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Systemic deficiency of mouse arachidonate 15-lipoxygenase induces defective erythropoiesis and transgenic expression of the human enzyme rescues this phenotype

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Abstract

Arachidonic acid 15-lipoxygenases (ALOX15) are lipid peroxidizing enzymes, which has previously been implicated in the maturational breakdown of intracellular organelles and plasma membrane remodeling during reticulocyte-erythrocyte transition. Conventional *Alox15*$^{-/-}$ mice are viable, develop normally but do not exhibit a major defective erythropoietic phenotype. To characterize the putative in vivo relevance of Alox15 for red blood cell development, we explored the impact of systemic inactivation of the *Alox15* gene on mouse erythropoiesis. We found that *Alox15*$^{-/-}$ mice exhibited reduced erythrocyte counts, elevated reticulocyte counts and red cell hyperchromia. The structure of the plasma membrane of *Alox15*$^{-/-}$ erythrocytes is altered and a significant share of the red cells was present as echinocytes and/or acanthocytes. An increased share of the Alox15$^{-/-}$ erythrocytes cells were annexin V positive, which indicates a loss of plasma membrane asymmetry. Erythrocytes of *Alox15*$^{-/-}$ mice were more susceptible to osmotic hemolysis and exhibited a reduced ex vivo life span. When we transgenically expressed human ALOX15 in *Alox15*$^{-/-}$ mice under the control of the aP2 promoter the defective erythropoietic system was rescued and the impaired osmotic resistance was normalized. Together these data suggest the involvement Alox15 in the maturational remodeling of the plasma membrane during red cell development.

KEYWORDS

biomembranes, differentiation, eicosanoids, lipid peroxidation, redox equilibrium, reticulocytes

1 | INTRODUCTION

Oxidative stress,[1] which has been implicated in the pathogenesis of inflammatory, hyperproliferative, and neurodegenerative diseases,[2-4] is defined as an imbalance of the cellular redox equilibrium. At low concentrations, reactive oxygen species (ROS) function as second messengers[5] and are essential for proper cell function. In contrast, at higher levels ROS induce cellular malfunction and even cell death, such as apoptosis and/or necroptosis.[6,7] Usually, the intracellular redox homeostasis is well balanced and 15-lipoxygenase (ALOX15) plays an important role in the regulation of this equilibrium.[8,9] However, in mammals ALOX isoforms have also been implicated in more

Abbreviations: Alox, arachidonate lipoxygenase; Alox15, murine arachidonate 15-lipoxygenase; ALOX15, 15 lipoxygenase; Epo, erythropoietin; HETE, hydroxy-eicosatetraenoic acid; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

specific cell functions, such as skin development[10,11] and erythropoiesis.[12-14]

In the human genome, six different ALOX genes (*ALOX15*, *ALOX15B*, *ALOX12*, *ALOX12B*, *ALOXE3*, and *ALOX5*) are present.[15,16] In addition, a number of corrupted pseudogenes and an ALOX12 antisense gene have been identified.[15] In mice, a single ortholog exists for each human ALOX paralog but in addition a functional Alox12e is expressed in the skin.[17] This mouse Alox paralog, which shares a high degree of evolutionary relatedness with mouse Alox15, is encoded for by the *Alox12e* gene that is a corrupted pseudogene in humans.[16] Targeted gene inactivation studies indicated that most of the mouse Alox paralogs exhibit different biological functions,[16] and thus, the genetic multiplicity may not be considered a sign of functional redundancy. The biological relevance of the different ALOX paralogs has been explored for many years[18,19] but for some paralogs the biological functions remain unclear.

In rabbits, ALOX15 has been implicated in erythropoiesis and detection of this enzyme in human,[20,21] mouse[13] and rat reticulocytes[14] suggested that this enzyme may also be relevant for erythropoiesis in other species. Mammalian red blood cells develop in the bone marrow from myeloid stem cells along the erythroid linage and reticulocytes are the immediate precursors of mature erythrocytes.[22] As most somatic cells early erythroid precursors show extensive subcellular compartmentation and carry a nucleus, functional ribosomes, and mitochondria as well as a regularly structured endoplasmic reticulum. Human reticulocytes do not carry nuclei any more since this central intracellular organelle is functionally inactivated and extruded from the cells at earlier maturation stages.[12,22] However, in most mammalian species young reticulocytes still contain functional mitochondria and rabbit reticolocytes generate than 80% of their ATP via cellular respiration.[22] In contrast, old reticulocytes and mature erythrocytes do not have functional mitochondria any more, and in these cells glycolysis constitutes the major source of ATP production.[22,23] These data suggest that at least in rabbits functional mitochondria are degraded during reticulocyte-erythrocyte transition and ALOX15 has been implicated in this process.[23,24] In reticulocyte, precursor cells as well as in young reticulocytes ALOX15 is not expressed,[25-27] but its mRNA is present as translationally inactive mRNA-protein particles (RNPs). In more mature reticulocytes, the inhibitory proteins are proteolytically degraded and the ALOX15 mRNA is translated during a narrow time window.[24,28] Once the enzyme has been synthesized it oxygenates cellular membrane lipids[29,30] and this reaction destabilizes the membrane structure. This enzymatic lipid peroxidation disturbs the hydrophobic interaction between the membrane phospholipids and renders the membranes prone to proteolytic degradation via ATP-dependent and ATP-independent mechanisms.[31,32] Membranes of rabbit reticulocytes contain large amounts of oxygenated phospholipids[33] and the degree of oxidation of the mitochondrial membrane lipids was higher than that of the plasma membrane.[34] Taken together, these data suggested a role of ALOX15 in the maturational breakdown of intracellular organelles during red blood cell development but also in restructuring of the plasma membranes.[34,35]

From these data, it was concluded that systemic inactivation of the *Alox15* gene should lead to defective erythropoiesis. However, when *Alox15*$^{-/-}$ mice became available these animals did not exhibit a defective erythropoietic phenotype.[36,37] All basic hematological parameters (counts of erythrocytes, leukocytes, thrombocytes and reticulocytes, hemoglobin, hematocrit) were in the normal range[36] and histological examination of internal organs did not show obvious structural abnormalities.[36] However, more recent investigations suggested a role of this enzyme in hematopoietic stem cell function,[38] but still the role of Alox15 in hematopoiesis remains a matter of discussion.

To contribute to this discussion and to further explore the potential role of Alox15 in mouse erythropoiesis, we first compared basic red blood cell parameters of Alox15$^{-/-}$-mice with those of wild-type animals and found that Alox15 deficiency induced a reduction of the peripheral erythrocyte counts with compensatory elevated reticulocyte numbers and hyperchromia. Alox15 deficient erythrocytes were less resistant to osmotic challenge and showed an attenuated in vitro life span. The number of peripheral echinocytes/acanthocytes was significantly elevated in *Alox15*$^{-/-}$ mice and annexin V staining indicated plasma membrane abnormalities. Transgenic expression of human ALOX15 in *Alox15*$^{-/-}$-mice rescued the alterations of the hematopoietic parameters and normalized the osmotic stability. Taken together, our data indicate a functional role of Alox15 in mouse erythropoiesis.

2 | MATERIALS AND METHODS

2.1 | Chemicals

The chemicals were from the following sources: HPLC standards of 5(±)-HETE, 12(±)-HETE, and 15(±)-HETE from Cayman Chem. (distributed by Spi Bio, Montigny le Bretonneux, France), sodium borohydride from Life Technologies, Inc (Eggenstein, Germany), HPLC solvents were from Baker (Deventer, Netherlands) or VWR International GmbH (Darmstadt, Germany), FITC-conjugated annexin V from ImmunoTools GmbH (Friesoythe, Germany). Oligonucleotide synthesis was performed at BioTez (Berlin, Germany). Other chemicals (NaCl, KCl, $MgSO_4$, HEPES, glucose, $CaCl_2$) were purchased from Invitrogen (Darmstadt, Germany).

2.2 | Animals

All the mice were bred and maintained in SPF free animal facility, with food and water ad libitum. Procedures were performed according to the EU Directive 2010/63/EU, Federation of Laboratory Animal Science Associations and the local guidelines. For our experiments, male mice were used at the age of 10-25 weeks. *Alox15* knockout mice (strain name: B6.129S2-*Alox15*^{tm1Fun}/J) were obtained from Jackson laboratory (Maine, USA),[36] and backcrossed for seven generations into Black six background (*Alox15*$^{-/-}$ mice). Transgenic mice overexpressing human ALOX15 under the control of the aP2 promoter (*aP2-ALOX15* mice) were created by transfection of embryonic stem cells with an ALOX15 minigene construct.[39] Transgenic stem cells were selected for blastocyst injection and several chimeric individuals were tested for germline transmission of the transgene.[39]

2.3 | PCR genotyping of the native Alox15 locus and the ALOX15 transgene

PCR was performed with the MyTaq Red Mix (BIOLINE, Germany) according to the vendor's instructions. For genotyping the *Alox15* gene locus the primer sequences were recommended by Jackson laboratory: oIMR0013 Neo: 5′-CTTGGGTGGAGAGGCTATTC-3′, oIMR0014 Neo: 5′-AGGTGAGATGACAGGAGATC-3′, oIMR0862- Exon3: 5′-CGTGGTTGAAGACTCTCAAGG-3′, oIMR0863- Exon4: 5′-CGAAATCGCTGGTCTACA GG-3′. The wild-type band is 229 bp and the knockout band is 280 bp. Homozygous allele carriers only show the 280 bp band. The primers used to detect the human *ALOX15* transgene were as follows: aP2 promotor up-5′-AGTCAAAACAGG-AACCTTTAAAATACTC-3′ and ALOX15 do-: 5′-TACTTCCACCTTGAATTCTGT-CTCCTT-3′. A band of 450 bp indicate the presence of the aP2 promotor driven *ALOX15* gene.[40]

2.4 | Blood collection and isolation of bone marrow cells

For diagnostic analysis, blood was collected from the facial vein plexus into EDTA-containing tubes and the quantification of the basic blood parameters was carried out at the Institute for Veterinarian Diagnostics (Berlin, Germany) using a hematology analyzer (Sysmex XT-2000iV). The total volume of the single blood withdrawal was about 6% of mouse body weight. For terminal blood removal mice were sacrificed by cervical dislocation after isoflurane anesthesia and blood was collected by cardiac puncture.

