitro* EBI formation by counting the number of surrounding EB associated with BM macrophages from CD169 WT and KO mice as shown in (E) (N=3). Data are shown as mean ± standard error of the mean; *P<0.05; **P<0.01; ***P<0.001.

formed to examine the binding affinity of CD169 to CD43. As shown in Figure 3C, their binding was dose-dependent and exhibited a fast association-dissociation process with a KD value of 107.1 nM. Meanwhile, imaging flow cytometry analysis showed that in a typical EBI structure one CD169$^+$F4/80$^+$ central macrophage was surrounded by several Ter119$^+$ EB, of which one or two were CD43$^+$ (Figure 3D). Furthermore, blockade of CD43 on BM EB with anti-CD43 antibody disrupted EBI formation *in vitro* (Figure 3E, F), which was consistent with the effect of CD169 blockade and CD169 deletion on macrophages (Figure 1E, F; Figure 2E, F). Taken together, our findings strongly suggest that CD43 is the counter receptor on EB for CD169 in EBI.

CD43 might be a novel marker for distinguishing erythroid differentiation

Given that not all EB express CD43, we next investigated whether the kinetics of CD43 expression are relevant to the specific developmental stages of erythroid cells. We

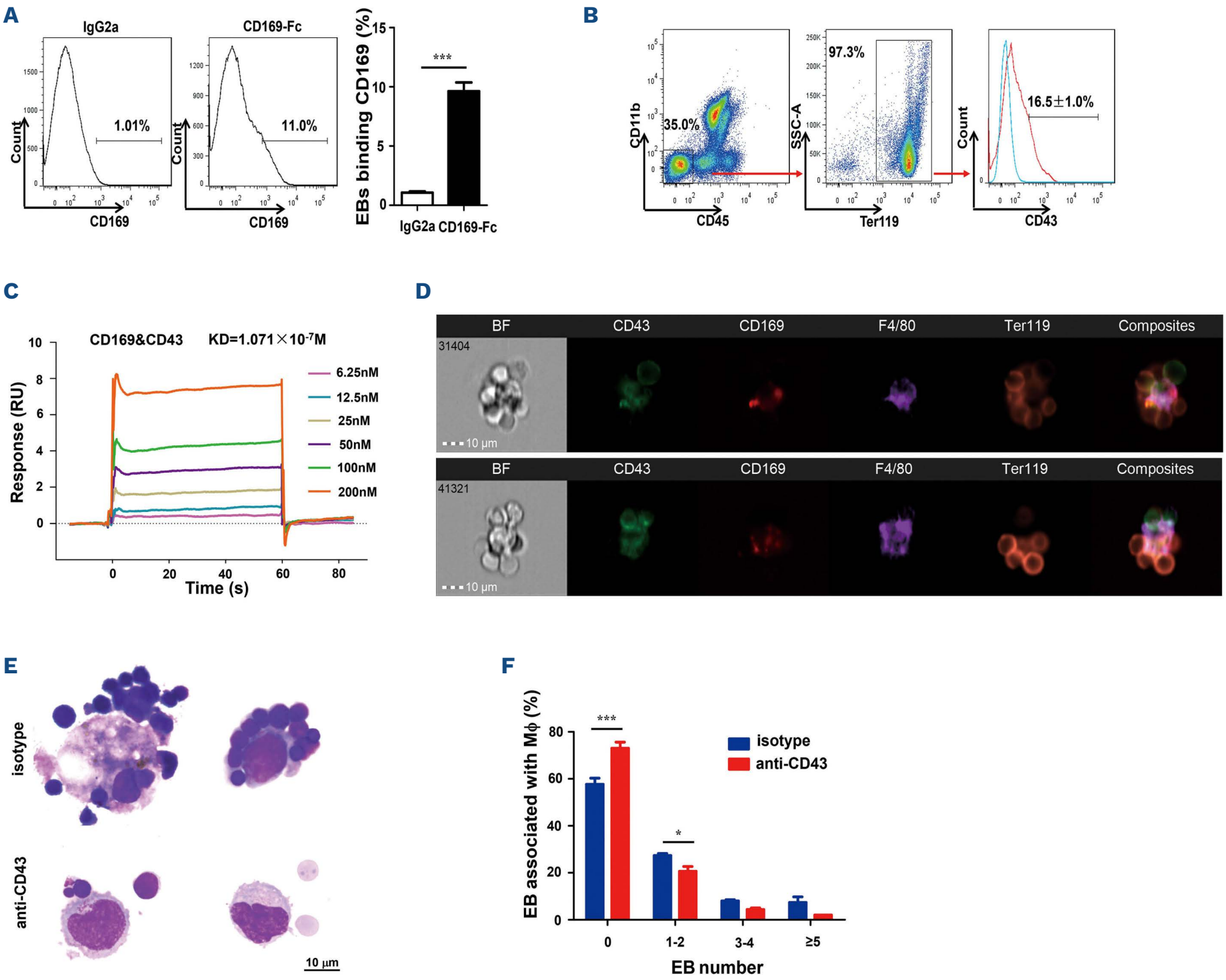
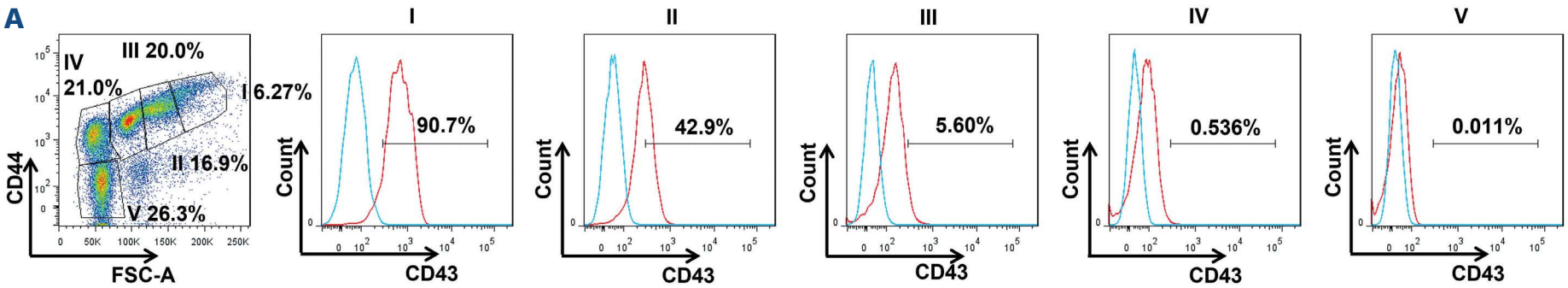
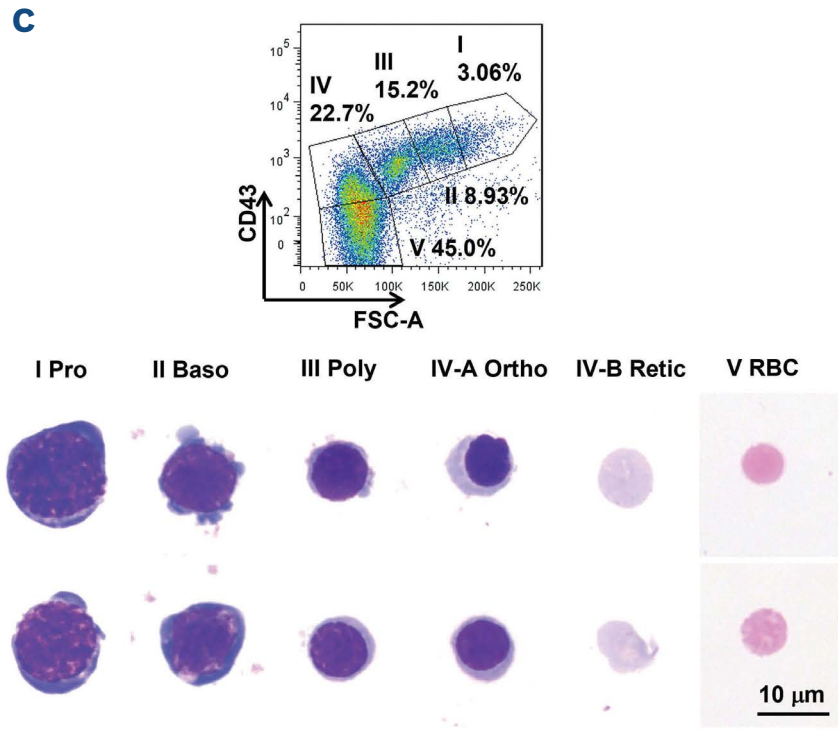
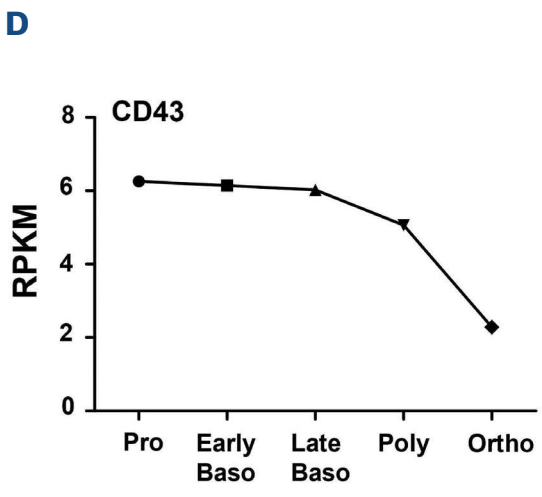


