Interferon regulatory factor-8–driven myeloid differentiation is regulated by 12/15-lipoxygenase–mediated redox signaling

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Objective. Several transcription factors determine the cell fate decision between granulocytes and monocytes, but the upstream signal transduction pathways that govern myelopoiesis are largely unknown. Based on our observation of aberrant myeloid cell representation in hematopoietic tissues of 12/15-lipoxygenase (12/15-LOX)-deficient (Alox15) mice, we tested the hypothesis that polyunsaturated fatty acid metabolism regulates myelopoiesis.

Materials and Methods. Multicolor flow cytometric analysis and methylcellulose assays were used to compare myelopoiesis and the differentiative capacity of progenitors from Alox15 and wild-type mice. Furthermore, we elucidated the mechanism by which 12/15-LOX is involved in regulation of myelopoiesis.

Results. Granulopoiesis in Alox15 mice is increased while monopoiesis is reduced. Moreover, there is an accumulation of granulocyte-macrophage progenitors that exhibit defective differentiation. Mechanistically, we demonstrate that transcriptional activity of interferon regulatory factor-8 (Irf8), which regulates myelopoiesis, is impaired in Alox15 progenitors and bone marrow-derived macrophages due to loss of 12/15-LOX-mediated redox regulation of Irf8 nuclear accumulation. Restoration of redox signaling in Alox15 bone marrow cells and granulocyte-macrophage progenitors reversed the defect in myeloid differentiation.

Conclusions. These data establish 12/15-LOX-mediated redox signaling as a novel regulator of myelopoiesis and Irf8.

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(12/15-LOX)—mediated signaling in macrophages and splenocytes [7,8]. However, the role of 12/15-LOX in myelopoiesis and the mechanism whereby 12/15-LOX promotes nuclear accumulation of Irf8 has not been elucidated.

The signaling pathways that regulate myeloid cell specification remain under active investigation. In this study, we demonstrate that 12/15-LOX—dependent signaling regulates myelopoiesis. 12/15-LOX is an oxidative enzyme that mediates polyunsaturated fatty acid metabolism of substrates, such as arachidonic and linoleic acids. 12/15-LOX—mediated reactions generate bioactive lipid metabolites, including 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), 15(S)-HETE, 13(S)-HODE, lipoxins, and hepoxilins [9]. Reactive oxygen species (ROS) generated by products of 12/15-LOX can function as second messengers by modifying thiol groups in cysteine residues. Oxidative modifications regulate the activity of proteins, including phosphatases, transcription factors, and signal transducers [10]. However, little is known about the consequences of 12/15-LOX signaling during myelopoiesis.

In view of our observations of reduced numbers of monocytes [11] and increased percentages of granulocytes [7] in 12/15-LOX—deficient (Alox15) mice, we hypothesized that 12/15-LOX regulates myeloid cell fate decisions. Herein, we establish that lack of 12/15-LOX in Alox15 mice results in accumulation of myeloid progenitors due to defective differentiation, which is skewed toward the granulocyte lineage. Loss of 12/15-LOX—dependent redox signaling in part mediates nuclear accumulation of Irf8 to drive monopoiesis and inhibit granulopoiesis.

**Materials and methods**

**Animals**

C57BL/6 (B6) and Alox15 8- to 10-week-old mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), and bred in the Wistar Institute. All procedures were approved by the Institutional Animal Care and Use Committee of the Wistar Institute.

**Flow cytometry**

Single-cell suspensions were prepared and red blood cell—lysed using ammonium chloride. Immunocytocychemistry reagents included cKit (Invitrogen, Carlsbad, CA, USA), streptavidin-conjugates (BD Bioscience, San Jose, CA, USA), and all others (eBioscience, San Diego, CA, USA). Lineage markers included antibodies against Gr-1, B220, CD3, interleukin (IL)-7Rα, Ter119, NK1.1, Mac1, and CD11c. Flow cytometric analysis was performed on FACSCalibur or LSR II and analyzed using Flowjo software (TreeStar, Ashland, OR, USA). For cell sorting, bone marrow (BM) cells from two to four mice were pooled, stained, and sorted on an Aria.

**BMM**

The 1 × 10⁶ BM cells/mL were cultured in 10% fetal calf serum, 10% L929 supernatant in RPMI on non—tissue-culture-treated plates. After 5 days, adherent cells were isolated. At least 90% of cells were F4/80⁺ by flow cytometric analysis.