To isolate bone marrow cells, the femur bones were removed. Both the ends of each femur bone were cut off using a scalpel, and the bone marrow cells were prepared by rinsing the bone marrow cavity of the femur with 10 mL phosphate-buffered saline (PBS). Cells were washed once with 1 mL of PBS, pelleted by centrifugation (800 g), and reconstituted in 0.5 mL of PBS. Aliquots of this suspension were used for cellular Alox activity assays and RNA preparation.

2.5 | RNA isolation and quantitative real-time PCR

Total RNA was extracted from different tissues using the NucleoSpin RNA Plus (MACHEREY-NAGEL, Düren, Germany) and from blood cells using the QIAamp RNA Blood Kit (QIAGEN, Hilden, Germany). Synthesis of the cDNAs was performed with 0.5-2 μg of the total RNA preparations using oligo (dT) primers and Tetro Reverse Transcriptase (BIOLINE, Luckenwalde, Germany). Quantitative real-time PCR (RT-PCR) was carried out with a Rotor Gene 3000 (Corbett Research) using the SensiFast SYBR PCR Kit (BIOLINE, Luckenwalde, Germany). The following primer sets were employed to quantify the Gapdh, Alox15, Alox12, Alox5, ALOX15, and Erythropoietin mRNA: Gapdh; 5′-CCATCACCATCTTCCAGGAGCGA-3′; 5′-GG ATGACCTTGCCCACAGC-CTTG-3′, Alox15; 5′-GCTGCA CCGTGGTTGAAGACTCT-3′; 5′-CTGTACAGACTCCTC CTTTCTTCC-3′, Alox12; 5′-GCGGCCATGTTCAGTTGC TTAC-3′; 5′-CATCGTCACGTCGTCCTTGCTG-3′, Alox5; 5′-TCG-AGTTCCCATGTTACCGCT-3′; 5′-CTGTGGTCACT GGGAGCTTCG-3′, ALOX15; 5′-ACTGAAATCGGG-C TGCAAGGGG-3′; 5′-TGGCCCACAGCCACCATAACGG-3′, and Epo; 5′-CACCCTGCTGCTTTTACTCT-CCTT-3′; 5′-CTT CTGCACAACCCATCGTGACAT-3′. The experimental raw data were analyzed with the Rotor-Gene Q software. To generate standard curves for quantification of gene expression levels, specific amplicons were used as external standards for each target gene. Gapdh mRNA was used as an internal standard to normalize expression of the target transcripts. All RNA preparations were analyzed at least in triplicate, and means ± standard deviation are given.

2.6 | Alox activity assay

Blood plasma was separated by centrifugation and peripheral red blood cells were washed twice with PBS. Cells were pelleted and 10 and 100 μL of packed red cells were resuspended in 0.5 mL of PBS. Arachidonic acid was added to reach a final concentration of 100 μM and the samples were incubated for 10 minutes at room temperature. Bone marrow cells were washed twice with PBS and 10^7 cells were used for the activity assays. Hydroperoxy lipids were reduced by the addition of solid sodium borohydride (1 mg) and after acidification (50 μL of concentrated acetic acid) the lipids

were extracted twice with 0.5 mL of ethyl acetate. The extracts were combined, the solvent was evaporated and the remaining lipids were reconstituted in 1 mL of HPLC column solvent. 0.3 mL of the lipid preparation were injected to RP-HPLC to quantify the amounts of 12S-HETE (hydroxyeicosatetraenoic acid), 15S-HETE, and 5S-HETE constituting the major arachidonic acid oxygenation product of mouse Alox15, Alox12, Alox5, and human ALOX15. To test the Alox activity 50 mg of tissue (wet weight) were added to 0.5 mL of PBS containing 100 μM arachidonic acid. The tissue was homogenized using a FastPrep-24 homogenizer (MP-Biomedicals, Eschwege, Germany) and the homogenates were incubated for 10 minutes at room temperature. The hydroperoxy lipids were reduced (addition of 1 mg $NaBH_4$) and after acidification (50 μL of acetic acid) the lipids were extracted twice with 0.5 mL of ethyl acetate. Solvents were evaporated and lipids were reconstituted in 1 mL of HPLC column solvent. 0.3 mL of the lipid preparation were injected to RP-HPLC to quantify 12-HETE amounts.

2.7 | HPLC analysis of the reaction products

HPLC analysis of the arachidonic acid oxygenation products was carried out as described in Kuhn et al.[18] Combined normal phase/chiral phase HPLC was performed on a Chiralpak AD-H column (250 × 4.6 mm, 5 μm particle size; Daicel Chem., Osaka, Japan), which was connected with a Nucleosil normal phase HPLC guard column (Marcherey-Nagel, Düren, Germany; KS-system, 35 × 4.4 mm, 5 μm particle size). A solvent system consisting of n-hexane/methanol/ethanol/acetic acid (96/3/1/0.1, by volume) was used at a flow rate of 1 mL/min. The absorbance at 235 nm was recorded.

2.8 | HPLC analysis of the PUFA composition of red blood cell membrane lipids

Mice were sacrificed by cervical dislocation after isoflurane anesthesia and blood was withdrawn from *Alox15* knockout mice (*Alox15*$^{-/-}$) and corresponding C57BL/6 wild-type controls (WT). Blood plasma was separated by centrifugation and peripheral blood cells were washed twice with PBS. Cells were pelleted and the membrane lipids were extracted[41] from 50 μL of packed red cells. The solvent was evaporated, the remaining lipids were reconstituted in 0.35 mL anaerobic methanol and 0.075 mL of anaerobic 40% KOH was added. The ester lipids were hydrolyzed under an argon atmosphere (20 minutes at 60°C) and the samples were neutralized by the addition of 0.075 mL of acetic acid. The precipitate was spun down and aliquots of the clear supernatant were injected to RP-HPLC analysis on a Nucleodur C18 Gravity column (Macherey-Nagel, Düren, Germany; 250 × 4 mm, 5 μm particle size). The free polyenoic fatty acids were eluted with a solvent system consisting of acetonitrile/water/acetic acid (70/30/0.1, by vol.) at a flow rate of 1 mL/min. Polyenoic fatty acids were analyzed recording the absorbance at 210 nm, whereas oxygenated polyenoic fatty acids were detected at 235 nm The retention times of different PUFA and hydroxyl PUFA standards were determined and chromatographic scale was calibrated by injecting known amounts of these standards.

2.9 | Determination of osmotic stability of erythrocytes

To assess the resistance of the red blood cells for osmotic stress, 2 μL of blood was diluted in 200 μL phosphate buffer (pH 7.4) containing NaCl at different concentrations (0%-0.85%) and the cells were incubated for 30 minutes at room temperature. The samples were centrifuged at 200 *g* for 10 minutes and the supernatant containing the free hemoglobin, which was released during hemolysis, was quantified by assaying the absorbance at 540 nm. The absorbance values were expressed as a percentage of complete hemolysis, which was achieved when the cells were incubated in the absence of NaCl. This absorbance was set 100%.

2.10 | Erythrocyte isolation from whole blood and ex vivo life span analysis

For blood removal, mice were sacrificed by cervical dislocation after isoflurane anesthesia and blood was collected by cardiac puncture. About 500 μL of whole blood was centrifuged at 700 *g* for 5 minutes and supernatant and the thin buffy coat were removed. Red blood cells were washed twice with 1 mL Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$; pH 7.4). Erythrocytes were incubated at a hematocrit of 0.4% at room temperature under continuous slow-moving.[42] After different incubation periods, 500 μL of the cell suspension were taken off and centrifuged at 700 *g* for 5 minutes. The supernatant containing the free hemoglobin, which was released during hemolysis, was quantified by assaying the absorbance at 540 nm. The absorbance values were expressed as a percentage of complete hemolysis, which was obtained when the cells were incubated in water. This absorbance represented 100% hemolysis. The cell pellet was used for FACS analysis.

2.11 | Flow cytometry analysis

Erythrocyte phosphatidylserine exposure was quantified by staining the cells with FITC-conjugated annexin V

(Immunotools, Friesoythe, Germany) at a dilution of 1:100 and an incubation time of 30 minutes at room temperature under the protection of light. Annexin-V fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACSCalibur (BD Biosciences, Heidelberg, Germany). A marker (M1) was placed to set an arbitrary threshold between annexin V binding cells and control cells. The same threshold was used for wild-type and Alox15 knockout mice.

2.12 | Live cell imaging

To evaluate the cell morphology and phosphatidylserine exposure on the erythrocyte surface at different time points, the annexin V stained erythrocyte suspensions were transferred into chambered coverslips (Thermo Fisher Scientific, Schwerte, Germany). Images (bright field and annexin V fluorescence) were captured and evaluated using an Axioskop microscope (Axiocam, Zeiss, Germany) at a magnification of 40-fold objective and processed using Axiovision software (SE64 Rel. 4.9, Zeiss, Jena, Germany). Several randomly selected frames from each sample were selected for morphological inspection and statistical evaluation. About 1000-1500 erythrocytes per incubation period were scored.

2.13 | Statistical analysis

Statistical analyses were carried out with SPSS 24 (IBM New York, USA) software package and the results are presented as means ± standard deviation. Nonparametric tests, most frequently the Mann-Whitney *U* test, was carried out. Two-tailed significances were accepted at $P < .05$. Means (black vertical lines in boxes) and the 25th and the 75th percentile are visualized.

3 | RESULTS

3.1 | Systemic inactivation of the *Alox15* gene induces reduction of peripheral red blood cell counts, hyperchromia and reticulocytosis

In rabbits, ALOX15 has been implicated in the maturational degradation of mitochondria during reticulocyte-erythrocyte transition[12] and the remodeling of the plasma membrane.[34] Although ALOX15 is present in reticulocytes of rabbit,[29] rats, mice,[13,14] and humans[21] systemic inactivation of the mouse *Alox15* gene did not induce an obvious hematopoietic phenotype.[16,36] These data challenged the previous hypothesis derived from experiments with rabbit reticulocytes that ALOX15 may play an erythropoietic role in mammals.[12] However, more recent studies with these mice revealed abnormal hematopoietic parameters and based on these data it was concluded that Alox15 might be of hematopoietic relevance.[38] To contribute to this discussion, we first compared basic red cell parameters of *Alox15*$^{-/-}$ mice with wild-type controls of identical genetic background and found that *Alox15*$^{-/-}$ mice suffer from reduced peripheral erythrocyte counts, hyperchromia, and reticulocytosis (Figure 1A-C). Comparison of the other erythrocyte parameters (hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin concentration) did not reveal significant differences (Figure 1D-G).