Figure 3. The counter receptor on erythroblasts for CD169 is CD43. (A) The binding ability of recombinant mouse CD169-Fc protein to sorted bone marrow (BM) erythroblasts (EB) was detected by fluorescence activated cell sorting (FACS) with recombinant mouse Fc protein as control stained by anti-mouse CD169 antibody (N=7). (B) The CD43 expression of CD45$^-$CD11b$^-$Ter119$^+$ BM EB was analyzed by FACS (N= 12). (C) The binding affinity of CD169 to CD43 was determined by surface plasmon resonance (SPR). (D) The typical erythroblastic island (EBI) structure was observed by using imaging flow cytometry (IFC): CD169$^+$F4/80$^+$ macrophage was surrounded by several Ter119$^+$ EB, among which 1 to 2 EB were CD43$^+$. (E) Representative May–Grunwald–Giemsa (MGG) staining images of *in vitro* EBI formation between sorted BM macrophages and sorted BM EB pretreated with isotype or anti-CD43 antibody (N=3). (F) Quantitative analyses of *in vitro* EBI formation by counting the number of surrounding EB pretreated with isotype or anti-CD43 antibody associated with macrophages (N=3). Data are shown as mean ± standard error of the mean; **P*<0.05; ***P*<0.01; ****P*<0.001.

adopted a putative method to identify distinct EB populations[34,35] and examined the expression of CD43 in different developmental stages. During the successive differentiation progress from immature EB to mature RBC, CD43 displayed a progressively decreasing pattern from region I (highest level) to region V (lowest level) (Figure 4A). In addition, the difference among the mean fluorescence intensity (MFI) of CD43 in distinct EB population was statistically significant (*Online Supplementary Figure S3A*), and the MFI of CD43 was positively correlated with the MFI of CD44 (*Online Supplementary Figure S3B*). Consistently, imaging flow cytometry also revealed the progressive decrease of CD43 fluorescence as well as CD44 from Pro-EB to RBC (Figure 4B). Then we used CD43 and FSC-A to sep-