**Immunoblot and quantitative polymerase chain reaction**

Lin—cKit⁺ cells were obtained using MACS kit according to manufacturer’s instructions (Miltenyi Biotech, Auburn, CA, USA). BMM were collected, counted, and replated in the presence or absence of Tiron (Mallinkrodt Baker, Inc., Phillipsburg, NJ, USA) or L-Buthionine-[S,R]-sulfoximine (BSO) (Sigma-Aldrich, St Louis, MO, USA). All experiments were performed under steady-state conditions. Nuclear extracts were prepared using Nucbuster kit (EMD Biosciences, San Diego, CA, USA). Lysates were normalized using Bradford assay (Pierce, Rockford, IL, USA), resolved by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and immunoblotted with antibodies specific for Irf8, actin, or retinoblastoma protein (Santa Cruz Biotecnoity, Santa Cruz, CA, USA) and secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA). RNA was isolated from BMM using TriZol (Invitrogen, Carlsbad, CA, USA) and treated with turbo DNase (Ambion, Austin, TX, USA). RNA was isolated from enriched progenitors using RNasea microRNA kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized using reverse transcriptase, and quantitative polymerase chain reaction was performed by normalizing to glyceraldehyde 3-phosphate dehydrogenase using Sybr Green Master Mix on ABI7000 Cycler (Applied Biosystems, Foster City, CA, USA). For primer sequences see Supplementary Methods (online only, available at www.exphem.org).

**ROS and lipid analysis**

ROS levels were determined by loading with 10 μM CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA) for 30 minutes at 37°C and analyzed by flow cytometry. For lipid analysis, BMM were loaded for 30 minutes with 50 μM arachidonic acid (Cayman Chemical, Ann Arbor, MI, USA). Supernatants were extracted and analyzed by stable isotope dilution, normal-phase chiral liquid chromatography coupled with electron capture atmospheric pressure chemical ionization/mass spectrometry.

**Methylcellulose assays**

The 1.5 × 10⁵ splenocytes, 1.5 × 10⁴ bone marrow cells, 5 × 10⁵ Lin—cKit⁺ “cKit⁺” cells (LSK), 5 × 10⁵ common myeloid progenitors (CMPs), and 10⁵ GMPs were plated in methylcellulose containing IL-3, IL-6, stem cell factor (SCF), and erythropoietin (M3434; Stem Cell Technologies, Vancouver, BC, Canada). The 5 × 10⁵ BM cells or 10³ GMPs were also plated in methylcellulose (M3234; Stem Cell Technologies) with 10 ng/mL macrophage colony-stimulating factor (CSF), granulocyte CSF (G-CSF), or granulocyte-macrophage CSF (GM-CSF) (Peprotech, Rocky Hill, NJ, USA). After 10 days, colonies were enumerated using light microscopy in a blinded fashion.

**Statistical analysis**

Student’s t tests were applied using Excel (Microsoft, Redmond, WA, USA).

**Results**

Alox15 progenitors exhibit excess granulopoiesis and reduced monopoiesis

We confirmed the decrease in Alox15 blood monocytes that we reported previously [11]. Although the numbers of
granulocytes were similar between B6 and Alox15 mice, there was a decrease in white blood cell number resulting in an increased percentage of granulocytes (Supplementary Figure E1A, B, online only, available at www.exphem.org) [7]. Moreover, we also confirmed the increased percentage of granulocytes in the Alox15 splenocytes (Supplementary Figure E1C, online only, available at www.exphem.org) [7]. 12/15-LOX was expressed in myeloid progenitors (Supplementary Figure E1D, online only, available at www.exphem.org), therefore, we hypothesized that the decrease in monocytes and increased percentage of granulocytes in Alox15 mice is due to defective myelopoiesis. To test this, we plated cells from B6 and Alox15 mice in methylcellulose assays containing IL-6, IL-3, SCF, and erythropoietin and compared the frequency of colony-forming units granulocyte (CFU-G) with that of CFU-M. We calculated the ratio of CFU-G/CFU-M to assess skewing toward differentiation independently of total colony number. BM cells from Alox15 mice generated an increased ratio of CFU-G/CFU-M compared to B6 (Fig. 1A). Alox15 BM gave rise to an increased number of total colonies, due to an increased number of CFU-G and CFU-granulocyte-macrophage (CFU-GM). We employed multicolor flow cytometric analysis to determine the effect of instructive cytokines, we plated B6 and Alox15 BM cells in methylcellulose in the presence of M-CSF or G-CSF. Interestingly, cells from Alox15 BM gave rise to an increased number of total colonies in the presence of both M-CSF and G-CSF compared to cells from B6 BM. However, in the presence of M-CSF, Alox15 BM cells generated more CFU-GM but not CFU-M. In the presence of G-CSF, Alox15 BM cells gave rise to an increased number of both CFU-GM and CFU-G compared to B6 BM cells (Fig. 1E). Taken together, these data demonstrate that Alox15 BM cells exhibit an increased frequency of CFU-GM and enhanced granulocytic differentiation.