3.2 | Systemic inactivation of the *Alox15* gene induces structural alterations in peripheral red blood cells

To explore whether Alox15 deficiency alters the structure of the peripheral red blood cells, we first carried out life cell imaging. Here, we found that in wild-type mice the vast majority of the red blood cells are regularly shaped biconcave discocytes (Figure 2A). Less than 1% echinocytes and/or acanthocytes were detected. In contrast, in *Alox15*$^{-/-}$ mice the relative share of echinocytes/acanthocytes was higher (Figure 2B) and the difference was statistically significant (Figure 2C). When we repeated life cell imaging after 3 days in vitro culturing period, the number of echinocytes/acanthocytes was elevated in both wild-type (Figure 2D) and *Alox15*$^{-/-}$ erythrocytes (Figure 2E). After the 3 days of culturing period, there was still a significant difference between the two genotypes (Figure 2F). These data indicate that in the absence of a functional Alox15 a share of the peripheral red blood cells are abnormally structured. To explore the molecular basis for the observed structural differences, we carried out annexin V staining. With this method cells are stained, which have elevated phosphatidylserine content in the outer leaflet of the plasma membrane (loss of membrane asymmetry) and such changes are an early indicator for various types of cell death.[43,44] To compare the staining intensity of peripheral red blood cells of wild-type control mice (Figure 2G) with those of *Alox15*$^{-/-}$ animals (Figure 2H), we first carried out immunohistochemical annexin V staining and counted the annexin V positive cells. Here, we found that the blood of *Alox15*$^{-/-}$ mice contained more annexin V positive (indicated by the arrows) cells than the blood of wild-type animals. For more precise quantification of the annexin V positive cells we combined annexin V staining with FACS analysis and found more intensive annexin V staining for *Alox15*$^{-/-}$ erythrocytes (Figure 2I). Statistical analysis of the experimental raw data indicated a significant difference between the erythrocytes of the two cell types (Figure 2J). Similar differences were observed when the erythrocytes of

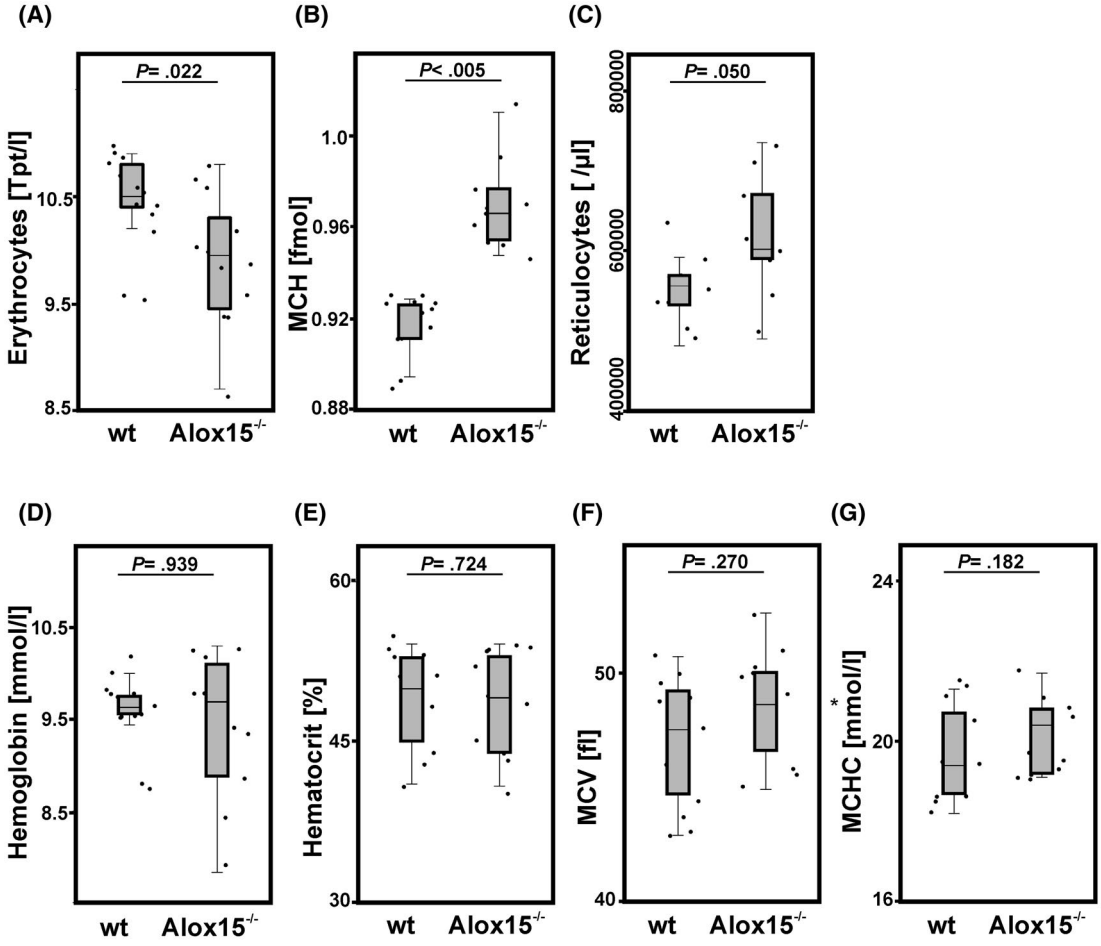

FIGURE 1 Systemic inactivation of the *Alox15* gene induces reduction of the peripheral erythrocyte counts, hyperchromia and elevated reticulocyte numbers. About 200 µL blood was withdrawn from male *Alox15* knockout (*Alox15*$^{-/-}$) and corresponding C57BL/6 wild-type (wt) control mice. For blood removal mice were sacrificed by cervical dislocation after isoflurane anesthesia and blood was collected by cardiac puncture. Coagulation was inhibited by EDTA and the basic red blood cell parameters were determined as described in "Material and Methods". A, erythrocyte counts. B, mean corpuscular hemoglobin content. C, reticulocyte counts. D, hemoglobin. E, hematocrit. F, mean corpuscular volume (MCV). Genotyping of wt or Alox15$^{-/-}$ mice: see "Material and Methods." Significances were calculated using Mann-Whitney *U* test. N = 9-13 animals were analyzed. Significances were accepted at $P < .05$

the two genotypes were analyzed after a three day in vitro culturing period (Figure 2K) and here again a significant difference was observed between the two genotypes (Figure 2L).

Taken together, these data indicate that Alox15 deficiency leads to the formation of structurally compromised erythrocytes, which are surrounded by a modified plasma membrane. This finding is consistent with our working hypothesis that Alox15 is involved in restructuring of the plasma membrane during reticulocyte-erythrocyte transition.

3.3 | Diagnostic blood removal induces expression of Alox15 in peripheral blood cells and spleen tissue

In rabbits expression of ALOX15 is strongly upregulated during the time course of experimental anemia.[12,24] To explore whether a single blood removal for diagnostic purpose (about 6% volume of mouse body weight) is sufficient to induce Alox15 expression, wild-type mice were sacrificed 24 hours after blood withdrawal and Alox15 expression was tested in different tissues (Figure 3A). Here, we detected significantly elevated levels of Alox15 mRNA in peripheral blood cells and the spleen. In bone marrow cells, Alox15 mRNA was slightly down and in the kidney no alterations were observed. To test whether this induction is also visible on the activity level, we carried out ex vivo activity assays. Using spleen tissue homogenates of bled mice as enzyme source (Figure 3B), we detected the formation of 12-HETE (major arachidonic acid oxygenation product of mouse Alox15). Much lower amounts of these products were observed in spleen tissue of unbled mice. Chiral phase HPLC indicated a strong preponderance of 12*S*-HETE indicating that the 12-HETE formed by spleen tissue of bled mice originated from the Alox15 pathway (Figure 3B, panel b). Similar analyses were carried out with peripheral blood cells (Figure 3C). Here, we used

10 µL of packed red cells as enzyme source and also detected 12S-HETE as major lipoxygenation product. When we compared the extent of 12S-HETE formation by spleen tissue and peripheral blood cells prepared from bled and unbled wild-type mice (Figure 3D) we observed significantly elevated 12S-HETE formation in bled mice. In contrast, there was no significant difference between unbled wild-type and bled *Alox15* deficient mice. From these data, it was concluded that a single diagnostic blood removal induced expression of Alox15 in spleen and circulating blood cells.