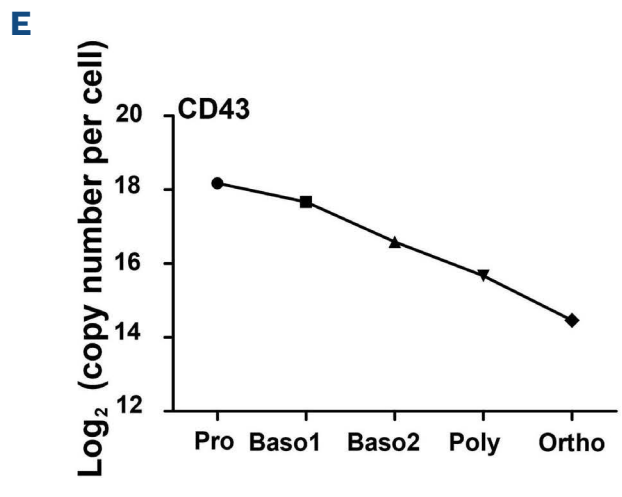


Figure 4. CD43 may be a novel marker for distinguishing erythroid differentiation. (A) Representative histograms showing CD43 expression of bone marrow (BM) erythroblasts (EB) at various stages gated by CD44 and FSC-A (I, pro-EB; II, basophilic EB; III, polychromatic EB; IV, orthochromatic EB and reticulocytes; V, mature red blood cells [RBC]). (B) Representative images of imaging flow cytometry (IFC) analysis showing the expression trend of CD43 as well as changes in cell size, CD44, and Ter119 from pro-EB to RBC. (C) Representative gating strategy using CD43 and FSC-A of $CD45^{-}CD11b^{-}Ter119^{+}$ BM EB and May–Grunwald–Giemsa (MGG) staining images of EB cytospins sorted from distinct clusters. (D) The expression level of CD43 during human erythroid differentiation was analyzed from published transcriptomic data (poly *vs*. ortho false discovery rate [FDR] =0.001). (E) CD43 expression during human erythroid differentiation from published proteomic analysis (ANOVA significant–FDR <0.05). RPKM: reads per kilobase million.

arate EB at distinct stages and observed similar morphology of EB staged by CD44 (Figure 4C). Moreover, both published transcriptomic[36] and proteomic[37] data proved that CD43 expression decreased during human erythroid differentiation (Figure 4D, E). Similarly, the protein level of CD43 progressively decreased in K562 cells induced by hemin towards erythroid differentiation, whereas the positive cell rate of benzidine staining and erythroid surface marker CD235a expression increased gradually (*Online Supplementary Figure S3C–F*). Collectively, these results indicate that CD43 might be a novel surface marker to distinguish erythroid differentiation.

CD169 plays a dispensable role in the bone marrow erythroblastic island niche *in vivo*

In order to further dissect the role of CD169 in BM EBI *in vivo*, we evaluated EBI formation and erythroid differentiation in CD169 WT and KO mice. Unexpectedly, we neither observed paler bones (Figure 5A) nor striking defects of BM EBI percentages in CD169 KO mice *versus* WT mice

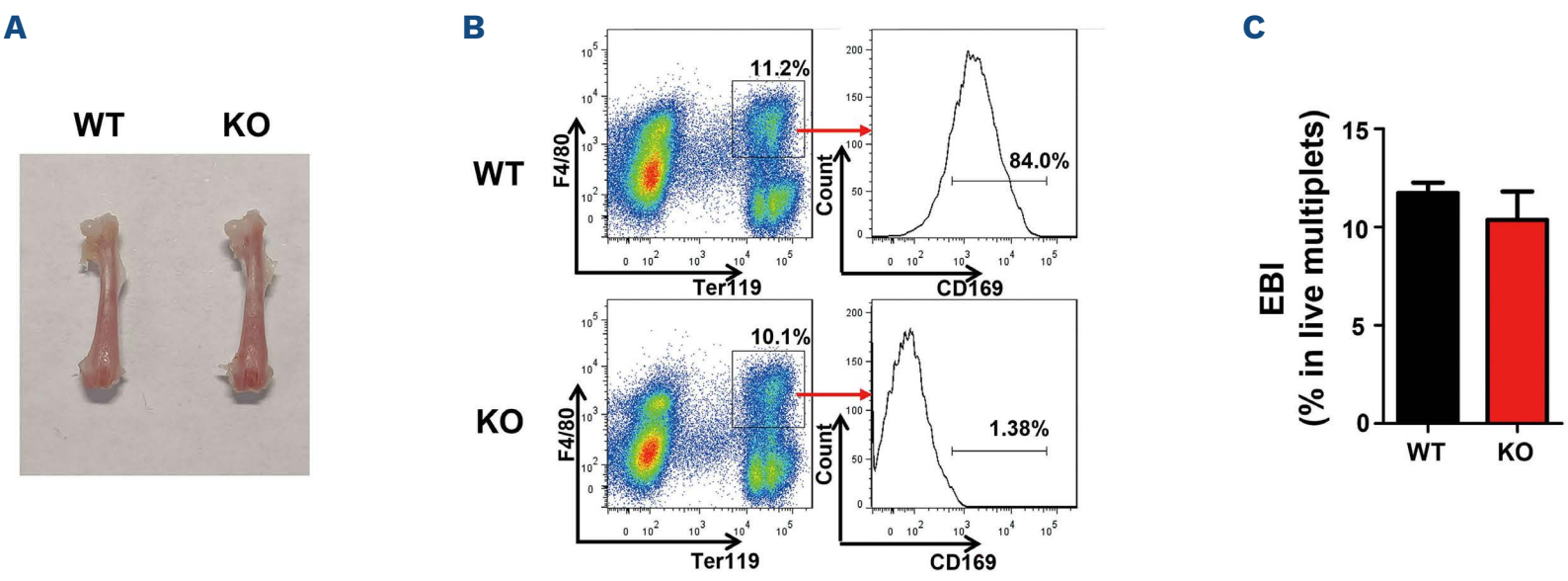


Figure 5. CD169 plays a dispensable role in the bone marrow erythroblastic island niche *in vivo*. (A) Representative photograph of femurs dissected from CD169 wild-type (WT) and knockout (KO) mice. (B) CD169 expression bone marrow (BM) erythroblastic islands (EBI) gated as Ter119^{+}F4/80^{+} live multiplets was determined by fluorescence activated cell sorting (FACS) from CD169 WT and KO mice. (C) The percentage of BM EBI as shown in (B) in CD169 WT and KO mice was quantitatively compared. (D) Representative flow cytometry gating strategy of BM EB from CD169 WT and KO mice. (E) The percentage and cell numbers of BM EB as shown in (D) were quantitatively compared between CD169 WT and KO mice. (F) The percentages of BM erythroblasts (EB) at distinct stages of EB differentiation in (D) were quantitatively compared (N=3). Data are shown as mean ± standard error of the mean.