The increased number of total myeloid colonies that developed from Alox15 cells suggested that there are increased numbers of myeloid progenitors present. Specifically, the increase in CFU-GM in the presence of all cytokines suggested that the GMP population is increased in Alox15 mice.

Accumulation of defective GMPs in Alox15 mice
We employed multicolor flow cytometric analysis to determine the numbers of phenotypic progenitor populations. At all ages tested, Alox15 BM had similar percentages of LSK, CMPs, and megakaryocyte erythroid progenitors (MEPs), but an increased percentage of GMPs compared with B6 (Fig. 2A–C). The increased percentage of GMPs was also evident in Alox15 spleen (Fig. 2D). In addition to an increase in relative percentage, the absolute frequency of Alox15 GMPs was increased (Fig. 2E).

There are several potential explanations for the increased number of GMP progenitors in Alox15 mice. First, CMPs may produce more GMPs at the expense of MEPs. However, this is not likely, as there were similar numbers of erythroid progenitors (burst-forming unit-erythroid) produced from Alox15 BM (Fig. 1A) and similar numbers of MEPs in B6 and Alox15 mice (Fig. 2). Second, Alox15 GMPs may expand by proliferating and/or through enhanced survival. Alternatively, Alox15 GMP differentiation may be decreased, resulting in accumulation of GMPs as they fail to progress from this differentiative stage.

To determine whether Alox15 GMPs exhibit enhanced proliferation, we performed cell cycle analysis ex vivo, which was comparable between B6 and Alox15 GMPs (Supplementary Figure E2A, online only, available at www.exphem.org). Moreover, cell survival was similar between B6 and Alox15 GMPs as measured by the percentage of cells binding Annexin-V ex vivo and after stimulation with SCF in vitro (Supplementary Figure E2B, C, online only, available at www.exphem.org). Therefore, Alox15 GMPs are not increased due to enhanced proliferation or cell survival.

To test whether GMPs are accumulated in Alox15 mice due to a decreased differentiation, we isolated enriched populations of LSK, CMPs, and GMPs from B6 and Alox15 BM by cell sorting prior to methylcellulose assays. Similar to whole BM cells, Alox15 LSK and CMPs generated increased numbers of CFU-G, but decreased numbers
Figure 1. Enhanced granulopoiesis at the expense of monopoiesis of Alox15 progenitors in methylcellulose assays. (A) B6 and Alox15 bone marrow (BM) cells were plated in methylcellulose in the presence of interleukin (IL)-3, IL-6, stem cell factor (SCF), and erythropoietin. After 10 days, colonies were enumerated using light microscopy. Shown are the ratio of colony-forming unit granulocyte (CFU-G)/CFU macrophage (CFU-M) and the numbers of identifiable colonies. (B) Cells from methylcellulose plates were recovered and analyzed for monocytic markers, Ly6C, F4/80, and CD115, and the granulocytic marker Ly6G by flow cytometry. (C) Wright-Giemsa stain from cytospins of representative B6 and Alox15 methylcellulose colonies. 20× magnification. (D) B6 and Alox15 BM cells were cultured in methylcellulose in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). Shown are a summary of three independent experiments as a ratio of CFU-G/CFU-M and numbers of colonies CFU-M (black), CFU-G (checkered), and CFU granulocyte-macrophage (CFU-GM) (white). (E) B6 and Alox15 BM cells were cultured in methylcellulose in the presence of M-CSF or G-CSF as indicated on the x-axis. Shown are numbers and phenotypes of colonies. n = 6 in three independent experiments for all data shown. *p < 0.05, **p < 0.01. Error bars represent ± standard error of the mean.
of CFU-M compared with B6, resulting in increased ratios of CFU-G/CFU-M compared with B6 (Fig. 3A, B).