3.4 | Diagnostic blood withdrawal induced similar erythropoietic alterations in wild-type animals and *Alox15*$^{-/-}$ mice

Figures 1 and 3 indicate that both, *Alox15* deficiency and analytical blood removal challenge the erythropoietic system. To compare the regulatory capacities of wild-type mice and *Alox15*$^{-/-}$ animals, we quantified the basic erythropoietic parameters of bled and unbled wild-type mice with those of bled and unbled *Alox15*-deficient animals 24 hours after

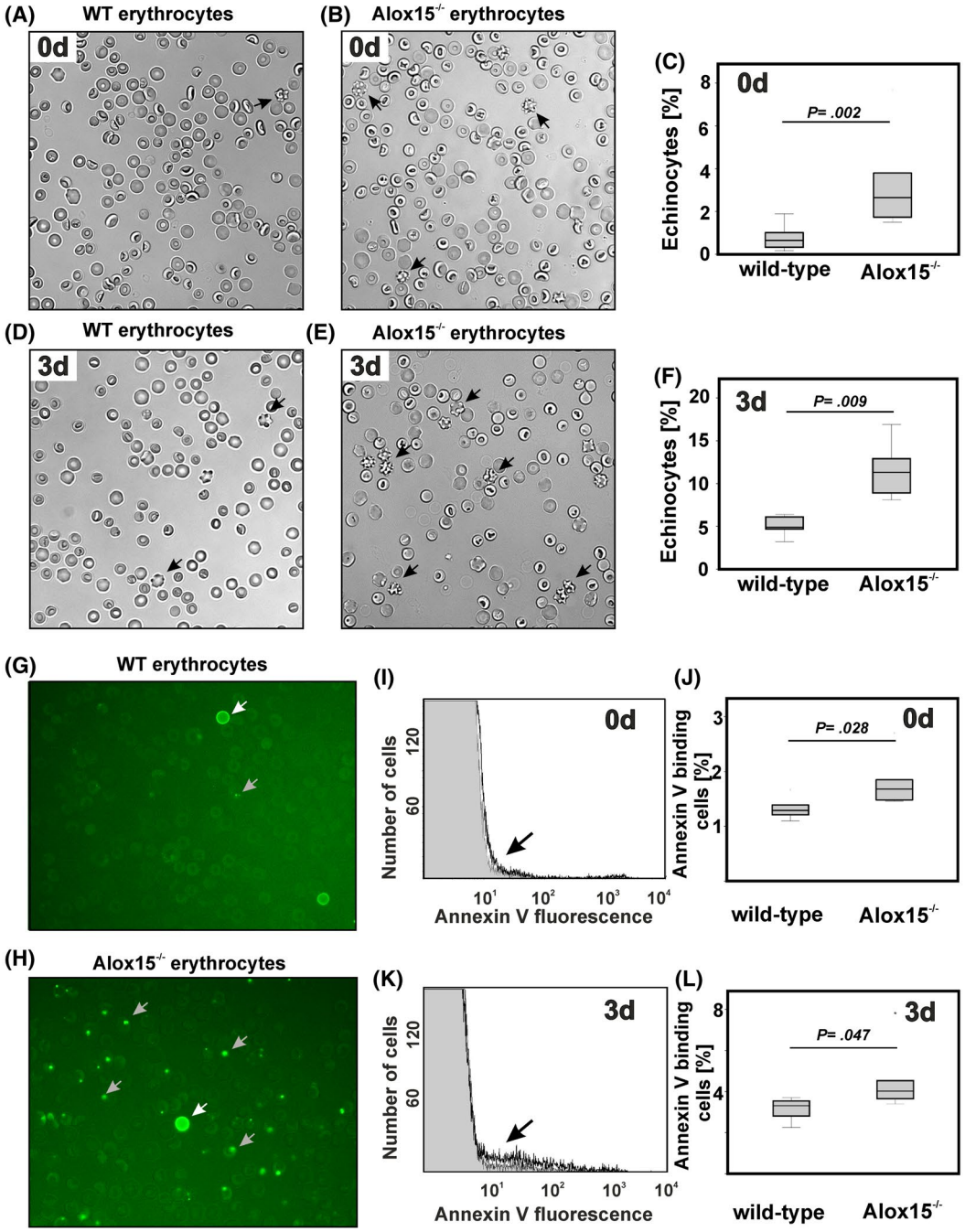

FIGURE 2 The blood of *Alox15*$^{-/-}$ mice contains an elevated share of structurally compromised erythrocytes. Erythrocytes were prepared from wild-type mice and Alox15-deficient animals (see Section 2) and life cell imaging was carried out (Figure 2A-F). The remaining cells were employed to set up in vitro erythrocyte cultures and to quantify the degree of annexin V staining in combination with FACS analysis (Figure 2G-L). A, Life cell imaging of erythrocytes prepared from wild-type mice immediately after the animals had been sacrificed. It can be seen that the vast majority of the cells were present as regularly shaped biconcave discocytes. Only a minor share of the cells was present as echinocytes and/or acanthocytes (black arrow). A representative life cell image is shown and corresponding images were taken for eight different wild-type individuals. B, Life cell imaging of erythrocytes prepared from *Alox15*$^{-/-}$ mice immediately after the animals had been sacrificed. An increased share of echinocytes/acanthocytes (black arrows) was observed. A representative life cell image is shown and corresponding images were taken for eight different *Alox15*$^{-/-}$ individuals. C, Quantification of the echinocyte/acanthocyte share in the peripheral blood of *Alox15*$^{-/-}$ mice and corresponding wild-type controls immediately after the animals had been sacrificed. A significantly elevated share of echinocyte/acanthocyte was detected in *Alox15*$^{-/-}$ mice. The relative shares of echinocytes/acanthocytes of eight different individuals of each genotype were used for statistic evaluation employing the Mann-Whitney *U* test. D, Life cell imaging of wild-type erythrocytes cultured for 3 days in vitro. Here again, the vast majority of the erythrocytes were present as regularly shaped biconcave discocytes, but the relative share of echinocytes/acanthocytes (black arrows) was somewhat higher than that for wild-type cells analyzed immediately after the death of the animals (panel A). A representative life cell image is shown and corresponding images were taken for cultures erythrocytes from five different wild-type individuals. E, Life cell imaging of *Alox15*$^{-/-}$ erythrocytes cultured for 3 days in vitro. When compared with cultured wild-type erythrocytes an increased share of echinocytes/acanthocytes (black arrows) was observed. A representative life cell image is shown and corresponding images were taken for five different *Alox15*$^{-/-}$ individuals. F, Quantification of the relative echinocyte/acanthocyte share in cultured *Alox15*$^{-/-}$ and wild-type erythrocytes. A significantly elevated share of echinocyte/acanthocyte was detected in *Alox15−/−* mice. The relative shares of echinocytes/acanthocytes of five different individuals for each genotype were used for statistic evaluation employing the Mann-Whitney *U* test. G, Quantification of annexin V positive cells in the blood of wild-type control mice immediately after the animals had been sacrificed. Annexin V staining (see Section 2) was carried out for five individuals and a representative image is shown. Only a few annexin V positive cells (arrows) were observed. H, Quantification of annexin V positive cells in the blood of *Alox15*$^{-/-}$ mice. Annexin V staining (see Section 2) was carried out for five individuals and a representative image is shown. An increased share of annexin V positive cells (arrows) were observed. I, To quantify the relative shares of annexin V positive cells present in the blood of *Alox15*$^{-/-}$ mice and corresponding wild-type controls more precisely FACS analysis was performed. The staining curve for the *Alox15*$^{-/-}$ erythrocytes was shifted to the right (arrow) indicating a more intense annexin V staining of these cells when compared with wild-type erythrocytes. Similar staining curves were taken for the erythrocytes of five different individuals and we always observed a similar shift of the curves. J, Statistic evaluation of the FACS data indicated a significantly higher share of echinocyte/acanthocyte content in *Alox15*$^{-/-}$ mice. K, Annexin V staining of *Alox15*$^{-/-}$ erythrocytes and wild-type control cells cultured for 3 days in vitro. Here again FACS analysis indicated a more intense fluorescence staining of *Alox15*$^{-/-}$ cells when compared with wild-type erythrocytes. Similar staining curves were obtained for the erythrocytes of five different individuals of each genotype and we always observed a similar shift of the curves in all cases. L, Statistic evaluation of the annexin V staining of *Alox15*$^{-/-}$ erythrocytes and wild-type control cells. The staining curves of five different individuals were employed for statistic evaluation and the relative shares of annexin V positive cells were compared with the Mann-Whitney *U* test

diagnostic blood removal. If the balancing capacity of the erythropoietic system would be impaired in *Alox15*$^{-/-}$ mice, one would expect more pronounced erythropoietic alterations in these animals. We found that diagnostic blood removal significantly reduced the erythrocyte counts in both, wild-type mice and *Alox15*$^{-/-}$ animals (Figure 4, left box plots), but there was no difference in the degree of reduction (Figure 4, right box plots). Similar alterations were seen when the reticulocyte counts were quantified (Figure 4B, left box plots) and here again, the degree of alterations was not significantly different (Figure 4B, right box plots). Taken together, these data suggest that under our experimental conditions the "repair capacity" of the erythropoietic system was not dramatically different when wild-type mice and *Alox15*$^{-/-}$-animals were compared.

3.5 | *Alox15*$^{-/-}$ mice have bigger spleens and kidneys

Similar to humans the bone marrow is the most important hematopoietic organ in adult mice.[45] However, under stress conditions[46] the spleen significantly contributes to erythropoiesis. Since *Alox15*-deficiency constitutes a mild erythropoietic stress (Figure 1), we quantified the spleen weights of *Alox15*-deficient mice and wild-type animals and found a subtle (1.4-fold) but significant ($P = .044$) increase in the spleen/body weight ratio (Figure 5A).

Because of the chronic character of the erythropoietic challenge permanent compensation reactions are required to maintain normal erythropoiesis. The most important regulator of erythropoiesis is erythropoietin (Epo), which is synthesized in the kidney. To explore whether Epo homeostasis is regulated in response to the genetic manipulations we first determined the kidney weights of the different genotypes (Figure 5B). Here, we found that the kidney/body weight ratio of *Alox15*$^{-/-}$ mice was significantly ($P < .005$) higher than that of wild-type animals. To test whether Epo synthesis is upregulated in the kidneys of the genetically modified animals, we quantified the steady state concentrations of Epo mRNA (Figure 5C). Here we observed no significant difference between *Alox15*$^{-/-}$ mice and wild-type controls when the mRNA copy number ratios of Epo/GAPDH were compared. Next, we challenged the erythropoietic systems of the mice by diagnostic blood removal and here we quantified