(Figure 5B, C). There was no significant difference in BM EB quantification between CD169 WT and KO mice, except for marginally reduced BM EB numbers in CD169 KO mice (Figure 5D, E). Moreover, CD169 WT *versus* KO mice showed almost no difference in BM EB maturation measured by CD44 (Figure 5F). Furthermore, CD43 expression of total EB in BM was not different, either (*Online Supplementary Figure S4A*). Notably, CD169 deletion did neither influence the expression of other adhesion molecules, such as VCAM-1 and integrin αv on BM EBI central macrophages (*Online Supplementary Figure S4B*), nor integrin α4 and integrin β1 on BM EB (*Online Supplementary Figure S4C, D*), implying that other adhesion molecules might compensate for the effect of CD169 deletion in EBI formation. In addition, spleen EB and macrophages were not significantly altered and the peripheral RBC compartments at steady state were also normal (*Online Supplementary Figure S4E–M*).

Next, in order to verify whether CD169 deficiency would affect the nursing cell phenotype of BM EBI macrophages, we compared the expression level of genes supporting erythropoiesis between CD169 WT and KO BM macrophages, such as adhesion molecules, nucleus engulfment, iron handling, transcription factors, growth factors, and membrane receptors according to the literature[25] and our RNA sequencing data (*data not shown*). Overall, almost no significant difference was observed on the nursing cell phenotype of EBI macrophages mentioned above except different expression levels of *Slc40a1* and *Spic* between CD169 WT and KO group, indicating that CD169 deletion might not affect the EBI nursing macrophage phenotype (*Online Supplementary Figure S5*).

Taken together, CD169 plays a dispensable role in the BM EBI niche *in vivo*, resembling the function of VCAM-1 in BM EBI formation *in vivo* as reported by Wei Q *et al.*[12]

CD169 slightly promotes bone marrow erythroid differentiation probably via CD43 in high-altitude polycythemia

Next, we investigated the functional role of CD169 in stress erythropoiesis. Similar to the stress erythropoiesis model induced by erythropoietin (EPO) treatment,[38] the HAPC model was chosen as our experimental model owing to the hypoxia-induced increase in EPO levels. After 7 days of exposure to high altitude corresponding to 5,000 m, mice exhibited an increase in RBC production with elevated BM EBI and EB, as well as enhanced spleen erythropoiesis (*Online Supplementary Figure S6*). We then induced HAPC in CD169 WT and KO mice and observed slightly paler bones in CD169 KO HAPC mice (*Online Supplementary Figure S7A*). CD169 KO HAPC mice showed a slight decrease in BM EBI (Figure 6A, B) and BM EB quantification with no significance (Figure 6C, D). However, further analysis to distinguish the EB subpopulation revealed that the percentage of regions I and II increased, while the percentage of region V decreased in CD169 KO HAPC mice (Figure 6E). These results indicate a partial blockade of differentiation at a relatively early EB stage in CD169 KO HAPC mice. Additionally, a small but significant increase in the CD43 level of BM total EB was observed in CD169 KO HAPC mice (Figure 6F). The BM macrophages and monocytes of CD169 KO HAPC mice did not expand significantly *versus* controls (*Online Supplementary Figure S7B, C*). Although stress erythropoiesis primarily occurs in the spleen,[39,40] in CD169 KO HAPC mice, mild splenomegaly (*Online Supplementary Figure S7D, E*) was accompanied by a faint increase in spleen EB (*Online Supplementary Figure S7F-I*) and slightly augmented spleen macrophages (*Online Supplementary Figure S7J, K*), but no alterations in the peripheral RBC parameters (*Online Supplementary Figure S7L*).

We then validated whether CD169 could promote erythroid differentiation in hemin-induced K562 cells. We observed a higher positive cell rate in benzidine staining in the hemin plus CD169 group during the first 2 days (*Online Supplementary Figure S8A*) and lower expression of CD43 in the hemin plus CD169 group *versus* the hemin group (*Online Supplementary Figure S8C, D*). However, CD235a percentage showed no remarkable changes (*Online Supplementary Figure S8B*), in accordance with no prominent differences in the BM EB percentage between CD169 WT and KO HAPC mice, suggesting that CD169 only weakly promotes erythroid differentiation. In contrast, CD43 expression exhibited significant alterations in both the HAPC mice model and hemin-induced K562 cells model.

Discussion

EBI are specialized niches for erythropoiesis, consisting of a central macrophage surrounded by developing EB.[2,3] EBI central macrophages, characterized by specific markers and transcriptional factors,[25,41–44] nurse and support erythroid development through adhesion molecule interaction pairs.[3–7] Previously, adhesion molecules were validated *in vitro* by neutralizing antibodies to elucidate their function in mediating macrophages-EB interaction.[8,9,13] However, the latest study on this declared that not all adhesion molecules are indispensable for EBI formation *in vivo* using conditional gene KO mice,[12] implicating the need to comprehensively revisit and reassess the functional role of adhesion molecules in EBI formation. CD169 is suggested to be such an adhesion molecule participating in this progress,[4,5,25,45] but its exact role and counter receptor in the EBI have not been deeply explored. In the current study, we find that CD169 exerts functions on EBI central macrophages by interacting with CD43 on EB, which is a novel marker to distinguish ery-