On the other hand, GMPs from Alox15 mice exhibit defective differentiation by generating fewer total colonies, significantly fewer CFU-M and subtle decreases in CFU-G and CFU-GM. There were no differences in the percentages of myeloid markers (Ly6C, Ly6G, CD115, F4/80) by flow cytometry, likely because of the decrease in all colony types (data not shown). In spite of the subtle decrease in CFU-G, the ratio of CFU-G to CFU-M that arose from Alox15 GMPs was increased compared with B6 (Fig. 3C). To further analyze the defective differentiation, we directed differentiation of B6 and Alox15 GMPs with the instructive cytokines M-CSF or G-CSF. B6 and Alox15 GMPs produced similar numbers of granulocytic colonies in response to G-CSF, demonstrating that Alox15 GMPs did not exhibit defective differentiation or cytokine hypersensitivity to G-CSF per se. However, Alox15 GMPs gave rise to fewer monocytic colonies in response to M-CSF than GMPs from B6 mice (Fig. 3D). Therefore, these data illustrate that Alox15 GMPs exhibit reduced monocytic differentiation when directed by instructive cytokines that may in part underlie Alox15 GMPs accumulation. In addition, the presence of immature granulocytes in CFU-G from Alox15 BM and the reduction of all colonies from GMPs further support reduced differentiation of Alox15 GMPs. Therefore, lack of 12/15-LOX results in an accumulation of GMPs, reduced numbers of monocytes, and increased numbers of mature and immature granulocytes (Fig. 3E).

**12/15-LOX regulates myelopoiesis by promoting GMP differentiation to monocytes and preventing the aberrant granulopoiesis that occurs in its absence.**

**Redox regulation of Irf-8 nuclear accumulation**

Because 12/15-LOX regulates Irf8 nuclear accumulation in mature myeloid cells [7,8] and because Irf8 critically

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**Figure 2.** Accumulation of granulocyte-macrophage progenitors (GMPs) in Alox15 mice. Bone marrow (BM) cells from 5- to 8-week (A), 12- to 16-week (B, E), 20+ -week (C), and spleens from 12- to 16-week-old (D) B6 and Alox15 mice were isolated and subjected to multicolor flow cytometric analysis to determine the relative frequency (A–D) and absolute number (E) of myeloid progenitors in tibias and fibulas of each mouse: LSK (Lin− Sca1− cKit+), common myeloid progenitors (CMPs) (Lin− Sca1− cKit+ CD34+ CD16/32lo), GMP (Lin− Sca1− cKit+ CD34+ CD16/32hi), and megakaryocyte erythroid progenitors (MEP) (Lin− Sca1− cKit+ CD34+ CD16/32hi). n = 3 independent experiments for all data shown. *p < 0.05, **p < 0.01. Error bars represent ± standard error of mean.
promotes monocyte differentiation and inhibits granulocyte development [3,5,6], we hypothesized that defective 12/15-LOX regulation of Irf8 in myeloid progenitor cells may underlie the skewed granulopoiesis and reduced monopoiesis in Alox15 mice. To test this, we determined Irf8 expression in Alox15 Lin− Sca1+cKit+ myeloid progenitors. Alox15 myeloid progenitors exhibited defective nuclear accumulation and total protein expression of Irf8 compared to B6 although the defect in Alox15 nuclear Irf8 was greater than the defect in total Irf8 (Fig. 4A, B). Meanwhile, Irf8 mRNA had a trend toward increased expression in the Alox15 progenitors compared with B6 (Fig. 4C). These data suggest that 12/15-LOX regulates Irf8 in a post-transcriptional manner.