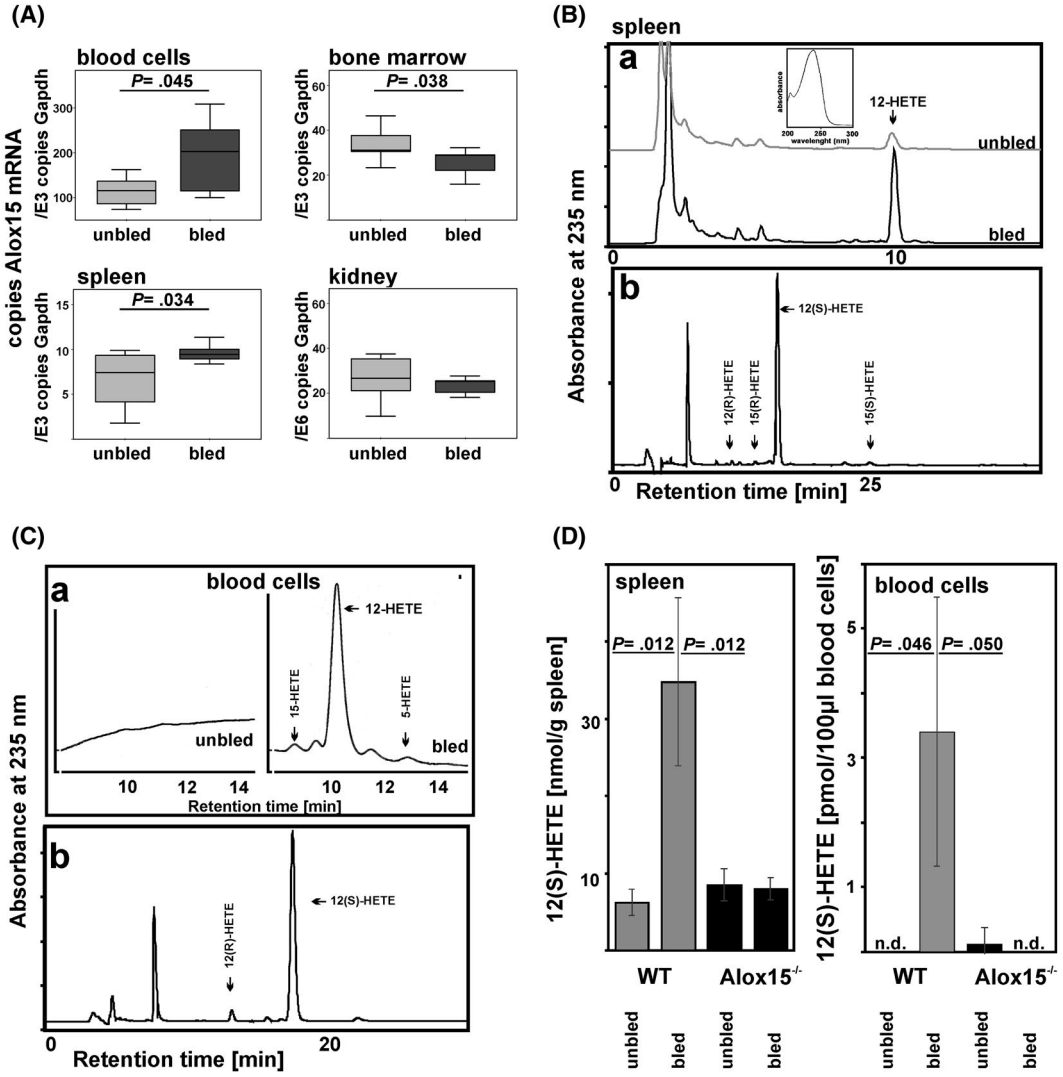

FIGURE 3 Blood removal for diagnostic purpose induces expression of Alox15 in peripheral blood cells and spleen. Male mice were sacrificed 24 hours after diagnostic blood removal by cervical dislocation after isoflurane anesthesia and expression of Alox15 was quantified by qRT-PCR and activity assays were carried out (see "Material and Methods"). As controls male mice without diagnostic blood removal were employed. RNA preparation, qRT-PCR and Alox15 activity assay were carried out as described in "Material and Methods." A, Expression of Alox15 mRNA. Total RNA was extracted from peripheral blood cells, bone marrow cells, spleen, and kidney of untreated (unbled mice) wild-type mice (C57BL/6) and of wild-type (C57BL/6) individuals, which underwent diagnostic blood removal 24 hours before (bled mice). A, qRT-PCR of Alox15 mRNA was carried out as described in "Material and Methods" using the specified amplification primers. For these experiments, n = 9 animals were sacrificed and each sample was quantified in triplicate. Significances were calculated using the Mann-Whitney *U* test and *P*-values < .05 were considered statistically significant. B, Lipoxygenase activity assay of spleen tissue. Homogenized spleen tissue was incubated with arachidonic acid (100 µM final concentration) for 15 minutes at room temperature. Lipid extraction and HPLC analysis were carried out as described in "Material and Methods." Panel a: RP-HPLC detection of 12-HETE as major arachidonic oxygenation product. The inset shows the UV-spectral properties of the 12-HETE peak. Panel b: Combined normal phase/chiral phase HPLC of the RP-HPLC purified 12-HETE peak confirming the chemical structure of the major Alox15 product as 12*S*-HETE. The corresponding 12*R*-enantiomer was hardly formed (inset). Four different animals were analyzed separately by RP-HPLC and combined normal phase/chiral phase HPLC was performed with the pooled 12-HETE RP-HPLC fractions. C, Lipoxygenase activity of peripheral blood cells. About 10 µL of packed blood cells were resuspended in PBS and incubated with arachidonic acid (100 µm) for 15 minutes at room temperature. Lipid extraction and HPLC analysis of the reaction products were carried out as described in "Material and Methods." Panel a: RP-HPLC detection of 12-HETE as major arachidonate oxygenation product in blood cells. Panel b: Combined normal phase/chiral phase HPLC of the major arachidonic acid oxygenation products formed (12-HETE) indicated a strong preponderance of 12*S*-HETE over its corresponding 12*R*-enantiomer. Four different animals were analyzed separately by RP-HPLC and combined normal phase/chiral phase HPLC was performed with the pooled 12-HETE RP-HPLC fractions. D, 12S-HETE production in bled and unbled animals. 12S-HETE production in spleen and blood cells was quantified by RP-HPLC (see above) and compared with each other. Chemicals: see "Material and Methods," Significances were calculated using Student's t test and *P*-values <.05 were considered statistically significant

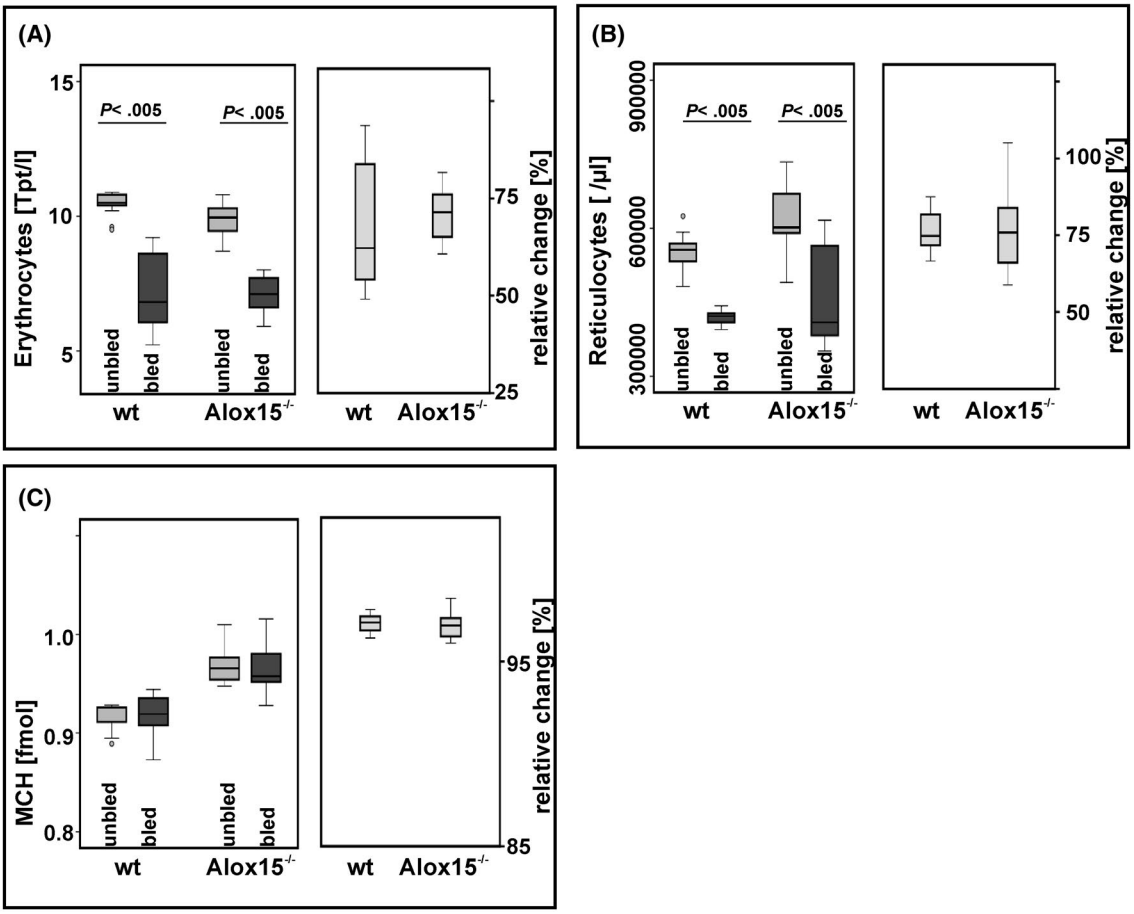

FIGURE 4 Diagnostic blood withdrawal induced similar alterations of red blood cell parameters in wild-type and *Alox15*$^{-/-}$ mice. Blood was withdrawn from male *Alox15* knockout mice (*Alox15*$^{-/-}$) and corresponding C57BL/6 wild-type controls (wt) from the facial vein plexus for analytic purpose and the basic red blood cell parameters (unbled mice) were determined (see "Material and Methods"). After 24 hours, the animals were sacrificed by cervical dislocation after isoflurane anesthesia and blood was collected by cardiac puncture and after the red blood cell parameters were again quantified (bled mice). A, erythrocyte counts. B, reticulocyte counts. C, mean corpuscular hemoglobin content. Significances were calculated using the Mann-Whitney *U* test. N = 9-13 animals were analyzed and *P*-values <.05 were considered statistically significant

significantly ($P < .005$) elevated Epo-mRNA levels in the kidneys of *Alox15*-deficient and wild-type mice (Figure 5C). Moreover, when we compared the relative fold change of Epo-mRNA expression a significant ($P < .005$) higher induction was observed in *Alox15*$^{-/-}$ kidneys after blood removal compared with wild-type controls (Figure 5D). Taken together, these data suggest that the impact of systemic inactivation of the *Alox15* gene on the erythropoietic system may not be strong enough to induce a counterbalancing upregulation of renal Epo synthesis. However, after additional stimulation of the erythropoietic system (diagnostic blood withdraw) there was a significant difference in the compensatory response (elevated Epo expression) between *Alox15*$^{-/-}$ mice and wild-type controls.