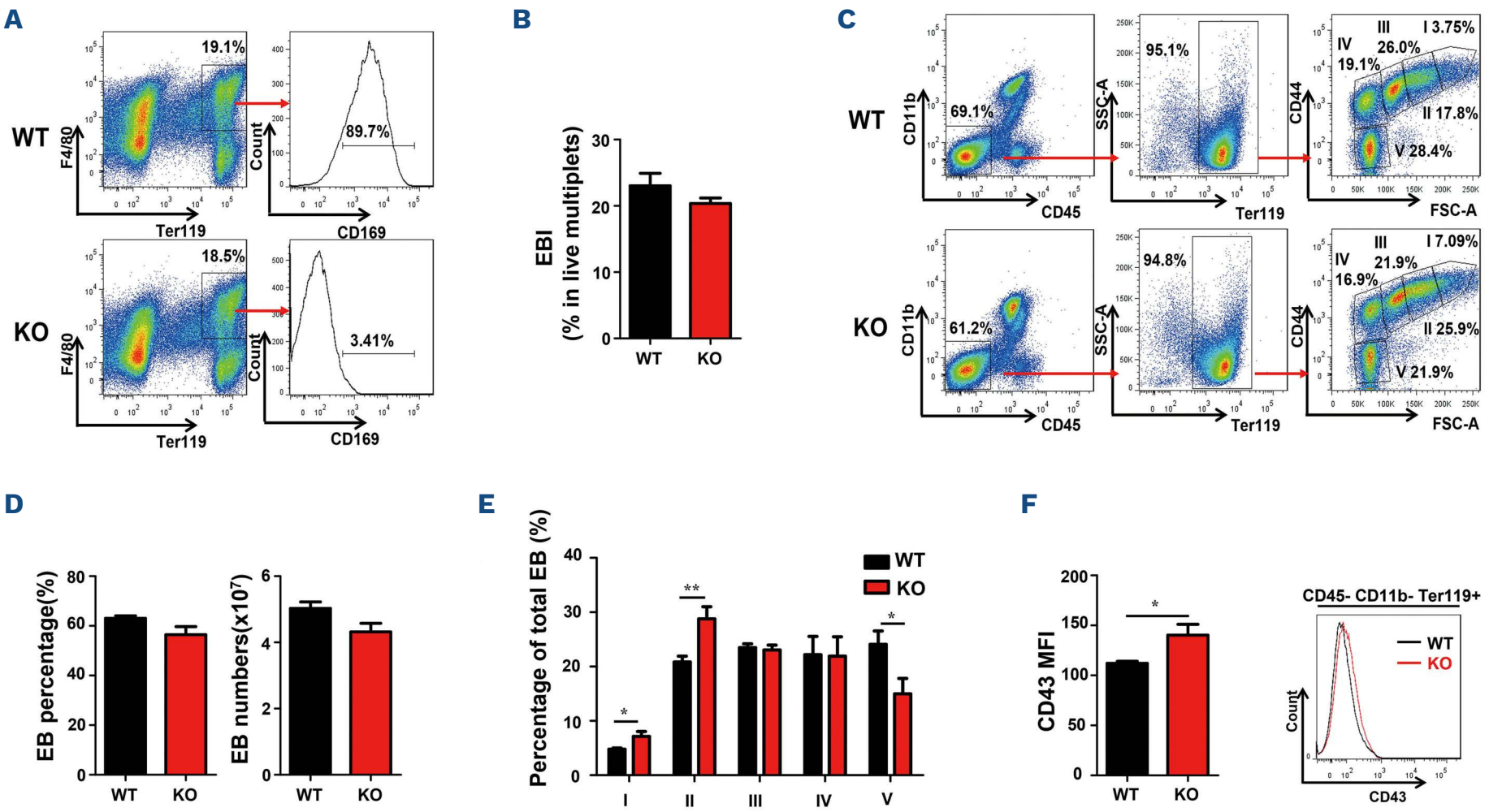


Figure 6. CD169 slightly promotes bone marrow erythroid differentiation probably via CD43 in high-altitude polycythemia. CD169 wild-type (WT) and knockout (KO) mice were exposed to hypoxia in a hypobaric chamber (altitude: 5,000 m) for 7 days to establish high-altitude polycythemia (HAPC) models. (A) CD169 expression in bone marrow (BM) erythroblastic island (EBI) was determined by fluorescence activated cell sorting (FACS) from CD169 WT and KO HAPC mice, respectively. (B) The percentage of BM EBI as shown in (A) in CD169 WT and KO HAPC mice was quantitatively compared (N=6). (C) Representative flow cytometry gating strategy of BM erythroblasts (EB) from CD169 WT and KO HAPC mice. (D) The percentage and cell numbers of BM EB as shown in (C) were quantitatively compared between CD169 WT and KO HAPC mice (N=6). (E) The percentage of BM EB at different stages of EB differentiation in (C) was quantitatively compared (N=6). (F) CD43 expression of $CD45^{-}CD11b^{-}Ter119^{+}$ BM EB as shown in (C) was detected by FACS from CD169 WT and KO HAPC mice, and the mean fluorescense intensisty (MFI) of CD43 was quantitatively compared (N=4). Data are shown as mean ± standard error of the mean; **P*<0.05; ***P*<0.01.

throid differentiation. This CD169-CD43 interaction might promote BM EB differentiation in stress erythropoiesis (Figure 7). Although CD169 deletion may not affect the EBI nursing macrophage phenotype with the exception of *Slc40a1* and *Spic*, which are reportedly involved in iron-recycling,[46] the role of CD169 in scavenging extruded EB nuclei and iron-recycling still awaits further investigation. This study evaluated the function of CD169 in EBI formation through both *in vitro* and *in vivo* studies. Blocking CD169 and CD169 deletion could impair EBI formation *in vitro*, but CD169 KO mice did not display defects in BM EBI function *in vivo*, indicating that other adhesion molecules may compensate for the effect of CD169 deletion in BM EBI formation *in vivo*. Furthermore, *in vitro* cultured murine BMDM lack VCAM-1 expression, which is consistent with the report of Li W *et al*. in which VCAM-1 expression is not observed in umbilical cord blood $CD34^{+}$ cell-derived macrophages *in vitro*.[25] Unexpectedly, overexpression of VCAM-1 on $CD169^{+}$ BMDM might not rescue the impairment of EBI formation upon blocking CD169 *in vitro*. However, whether VCAM-1 can synergize with CD169 on nursing macrophages for EBI formation *in vivo* still needs more experiments to verify.