Figure 3. Alox15 granulocyte-macrophage progenitors (GMPs) exhibit defective differentiation. B6 and Alox15 bone marrow (BM) were isolated and sorted for myeloid progenitors. Lin− Sca1+cKit+ cells (LSK) (A), common myeloid progenitors (CMPs) (B), and granulocyte-macrophage progenitors (GMPs) (C) were plated in methylcellulose in the presence of interleukin (IL)-3, IL-6, stem cell factor (SCF), and erythropoietin (Epo). Shown are a summary of three experiments as colonies enumerated and the colony-forming unit granulocyte (CFU-G)/CFU macrophage (CFU-M) ratio. (D) B6 and Alox15 GMPs were plated in the presence of macrophage colony-stimulating factor (M-CSF) or granulocyte CSF (G-CSF) in methylcellulose assays. n = 6 in three independent experiments for all data shown. *p < 0.05, **p < 0.01 Error bars represent ± standard error of mean. (E) Diagram of GMP differentiation in Alox15 mice. There is an accumulation of Alox15 GMPs, which exhibit reduced monocyte differentiation and enhanced granulopoiesis.
To determine the impact of decreased Irf8 nuclear accumulation on its transcriptional activity, we analyzed gene expression in myeloid progenitors. CMPs and GMPs from Alox15 mice exhibited decreased expression of Irf8-regulated genes \( Nf1 \) [12] and \( Egr1 \) [3] compared to B6 (Fig. 4D), but similar expression of \( Pu.1 \) and \( C/ebpA \) [3], which are important in myeloid differentiation, but not regulated by Irf8 (Fig. 4D), indicating a selective defect in Irf8-mediated gene transcription. The defect in Irf8-mediated gene transcription was not due to decreased transcription of Irf8 itself, which was similar between B6 and Alox15 CMPs and GMPs (Fig. 4D).

To determine the mechanism whereby 12/15-LOX regulates Irf8 transcriptional activity in developing monocytes, we utilized B6 and Alox15 BMM, which recapitulated the defect in \( Nf1 \) gene expression found in Alox15 CMPs and GMPs and expressed Irf8 mRNA similarly (Fig. 5A). Although B6 and Alox15 BMM exhibit comparable gene expression was normalized by glyceraldehyde 3-phosphate dehydrogenase (\( Gapdh \)). (\( n = 3 \) independent experiments for all experiments shown). *\( p < 0.05 \), **\( p < 0.01 \). Error bars represent ± standard error of mean.

Figure 4. Decreased interferon regulatory factor-8 (Irf-8) nuclear accumulation and transcriptional activity in Alox15 myeloid progenitors. (A) Decreased accumulation of Irf8 in nuclear lysates of Alox15 Lin− cKit+ myeloid progenitors determined by immunoblot. Shown are a representative experiment and a summary of three experiments as Irf8/retinoblastoma protein (Rb) relative to wild-type. (B) Immunoblot of total lysates from B6 and Alox15 Lin− cKit+ cells demonstrating decreased levels of Irf8. Shown are a representative blot and a summary of three experiments as Irf8/actin relative to wild-type. (C) Real-time analysis of Irf8 messenger RNA in B6 and Alox15 Lin− cKit+ cells (\( n = 3 \) independent experiments). (D) Real-time analysis of enriched B6 and Alox15 common myeloid progenitors (CMP) and granulocyte-macrophage progenitors (GMP) demonstrate that Alox15 myeloid progenitors exhibit decreased expression of the Irf8-mediated transcripts \( Nf1 \) and \( Egr1 \) and similar levels of \( Pu.1 \), \( C/ebpA \), and Irf8. Gene expression was normalized by glyceraldehyde 3-phosphate dehydrogenase (\( Gapdh \)). (\( n = 3 \) independent experiments for all experiments shown). *\( p < 0.05 \), **\( p < 0.01 \). Error bars represent ± standard error of mean.
expression of total Irf8 protein (Fig. 5B), there is a decrease in the nuclear accumulation of Irf8 in Alox15 BMM (Fig. 5C). To delineate the mechanism that may regulate Irf8-mediated gene transcription and nuclear accumulation, we compared 12/15-LOX products. Alox15 BMM were defective in 12(S)-HETE and in basal levels of ROS compared with B6 (Fig. 5D, E). Alox15 BMM also produced more of the 5-LOX product 5(S)-HETE (Fig. 5D), suggesting substrate redirevation into the 5-LOX pathway.