3.6 | Erythrocytes of *Alox15*$^{-/-}$ mice are more susceptible to osmotic hemolysis

Resistance against osmotic hemolysis is a frequently employed functional measure for the quality of erythrocytes and a reduced osmotic resistance suggests impaired stability of the red blood cells.[47] When we compared the osmotic resistance of wild-type and *Alox15*$^{-/-}$ erythrocytes (Figure 6) we found that between 0.50% and 0.45% NaCl the degree of hemolysis of *Alox15*$^{-/-}$ erythrocytes was elevated. Statistic evaluation of the osmotic resistance curves with the sign-test revealed no significant differences between the two genotypes in the NaCl concentration range between 0.85% and 0.55%. However, in the concentration range between 0.500% and 0.425% a highly significant difference ($P = .004$) was observed. Similar osmotic hemolysis curves were previously reported for erythrocytes prepared from mice lacking the functional mitogen- and stress-activated protein kinase MSK1/2.[48]

3.7 | Erythrocytes of *Alox15*$^{-/-}$ mice have a reduced ex vivo life spans

The reduced osmotic resistance of Alox15-deficient erythrocytes suggested an impaired mechanic stability of the

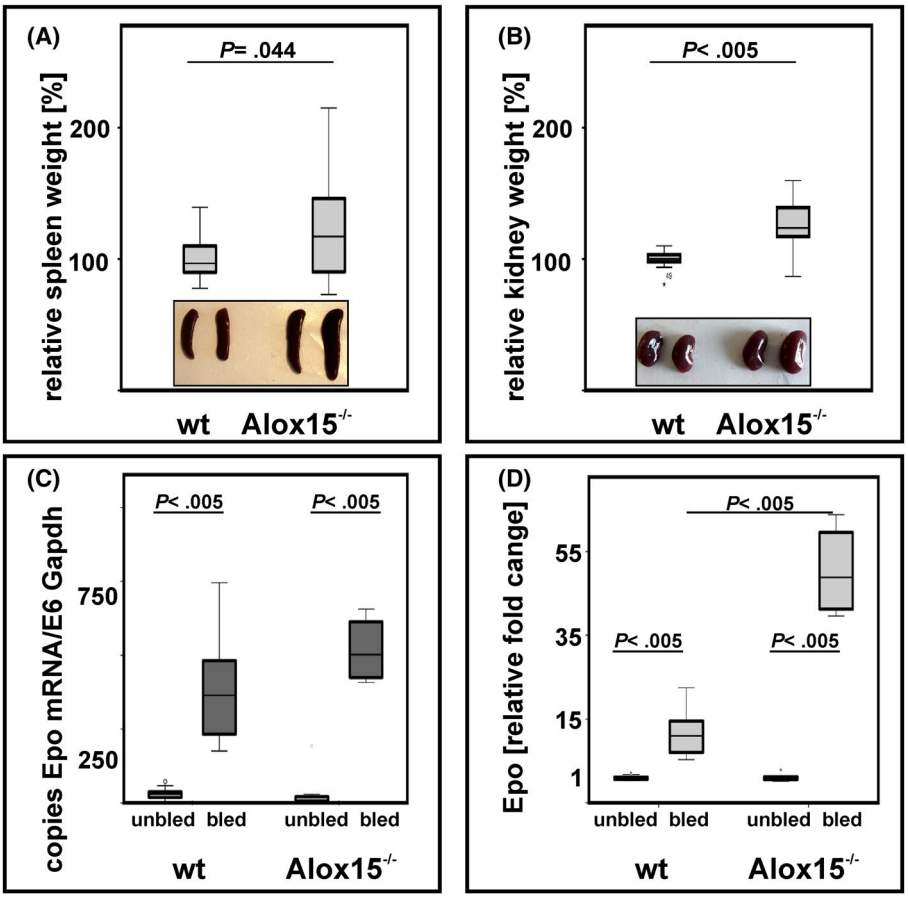

FIGURE 5 *Alox15*-deficient mice have bigger spleens and kidneys when compared with corresponding wild-type controls. The wet weights of spleens and kidneys of male *Alox15* knockout mice (Alox15$^{-/-}$) and the corresponding C57BL/6 wild-type controls (wt) were determined and the body weight/organ weight ratios were calculated. A, Spleen/body weight ratios (B) and kidney/body weight ratios. Significances were calculated using the Mann-Whitney *U* test. N = 19-22 animals were analyzed and *P*-values <.05 were considered statistically significant. Insets: Representative images of spleens and kidneys of wild-type mice and *Alox15*-deficient animals. C, Renal expression of erythropoietin (Epo) mRNA. Total RNA was extracted from kidney of unbled and bled (24 hours after blood removal) wild-type (wt) and *Alox15*-deficient (Alox15$^{-/-}$) mice. Epo mRNA expression levels were quantified by qRT-PCR (see "Material and Methods"). For these experiments, n = 8-14 animals were sacrificed and each sample was quantified in triplicate. Significances were calculated using the Mann-Whitney *U* test and *P*-values <.05 were considered statistically significant. D, Induction of Epo mRNA expression after diagnostic blood removal. Epo mRNA levels in kidneys after diagnostic blood removal (bled) of wild-type (wt) and *Alox15*-deficient (Alox15$^{-/-}$) mice were related to Epo mRNA expression of the corresponding mice without blood withdraw (unbled)

plasma membrane. If this is the case, the cells are likely to exhibit a reduced ex vivo life span. To test this hypothesis, we cultured erythrocytes prepared from wild-type control mice and *Alox15*$^{-/-}$ animals for 11 days and assayed the degrees of spontaneous hemolysis at different time points. From Figure 7, it can be seen that wild-type and *Alox15*$^{-/-}$ erythrocytes exhibit a similarly low degree of spontaneous hemolysis when kept in culture for up to 7 days. At day 8, *Alox15*$^{-/-}$ erythrocytes exhibited an increased hemolysis although the difference to wild-type cells did not reach the level of statistical significance ($P = .175$). However, at days 10 and 11 significantly enhanced degrees of spontaneous hemolysis were observed for *Alox15*$^{-/-}$ erythrocytes. These data indicate that *Alox15*$^{-/-}$ erythrocytes exhibit a reduced ex vivo life span when compared with wild-type cells.

3.8 | Transgenic expression of human ALOX15 rescues the defective erythropoietic phenotype of *Alox15*$^{-/-}$ mice

To explore whether transgenic expression of human ALOX15 can rescue the defective erythropoietic phenotype of *Alox15*$^{-/-}$ mice, we crossed transgenic mice, which express human ALOX15 under the control of the aP2 promoter (*aP2-ALOX15* mice) with *Alox15*$^{-/-}$ animals and characterized the erythropoietic system of the resulting

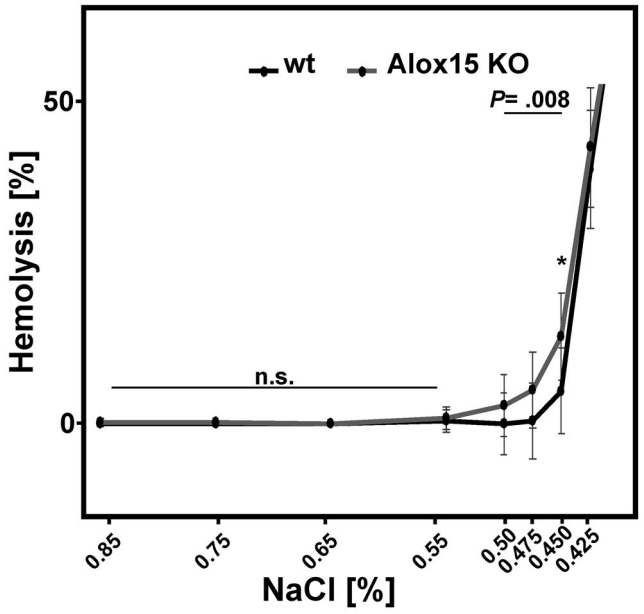

FIGURE 6 Erythrocytes of *Alox*15$^{-/-}$ mice are more susceptible to osmotic hemolysis when compared with corresponding wild-type control cells. Blood cells prepared from *Alox15*-deficient mice (Alox15$^{-/-}$) and corresponding wild-type controls with the same genetic background were incubated at different concentrations of NaCl as described in "Material and Methods." The degree of hemolysis was quantified for each NaCl concentration and the hemolysis curves were overlaid. Results are expressed as means ± SEM. Eight to nine animals in each group were included. The hemolysis data at each NaCl concentration were compared using the Student's *t* test. The two different parts of the hemolysis curves (0.850%-0.550%, curves are completely overlaid; 0.500%-0.425%, curves deviate from each other) were compared with the sign-test and *P*-values <.05 (*) were considered statistically significant

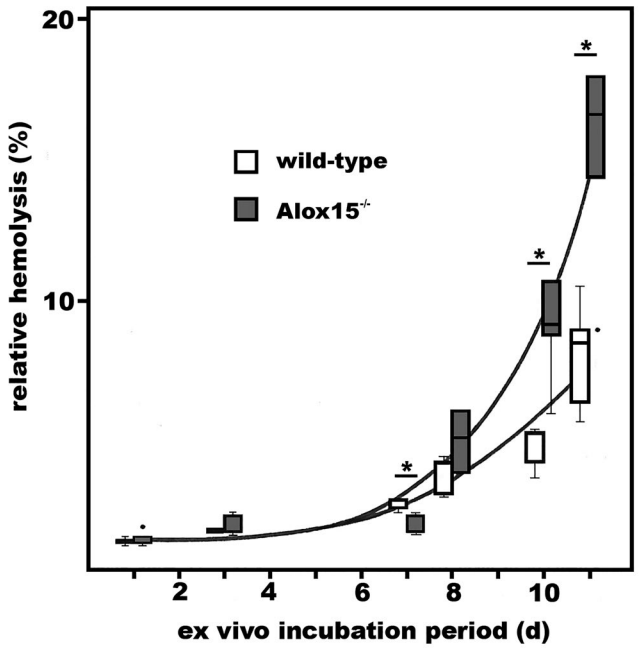

FIGURE 7 Hemolysis kinetics of wild-type and Alox15$^{-/-}$ erythrocytes during in vitro cell cultures. Erythrocytes were prepared from wild-type mice and Alox15$^{-/-}$ animals and cultured in vitro as described in Section 2. At the time points indicated aliquots of the culture suspension were removed and the degree of hemolysis was quantified (see Section 2). Five individuals in each group were analyzed at each time point and statistical significances were calculated using the Mann-Whitney *U* test