The counter receptor on EB for CD169 in EBI formation has been unknown in this field. MUC1 is anticipated to bind CD169, as evidenced from the study of human erythroleukemia K562 cells.[32] However, murine BM EB do not express MUC1. Therefore, we turned to another possible candidate, CD43 (also called sialophorin [Spn]), which was originally identified as the counter receptor for CD169 on T cells.[33] It has been reported that CD43 is expressed in hematopoietic cells, including T lymphocytes, B-cell subpopulations, and natural killer cells,[47] but it had so far remained unclear whether CD43 is expressed by EB. We confirmed the expression of CD43 on BM EB and found that the proportion of $CD43^{+}$ BM EB bore a striking similarity to that of recombinant CD169 protein binding to BM EB. Using multiple approaches, we have identified CD43 as the counter receptor for CD169 in the EBI. Our current results strongly authenticate the new finding that CD169 on macrophages and CD43 expressed by both classical and immune-prone erythroid cells are involved in the EBI based

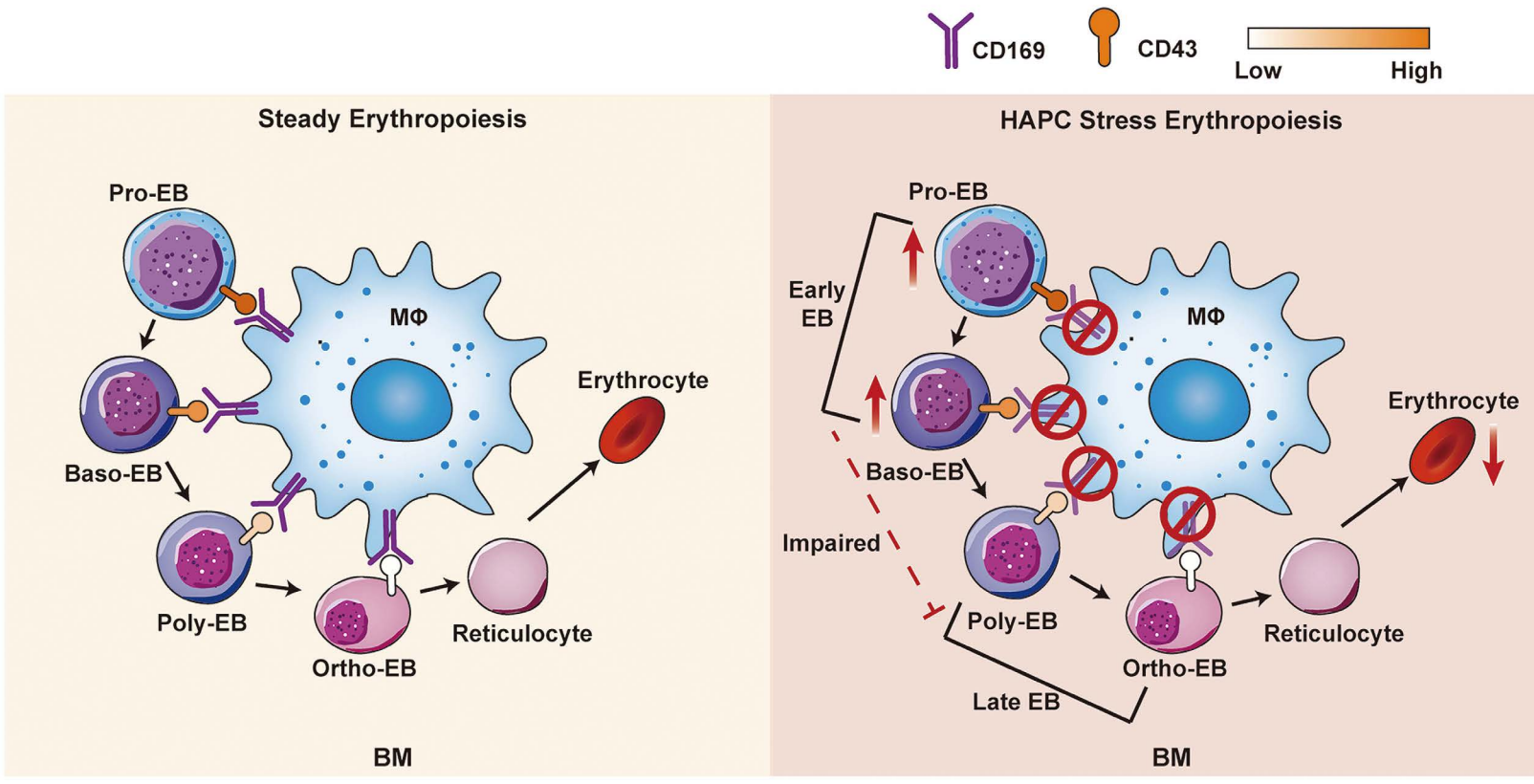


Figure 7. Schematic model of CD169–CD43 interaction in bone marrow erythroblastic island formation and erythroid differentiation. CD43 is identified as the counter receptor on erythroblasts (EB) for CD169 expressed by macrophages in erythroblastic island (EBI). As bone marrow (BM) EB mature, they gradually reduce and even lose CD43 expression, and their binding abilities to CD169 correspondingly attenuate in steady erythropoiesis (left). However, in high-altitude polycythemia (HAPC) stress erythropoiesis, CD169 deficiency impedes BM erythroid differentiation probably via CD43, with the increased percentage of pro-EB and baso-EB and decreased percentage of erythrocytes (right).

on single-cell RNA sequencing combined with CellPhoneDB analysis.[48] Additionally, we observed a progressive decrease of CD43 expression during murine EB maturation similar to the decrease of CD44. Moreover, both published transcriptomic[36] and proteomic[37] data also showed reduced CD43 expression during human erythroid differentiation, which needs further proof to determine whether CD43 could indicate human BM erythroid differentiation, especially by using human BM cell samples coupled with integrin α4 and band 3 antibodies.[49] Collectively, these findings suggest the potential of CD43 as a new marker for distinguishing erythroid maturation. However, it remains to be addressed whether CD43 deletion affects erythroid differentiation by generating CD43 KO mice.