To determine the potential impact of the reduced levels of ROS on Irf8-mediated gene transcription, we treated B6 BMM overnight with an ROS scavenger, Tiron, and measured transcription of the Irf8 target gene Nf1. Conversely, we treated Alox15 BMM with BSO, which increases ROS by inhibiting synthesis of the cellular antioxidant glutathione. These treatments caused changes in ROS levels, but did not cause apoptosis (Supplementary Figure E3A, B, online only, available at www.exphem.org). Interestingly, Nf1 gene expression was regulated in part by ROS levels. The ROS scavenger Tiron decreased Nf1 expression in B6 BMM, while the ROS inducer, BSO, restored Nf1 transcription in Alox15 BMM (Fig. 5F). We determined whether ROS-signaling alters Nf1 transcription by regulating Irf8 nuclear accumulation. Both B6 BMM treated with Tiron and Alox15 BMM had decreased Irf8 nuclear accumulation compared to untreated B6 BMM, indicating that low ROS levels decrease nuclear accumulation of Irf8 (Fig. 5G). Conversely, elevation of ROS levels in Alox15 BMM by the addition of BSO increased Irf8 nuclear accumulation (Fig. 5G). This regulation was not due to alterations in Irf8 gene expression (data not shown) or total protein expression (Supplementary Figure E3C, online only, available at www.exphem.org). The addition of BSO in Alox15 BMM restored nuclear Irf-8 protein expression to untreated B6 BMM, suggesting that redox signaling is the primary mediator of 12/15-LOX-dependent activation of Irf8. These data indicate that 12/15-LOX regulates Irf8 nuclear accumulation and transcriptional activity through ROS-mediated signaling.

Figure 5. ROS signaling regulates interferon regulatory factor-8 (Irf-8) nuclear accumulation in Alox15 cells. (A) Alox15 BMM have decreased expression of Nf1 and similar levels of Irf8 compared to B6 by real-time analysis normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (n = 3 independent experiments). (B, C) Total and nuclear lysates were isolated from B6 and Alox15 BMM and immunoblotted for Irf8 demonstrating (B) Alox15 exhibit comparable expression of total Irf8 protein but (C) decreased expression of nuclear Irf8 protein (n = 3 independent experiments). (D) Lipid product formation in B6 and Alox15 BMM after stimulation with 50 μM arachidonic acid demonstrating decreased 12/5)-hydroxyeicosatetraenoic acid (12(S)-HETE) and increased 5(S) HETE in Alox15 BMM compared with B6. (n = 4 in two independent experiments). (E) Flow cytometric analysis of B6 and Alox15 BMM loaded with the ROS-sensitive dye H2DCFDA demonstrate decreased levels of ROS in Alox15 BMM. Shown are a representative experiment and a summary of five experiments.
Redox regulation of monocyte development in Alox15 progenitors

If 12/15-LOX regulates nuclear Irf8 accumulation through ROS-mediated signaling, then increasing ROS signaling would restore Irf8 nuclear accumulation, thereby enhancing monocyte development and inhibiting aberrant granulocyte development in Alox15 progenitors. To determine the impact of redox signaling on Alox15 myeloid development, we plated BM cells from B6 and Alox15 mice in methylcellulose in the presence or absence of BSO. Interestingly, the presence of BSO increased Alox15 monocyte development (CFU-M) and decreased granulocyte development (CFU-G) resulting in a decreased ratio of CFU-G/CFU-M (Fig. 6 A). On the other hand, the presence of BSO had no effect on B6 myeloid development (Fig. 6 A), suggesting that addition of BSO selectively rescues Alox15 myeloid cell development. Treatment of BSO also decreased the percentage of granulocytic Ly6G+ cells in Alox15 plates, confirming the shift from granulocyte development (Fig. 6B). Addition of the 12/15-LOX product 12(S)-HETE or the 5-LOX inhibitor MK-886 had no effect on B6 and Alox15 myeloid cell differentiation (Supplementary Figure E4; online only, available at www.exphem.org). GMPs from Alox15 mice plated in the presence of BSO also generated more CFU-M colonies resulting in a decreased ratio of CFU-G/CFU-M (Fig. 6C). Thus, 12/15-LOX regulates myelopoiesis at least in part through redox signaling and restoration of redox signaling in Alox15 progenitors restored monocyte development.