Alox15$^{-/-}$+*aP2-ALOX15* mice. First, we explored whether the human ALOX15 transgene is expressed in hematopoietic cells. Here we found (Figure 8A) that the human ALOX15 transgene is not expressed in bone marrow cells of wild-type animals. In contrast, we detected the expression of other lipoxygenase paralogs (Alox15, Alox12, and Alox5). In bone marrow cells of *Alox15*$^{-/-}$+*aP2-ALOX15* mice, the human ALOX15 transgene is expressed but as expected, we did not observe expression of the endogenous mouse enzyme, which is consistent with systemic inactivation of the endogenous *Alox15* gene. Interestingly, the expression level of the human ALOX15 transgene in bone marrow cells was comparable with that of the endogenous mouse Alox15 in wild-type bone marrow cells. Thus, expression of the human transgene does compensate the lacking expression of the endogenous mouse Alox15, but there was no overcompensation.

Next, we carried out ALOX activity assays with bone marrow cells (Figure 8B) and quantified the relative shares of 12-HETE (major arachidonic acid oxygenation product of mouse Alox12 and mouse Alox15), 15-HETE (major reaction product of transgenic human ALOX15), and 5-HETE (major reaction product of mouse Alox5). Here, we found that 12-HETE was the major arachidonic acid oxygenation product of wild-type bone marrow cells and this product formation may be due to the combined catalytic activity of endogenous mouse Alox12 and mouse Alox15. Surprisingly, 5-HETE formation was minimal although Alox5 mRNA was expressed at high levels in bone marrow cells. When we employed bone marrow cells of *Alox15*$^{-/-}$+*aP2-ALOX15* mice for our activity assays the formation of 12-HETE was significantly reduced, which is consistent with the lack of the endogenous mouse Alox15. Most importantly, however, the relative share of 15-HETE formation was significantly upregulated and this data can be explained by expression of transgenic human ALOX15. Taken together, these data indicate expression silencing of endogenous mouse Alox15 and transgenic expression of human ALOX15 in bone marrow cells of *Alox15*$^{-/-}$+*aP2-ALOX15* mice.

To explore whether transgenic expression of human ALOX15 rescues the erythropoietic phenotype of *Alox15*-deficient animals, we compared the basic red blood cell parameters of *Alox15*$^{-/-}$- and *Alox15*$^{-/-}$+*aP2-ALOX15* mice. Here, we found that the erythrocyte counts (Figure 8C) and the reticulocyte counts (Figure 8D) were not significantly different between the two genotypes. These data indicate that the

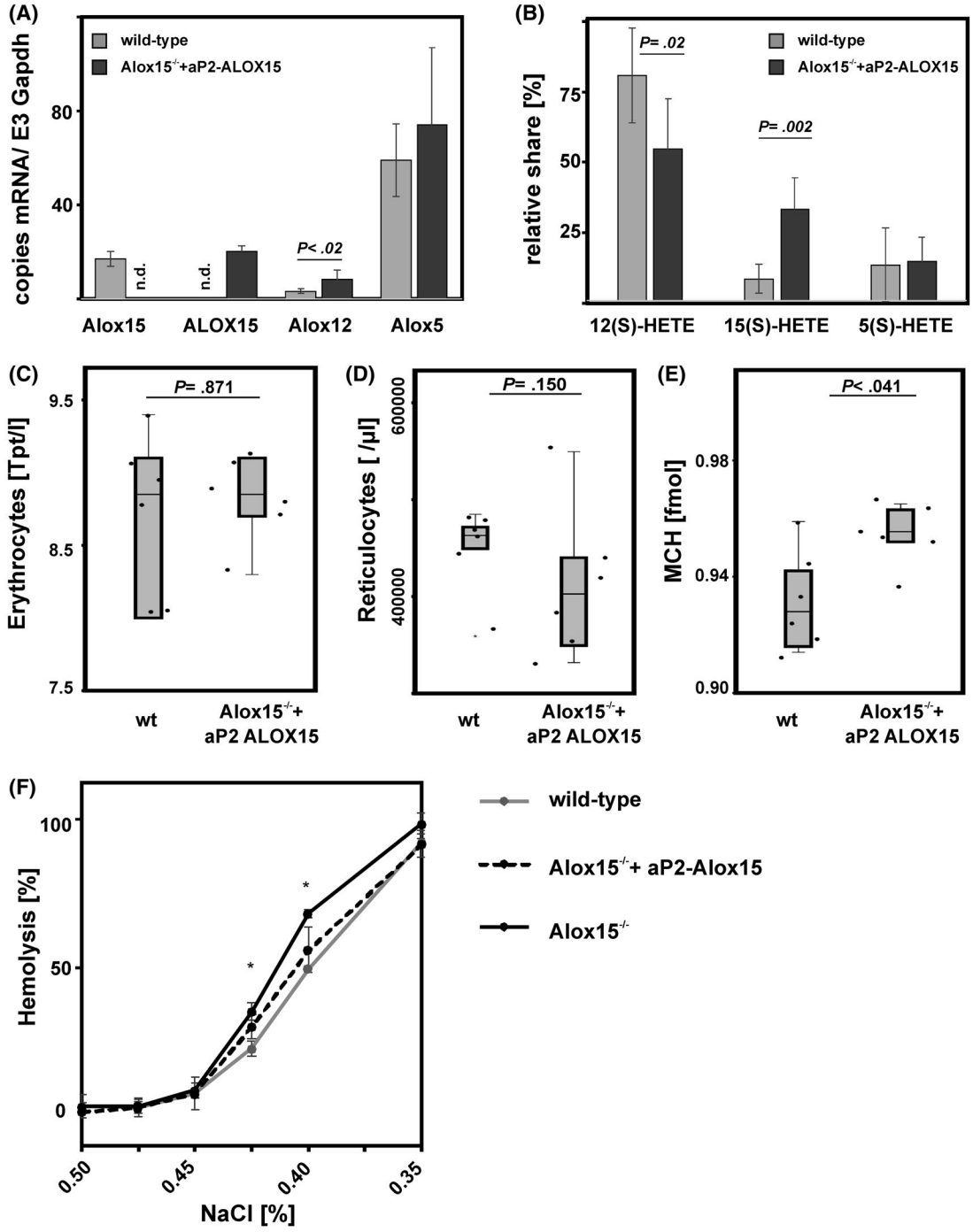

FIGURE 8 Transgenic expression of human ALOX15 compensates the erythropoietic defects induced by systemic inactivation of the *Alox15* gene. A, Expression of different ALOX-paralogs was quantified by qRT-PCR (see "Material and Methods") in bone marrow cells prepared from seven wild-type mice (n = 7) and Alox15$^{-/-}$+aP2ALOX15 (n = 6) animals. B, ALOX-activity assays (see "Material and Methods") of bone marrow cells prepared from wild-type mice (n = 8) and *Alox15*$^{-/-}$+*aP2ALOX15* animals (n = 6). 10^7 cells were used for the activity assays. C, Erythrocyte counts of wild-type mice (n = 6) and *Alox15*$^{-/-}$+*aP2ALOX15* animals (n = 6). D, MCH of peripheral red blood cells wild-type mice and *Alox15*$^{-/-}$+*aP2ALOX15* animals. D, Reticulocyte counts of wild-type mice and *Alox15*$^{-/-}$+*aP2ALOX15* animals. E, Osmotic resistance of peripheral red blood cells prepared from wild-type mice, *Alox15*$^{-/-}$ mice and *Alox15*$^{-/-}$+*aP2ALOX15* animals (n = 2 of each group, all samples were analyzed in triplicate) was determined as described in "Materials and Methods." Significances were calculated using Student's t test and *P*-values <.05 were considered statistically significant. (*) indicate significant differences ($P < .05$) between wild-type and *Alox15*$^{-/-}$ mice; n.d., no detectable

reduced erythrocyte count of *Alox15*-deficient mice was normalized by transgenic expression of human ALOX15 and a similar conclusion could be drawn for the reticulocyte count. However, the hyperchromic effect of *Alox15*-deficiency was not reversed by human ALOX15 expression (Figure 8E).

As shown in Figure 6 erythrocytes of *Alox15*-deficient mice are more susceptible to osmotic hemolysis. To test whether this functional red blood cell parameter can also be normalized by transgenic expression of human ALOX15, we compared the osmotic resistance of red blood cells prepared from mice of the three different genotypes under strictly comparable conditions (Figure 8F). Here, we confirmed that *Alox15*-deficient erythrocytes are more susceptible to osmotic hemolysis in an independent experimental setup. This difference was strongly reduced when red cells of wild-type mice and *Alox15*$^{-/-}$+*aP2-ALOX15* mice were compared and these data suggest functional compensation of *Alox15* deficiency by transgenic expression of human ALOX15.