Although CD169 plays a dispensable role in the EBI formation *in vivo*, CD169 deletion impedes erythroid differentiation under stress erythropoiesis, as seen in our mice HAPC model. In line with this, in a burn injury-associated anemia model, Hasan *et al*. highlighted that stagnant early EB of BM may be attributed to CD169 downregulation of EBI central macrophages, leading to the retardation of late EB and reticulocyte differentiation.[50] Our findings provide further evidence to uncover the underlying mechanism behind the phenomenon that CD169 deletion or downregulation of macrophages hinders BM erythroid differentiation probably via upregulated CD43 on EB in stress erythropoiesis. However, in our HAPC model, compensatory splenic erythropoiesis of CD169 KO mice might mask the BM deficit. Similarly, recombinant CD169 protein could enhance hemin-induced K562 erythroid differentiation, along with reduced CD43 expression. However, the mechanism of CD169 promoting erythroid differentiation remains to be determined. Combined with previous studies using CD169-DTR mice in a polycythemia vera model,[23] our current findings may indicate that the effect of CD169 molecule deletion in stress erythropoiesis is weaker than that of CD169$^+$ marcrophage depletion.

In summary, we have demonstrated that CD169 participates in the interaction between macrophages and EB. CD43 is also identified to be the counter receptor on EB for CD169 expressed by EBI central macrophages. More importantly, CD43 might be a novel indicator of erythroid differentiation and maturation. Despite the dispensable role of CD169 in EBI formation *in vivo*, CD169 could promote erythroid differentiation probably via CD43 under stress erythropoiesis, which might be a promising target for the treatment of erythroid disorders.

Disclosures

No conflicts of interest to disclose.

Contributions

JB, FF, CG and SL performed most of the experiments. TW, SC, JY and LZ helped perform the FACS experiments. CZ and JZ performed data analyses. WL, JY, and LF provided technical support. HQ supervised the study. JB and HQ

drafted the manuscript. All authors discussed the results and contributed to the manuscript.

Acknowledgments

We would like to thank the Military Medical Innovation Center, Fourth Military Medical University for providing SPR and IFC instruments. We thank Jinmei Xu and Ning An from the Department of Transfusion Medicine, Xijing Hospital, Fourth Military Medical University for technical assistance. We also thank Tingting Liu, Ying He, Xingyue An, and Ting Bian from the Department of Neurology, Xijing Hospital, Fourth Military Medical University for cytospin and MGG staining.

Funding

This work was supported by grants from the National Natural Science Foundation of China (81530018, 31970829, 82102224, and 82173082); Shaanxi Science and Technology Program (2020ZDLSF03-05); Shaanxi Science and Technology Innovation Team Program (2021TD-36).

Data-sharing statement

All data generated in this study are included in the present article and its Online Supplementary Appendix, which are available from the corresponding author on reasonable request.

References

1. Palis J. Primitive and definitive erythropoiesis in mammals. Front Physiol. 2014;5:3.
2. Bessis M. Erythroblastic island, functional unity of bone marrow. Rev Hematol. 1958;13(1):8-11.
3. Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. Blood. 2008;112(3):470-478.
4. Li W, Guo R, Song Y, Jiang Z. Erythroblastic island macrophages shape normal erythropoiesis and drive associated disorders in erythroid hematopoietic diseases. Front Cell Dev Biol. 2020;8:613885.
5. Klei TRL, Meinderts SM, van den Berg TK, van Bruggen R. From the cradle to the grave: the role of macrophages in erythropoiesis and erythrophagocytosis. Front Immunol. 2017;8:73.
6. Lévesque J-P, Summers KM, Bisht K, Millard SM, Winkler IG, Pettit AR. Macrophages form erythropoietic niches and regulate iron homeostasis to adapt erythropoiesis in response to infections and inflammation. Exp Hematol. 2021;103:1-14.
7. May A, Forrester LM. The erythroblastic island niche: modeling in health, stress, and disease. Exp Hematol. 2020;91:10-21.
8. Hanspal M, Hanspal JS. The association of erythroblasts with macrophages promotes erythroid proliferation and maturation: a 30-kD heparin-binding protein is involved in this contact. Blood. 1994;84(10):3494-3504.
9. Hanspal M, Smockova Y, Uong Q. Molecular identification and functional characterization of a novel protein that mediates the attachment of erythroblasts to macrophages. Blood. 1998;92(8):2940-2950.
10. Soni S, Bala S, Gwynn B, Sahr KE, Peters LL, Hanspal M. Absence of erythroblast macrophage protein (Emp) leads to failure of erythroblast nuclear extrusion. J Biol Chem. 2006;281(29):20181-20189.
11. Javan GT, Salhotra A, Finley SJ, Soni S. Erythroblast macrophage protein (Emp): past, present, and future. Eur J Haematol. 2018;100(1):3-9.
12. Wei Q, Boulais PE, Zhang D, Pinho S, Tanaka M, Frenette PS. Maea expressed by macrophages, but not erythroblasts, maintains postnatal murine bone marrow erythroblastic islands. Blood. 2019;133(11):1222-1232.
13. Sadahira Y, Yoshino T, Monobe Y. Very late activation antigen 4-vascular cell adhesion molecule 1 interaction is involved in the formation of erythroblastic islands. J Exp Med. 1995;181(1):411-415.
14. Hamamura K, Matsuda H, Takeuchi Y, Habu S, Yagita H, Okumura K. A critical role of VLA-4 in erythropoiesis in vivo. Blood. 1996;87(6):2513-2517.
15. Lee G, Lo A, Short SA, et al. Targeted gene deletion demonstrates that the cell adhesion molecule ICAM-4 is critical for erythroblastic island formation. Blood. 2006;108(6):2064-2071.
16. Lee G, Spring FA, Parsons SF, et al. Novel secreted isoform of adhesion molecule ICAM-4: potential regulator of membrane-associated ICAM-4 interactions. Blood. 2003;101(5):1790-1797.
17. Fabriek BO, Polfliet MMJ, Vloet RPM, et al. The macrophage CD163 surface glycoprotein is an erythroblast adhesion receptor. Blood. 2007;109(12):5223-5229.
18. O'Neill ASG, van den Berg TK, Mullen GED. Sialoadhesin - a macrophage-restricted marker of immunoregulation and inflammation. Immunology. 2013;138(3):198-207.
19. Macauley MS, Crocker PR, Paulson JC. Siglec-mediated regulation of immune cell function in disease. Nat Rev Immunol. 2014;14(10):653-666.
20. Crocker PR, Gordon S. Properties and distribution of a lectin-like hemagglutinin differentially expressed by murine stromal tissue macrophages. J Exp Med. 1986;164(6):1862-1875.
21. Crocker PR, Gordon S. Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialylated glycoconjugates characterized by a monoclonal antibody. J Exp Med. 1989;169(4):1333-1346.
22. Crocker PR, Werb Z, Gordon S, Bainton DF. Ultrastructural localization of a macrophage-restricted sialic acid binding hemagglutinin, SER, in macrophage-hematopoietic cell clusters. Blood. 1990;76(6):1131-1138.
23. Chow A, Huggins M, Ahmed J, et al. CD169+ macrophages provide a niche promoting erythropoiesis under homeostasis and stress. Nat Med. 2013;19(4):429-436.
24. Ramos P, Casu C, Gardenghi S, et al. Macrophages support pathological erythropoiesis in polycythemia vera and β-thalassemia. Nat Med. 2013;19(4):437-445.
25. Li W, Wang Y, Zhao H, et al. Identification and transcriptome analysis of erythroblastic island macrophages. Blood. 2019;134(5):480-491.
26. Li P, Huang J, Tian H, Huang Q, Jiang C, Gao Y. Regulation of bone marrow hematopoietic stem cell is involved in high-altitude erythrocytosis. Exp Hematol. 2011;39(1):37-46.
27. Ma P-F, Gao C-C, Yi J, et al. Cytotherapy with M1-polarized macrophages ameliorates liver fibrosis by modulating immune microenvironment in mice. J Hepatol. 2017;67(4):770-779.
28. Cao W, Fan W, Wang F, et al. GM-CSF impairs erythropoiesis by