4 | DISCUSSION

4.1 | Alox15 plays a role in mouse erythropoiesis

Erythropoiesis is a complex developmental process, in which myeloid precursor cells differentiate to mature erythrocytes.[45] In humans, this process proceeds in the bone marrow, but in mice extramedullary sites such as spleen and liver may also contribute.[49] The late steps in erythrocyte maturation, in particular the reticulocyte-erythrocyte transition, involve dramatic structural alterations as well as pronounced changes in the energy metabolism. Young rabbit reticulocytes contain functional mitochondria and generate more than 80% of the ATP required for survival by mitochondrial respiration.[12] In contrast, mature erythrocytes do not contain functional mitochondria and the major energy source of these cells is aerobic glycolysis yielding large quantities of lactate. There are different mechanisms for the intracellular breakdown of the mitochondria and other organelles and one of them is initiated by ALOX15-dependent oxygenation of the mitochondrial membranes lipids. This enzymatic lipid peroxidation, which interrupts the electron transfer chain, initiates the breakdown of intracellular organelles and structural remodeling of the plasma membrane.[12,29] The hydroperoxy lipids formed during the catalytic activity of Alox15 induce co-oxidative modification of membrane proteins and these oxidatively modified proteins are targeted for degradation by intracellular proteases. During in vitro maturation of rabbit reticulocytes, the kinetics of ALOX15 biosynthesis anti-parallels the maturational decline of cellular respiration[24,28] and an ALOX15 inhibitor retards this developmental process.[12] Taken together, these data suggested a role of ALOX15 in reticulocyte-erythrocyte transition.

However, when *Alox15*$^{-/-}$ mice became available these mice did not exhibit an erythropoietic phenotype[36,37] and these results challenged the role of Alox15 in erythropoiesis. To re-explore the putative role of this enzyme in red blood cell differentiation, we searched in detail for structural and functional differences between wild-type erythrocytes and *Alox15*$^{-/-}$ cells. We found that *Alox15*$^{-/-}$-mice suffered from reduced erythrocyte counts and this alteration was paralleled by a counterbalancing increase in MCH (hyperchromia) and by an elevated reticulocyte count. Although all parameters were in the normal range[50] there were significant differences between the two genotypes. In addition, we found that the blood of *Alox15*$^{-/-}$ mice involved an increased share of structurally modified erythrocytes (echinocytes/acanthocytes) and that the cells were more intensely stained with annexin V. These staining data suggested a compromised asymmetry of the plasma membrane suggesting a reduced cellular life span. Although we did not directly quantify the in vivo life span of *Alox15*$^{-/-}$ erythrocytes comparative ex vivo hemolysis studies indicated a reduced survival time of *Alox15−/−* erythrocytes (Figure 7). The structural and functional differences between wild-type red blood cells and *Alox15*$^{-/-}$ erythrocytes are, although statistically significant, not very dramatic. However, considering the previous observations that Alox15 independent mechanisms may also contribute to the maturational breakdown of intracellular organelles and plasma membrane remodeling[12,22] more pronounced effects have not been expected. Proper erythropoiesis is essential for the survival of blood containing animals, and therefore, this process is covered by a functional redundancy of different mechanisms. When the Alox15-dependent mechanisms are defective, as it is the case in *Alox15*$^{-/-}$ mice, Alox15-independent mechanisms may be upregulated to keep the functional consequences as low as possible.

A critical point in our research strategy was the fact that we did not employ wild-type littermates as control animals. Instead, we back-crossed *Alox15*$^{-/-}$ mice with mixed genetic background (B6.129S2-*Alox15*^{tm1Fun}/J) 7 times into C57/BL6 mice and then employed commercial C57/BL6 animals for control purpose. Although wild-type littermates are probably better control animals, we think that for our purpose non-littermate wild-type controls are sufficient because of the following reasons. (i) A similar control procedure (use of non-littermate wild-type controls) was employed in previous studies suggesting that Alox15 might not be relevant for erythropoiesis (36). To ensure strict comparability of the two studies, we wanted to keep as close as possible to the previously described experimental protocol. (ii) A similar mildly defective hematopoietic phenotype was observed in a third colony of *Alox15*$^{-/-}$ mice and here again the hematopoietic parameters were compared

between back-crossed *Alox15*$^{-/-}$ and non-littermate wild-type controls.[38] (iii) We were able to rescue the defective hematopoietic phenotype of *Alox15*-deficiency by transgenic overexpression of the human ortholog. If the phenotype would be due to mutations acquired during the breeding process, such rescue can hardly be explained. Taken together, these data confirm the previous conclusion that Alox15 is not essential for mouse erythropoiesis,[36] but they do suggest that the enzyme is involved in this process.

In previous studies, it has been suggested that Alox15 catalyzed oxidation of membrane lipids triggers the maturational breakdown of organelles during reticulocyte-erythrocyte transition and restructuring of the plasma membrane.[12,23] The mitochondrial membranes of rabbit reticulocytes contain specific Alox15 products[33] and such products were also present in the plasma membranes.[34] To test this molecular mechanism, we searched for specific Alox15 products in the membrane lipids of peripheral red blood cells. Although these cells do not contain any mitochondria any more there was still a chance to detect Alox15 products in the plasma membrane lipids. Unfortunately, we were not able to detect specific Alox15 products in the membrane lipids of peripheral red blood cells (Figure S1). Next, we compared the patterns of the major polyenoic fatty acids in the membrane ester lipids of *Alox15*$^{-/-}$ mice and corresponding wild-type controls. The rationale for these experiments was that if the enzyme was involved in membrane remodeling during erythropoiesis there was a chance that certain polyenoic fatty acids were preferentially oxygenated by Alox15, and thus, lower concentrations of these fatty acids should be present in the membrane ester lipids. However, when we compared the relative shares of the three major polyenoic fatty acids (arachidonic acid, docosahexaenoic acid, and linoleic acid) detected in the membrane ester lipids of wild-type and *Alox15*-deficient red blood cells we did not observe significant differences. Taken together, one may conclude that Alox15 did not leave major footprints for its catalytic activity in the membrane ester lipids of peripheral red blood cells. Although disappointing this finding was not surprising since the enzyme is only present in reticulocytes during a narrow time window. Moreover, after the mitochondria are degraded or expelled from the maturing cells and the plasma membrane is strongly restructured during later steps of the maturation process and this remodeling might wipe out the structural Alox15 footprints.

To explore whether Alox15 leaves functional footprints in *Alox15*-deficient erythrocytes, we quantified the osmotic hemolysis resistance of these cells. Here we found that *Alox15*-deficient erythrocytes are more susceptible to osmotic stress (Figure 6) and these data indicate defective erythropoiesis. To judge the extent of functional alterations, we compared the hemolysis curves of *Alox15*$^{-/-}$ erythrocytes with those of *msk*-deficient cells. For *msk*-deficient red blood cells, very similar hemolysis curves were obtained.[48] These data suggest a similar degree of osmotic stability alterations in *msk*$^{-/-}$ and *Alox15*$^{-/-}$ peripheral red blood cells. Thus, the functionality of *Alox15*$^{-/-}$ erythrocyte is significantly impaired and reduced red blood cell life spans are likely.

4.2 | Expression of human ALOX15 partially rescues defective erythropoiesis

When we expressed human ALOX15 under the control of the aP2 promoter expression of the transgenic human enzyme was detected by qRT-PCR and activity assays. In bone marrow cells, the human transgene was expressed at similar levels as the endogenous mouse Alox15 (Figure 8). In other words, expression of the human transgene compensates the silenced expression of mouse Alox15 in a rather balanced way since no dramatic overexpression was seen. This observation may be of cell physiological relevance. In fact, because of its membrane oxygenase activity[29] ALOX15 might be deleterious to cells when present at high levels.[51]

Although mouse and human ALOX15 orthologs share a number of catalytic properties, such as high degree of amino acid conservation and membrane oxygenase activity, they exhibit different reaction specificities. Mouse Alox15 oxygenates arachidonic acid mainly to its 12-hydroperoxy derivative (12-HpETE), whereas 15-HpETE is the major product of human ALOX15 catalyzed arachidonic acid oxygenation. A difference in the reaction specificity of these two enzyme orthologs was also observed for other polyenoic fatty acids.[52] Our observation that transgenic expression of human ALOX15 partially compensates the defects induced by systemic inactivation of the mouse *Alox15* gene suggests that the reaction specificity, and thus, the pattern of the oxygenation products might not be of major relevance for the erythropoietic role of this enzyme. In fact, according to the original hypothesis that ALOX15 orthologs play a role in the degradation of mitochondria during reticulocyte-erythrocyte transition[23,51] the membrane oxygenase activity of these enzyme orthologs may be much more important. All mammalian ALOX15 orthologs tested so far including human and mouse ALOX15 share these catalytic properties.

4.3 | *Alox15* deficiency leads to subtle splenomegalia

Alox15 has previously been suggested to function as a suppressor of myeloproliferative diseases.[53] In that study the

authors observed a small but significantly reduced life span of *Alox15*-deficient mice and a pronounced splenomegalia. Although a reduced life span of the animals and such a pronounced splenomegalia could not be confirmed in other colonies of *Alox15*$^{-/-}$ mice,[54] we observed a subtle but statistically significant increase in the spleen weight/body mass ratio in our study (Figure 5A). This subtle increase might be interpreted as a sign of a compensatory activation of the hematopoietic system induced by *Alox15*-deficiency. Interestingly, genetic manipulations of other gene loci of the murine genome, which lead to reduced osmotic resistance did also induce subtle splenomegalia.[55,56]

4.4 | Diagnostic blood removal constitute hematopoietic stress

In rabbits, repeated blood removal induces ALOX15 expression in erythroid cells.[12] In the standard bleeding protocol, 50 mL of peripheral blood was removed from the ear vein every other day for 6 days. This procedure induces severe anemia with HK values dropping down to 20% or lower.[12,24] Under these experimental conditions, the peripheral reticulocyte counts increase to more than 50% and Alox15 is expressed at high levels in peripheral red blood cells. For this study, we removed about 6% blood volume of mice body weight for diagnostic purposes (determination of hematopoietic parameters) and were surprised to observe that this rather mild hematopoietic manipulation was sufficient to induce Alox15 expression in spleen and peripheral red blood cells. This finding indicates that a single removal of about 180 µL of blood/30 g mouse body weight must be considered as an erythropoietic challenge.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

All co-authors contributed to designing the research strategy. M. Rademacher performed most of the experiments, analyzed the data, and contributed to writing the manuscript; A. Borchert co-designed and performed experiments, analyzed thse data, constructed the figures, and contributed to writing the manuscript; H. Kuhn supervised the study, co-designed the experiments, contributed to lipid analysis, and drafted the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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