disrupting erythroblastic island formation via macrophages. J Transl Med. 2022;20(1):11.
29. Zhang Y, Li J-Q, Jiang Z-Z, Li L, Wu Y, Zheng L. CD169 identifies an anti-tumour macrophage subpopulation in human hepatocellular carcinoma. J Pathol. 2016;239(2):231-241.
30. Chow A, Lucas D, Hidalgo A, et al. Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. J Exp Med. 2011;208(2):261-271.
31. Zhang D, Gao X, Li H, et al. The microbiota regulates hematopoietic stem cell fate decisions by controlling iron availability in bone marrow. Cell Stem Cell. 2022;29(2):232-247.
32. Rughetti A, Biffoni M, Pierelli L, et al. Regulated expression of MUC1 epithelial antigen in erythropoiesis. Br J Haematol. 2003;120(2):344-352.
33. van den Berg TK, Nath D, Ziltener HJ, et al. Cutting edge: CD43 functions as a T cell counterreceptor for the macrophage adhesion receptor sialoadhesin (Siglec-1). J Immunol. 2001;166(6):3637-3640.
34. Chen K, Liu J, Heck S, Chasis JA, An X, Mohandas N. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc Natl Acad Sci U S A. 2009;106(41):17413-17418.
35. Liu J, Zhang J, Ginzburg Y, et al. Quantitative analysis of murine terminal erythroid differentiation in vivo: novel method to study normal and disordered erythropoiesis. Blood. 2013;121(8):e43-e49.
36. An X, Schulz VP, Li J, et al. Global transcriptome analyses of human and murine terminal erythroid differentiation. Blood. 2014;123(22):3466-3477.
37. Gautier E-F, Ducamp S, Leduc M, et al. Comprehensive proteomic analysis of human erythropoiesis. Cell Rep. 2016;16(5):1470-1484.
38. Wang J, Hayashi Y, Yokota A, et al. Expansion of EPOR-negative macrophages besides erythroblasts by elevated EPOR signaling in erythrocytosis mouse models. Haematologica. 2018;103(1):40-50.
39. Chen Y, Xiang J, Qian F, et al. Epo receptor signaling in macrophages alters the splenic niche to promote erythroid differentiation. Blood. 2020;136(2):235-246.
40. Liao C, Prabhu KS, Paulson RF. Monocyte-derived macrophages expand the murine stress erythropoietic niche during the recovery from anemia. Blood. 2018;132(24):2580-2593.
41. Mukherjee K, Xue L, Planutis A, Gnanapragasam MN, Chess A, Bieker JJ. EKLF/KLF1 expression defines a unique macrophage subset during mouse erythropoiesis. Elife. 2021;10:e61070.
42. Mukherjee K, Bieker JJ. Transcriptional control of gene expression and the heterogeneous cellular identity of erythroblastic island macrophages. Front Genet. 2021;12:756028.
43. Okreglicka K, Iten I, Pohlmeier L, et al. PPARγ is essential for the development of bone marrow erythroblastic island macrophages and splenic red pulp macrophages. J Exp Med. 2021;218(5):e20191314.
44. Seu KG, Papoin J, Fessler R, et al. Unraveling macrophage heterogeneity in erythroblastic islands. Front Immunol. 2017;8:1140.
45. Falchi M, Varricchio L, Martelli F, et al. Dexamethasone targeted directly to macrophages induces macrophage niches that promote erythroid expansion. Haematologica. 2015;100(2):178-187.
46. Haldar M, Kohyama M, So AY-L, et al. Heme-mediated SPI-C induction promotes monocyte differentiation into iron-recycling macrophages. Cell. 2014;156(6):1223-1234.
47. Tuccillo FM, de Laurentiis A, Palmieri C, et al. Aberrant glycosylation as biomarker for cancer: focus on CD43. Biomed Res Int. 2014;2014:742831.
48. Xu C, He J, Wang H, et al. Single-cell transcriptomic analysis identifies an immune-prone population in erythroid precursors during human ontogenesis. Nat Immunol. 2022;23(7):1109-1120.
49. Hu J, Liu J, Xue F, et al. Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis in vivo. Blood. 2013;121(16):3246-3253.
50. Hasan S, Johnson MC, Kini AR, Baldea AJ, Muthumalaiappan K. A shift in myeloid cell phenotype via down regulation of Siglec-1 in island macrophages of bone marrow is associated with decreased late erythroblasts seen in anemia of critical illness. Front Med (Lausanne). 2019;6:260.