Discussion

This study demonstrates that 12/15-LOX promotes myelopoiesis through redox regulation of Irf-8. Lack of 12/15-LOX results in accumulation of GMPs, increased numbers of mature and immature granulocytes, and reduced numbers of monocytes in Alox15 mice. The accumulation of functional (CFU-GM) and phenotypic (GMPs) myeloid progenitors in Alox15 mice is accompanied by defective differentiation, which is likely a result of decreased Irf8-mediated gene transcription. We show that 12/15-LOX-mediated redox signaling regulates Irf8 nuclear accumulation, which subsequently promotes monocyte differentiation and inhibits granulocyte differentiation.

Similar to Alox15 BM cells, Alox15 LSK and CMPs exhibited decreased numbers of CFU-M and increased numbers of CFU-G compared with B6. However, Alox15 CMPs exhibited similar numbers of CFU-GM as B6. Alox15 GMPs had a decreased capacity to differentiate. Interestingly, we previously demonstrated that Alox15
HSC have a reduced capacity to self-renew and an increased propensity to differentiate [11]. The increased ability of Alox15 HSC to differentiate and 12/15-LOX regulation of Irf8 in CMPs (Fig. 4D) may also contribute to the accumulation of Alox15 GMPs.

12/15-LOX supports myeloid differentiation toward monocyte development, but is not absolutely required for monocyte differentiation. Rather, 12/15-LOX generates signaling intermediates that regulate Irf8, which in turn govern myeloid cell development. In the absence of 12/15-LOX, Irf8 still functions, albeit at reduced levels (Figs. 4 and 5). Importantly, there is a slight reduction of CFU-M and monocytes in Alox15 mice, which appear phenotypically normal (Fig. 1C). The normal number of Alox15 splenic macrophages may be due to homeostatic regulation.

A redox-dependent mechanism for Irf8 nuclear accumulation suggests that other mediators of redox signaling may also regulate myelopoiesis. Although increased levels of ROS promote monocyte to macrophage transition [13], our studies implicate an additional role for ROS signaling at an earlier stage of myeloid differentiation. Our finding that increasing ROS levels by the addition of BSO in Alox15 BM decreased granulocyte development is in agreement with a previous study that demonstrated ROS signaling, generated by dominant negative nuclear factor-kB, inhibits granulocyte differentiation [14]. However, that study did not consider potential effects on monocyte development and the ROS-generated mediated apoptosis. On the other hand, ROS generated in our studies did not cause apoptosis (Supplementary Figure E3, online only, available at www.exphem.org). ROS signaling is known to promote cytokine receptor signal transduction, including downstream of the cytokines erythropoietin and GM-CSF [15,16]. Therefore, 12/15-LOX redox signaling may also regulate other mediators in addition to Irf8 to regulate myeloid differentiation.

Because leukemia can result from aberrant myeloid cell development, elucidation of the processes that govern myelopoiesis may provide insight into leukemogenesis. Although excluded from these studies, a small percentage of Alox15 mice develop a myeloproliferative disease over the course of a year [7]. Irf8-deficient mice, which exhibited reduced monocyte development and enhanced granulocyte development, developed a similar myeloproliferative disease [17]. Reduced monocyte development coupled with a skewing toward granulocyte development in Alox15 and Irf8-deficient...
mice may contribute to or provide for a favorable environment for granulocytic leukemogenesis.

In summary, we demonstrate that ROS-signaling regulates Irf8 nuclear accumulation and subsequent myeloid cell differentiation. This results in an accumulation of defective GMPs and a skewing toward granulopoiesis at the expense of monopoiesis in Alox15 mice. Restoration of ROS rescues the aberrant Alox15 myeloid differentiation in vitro. These data establish a novel role for redox-signaling in the regulation of nuclear accumulation and gene transcription by Irf8 and in myeloid cell differentiation. These findings have implications for leukemogenesis.

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Conflict of Interest Disclosure
The authors have no conflicting financial interests.

References
Supplementary methods
Primer Sequences were designed using Primer Express (Applied Biosystems, Foster City, CA, USA) (Supplementary Methods).

Gapdh (forward) 5’—GACGCGCCGCACTTTCTTTGT and (reverse) 5’—CACACGACCTTCACTTTT TT-3’; β-actin, (forward) 5’—TCAGCAAGCAGGGTACGATG-3’ and (reverse) 5’—AACA GTCCGCCTTGAAGCCTTT-3’; Icsbp/Irf8 (forward) 5’TGGGCAGTTTTAAGGGAAGTT-3’ and (reverse) 5’—ACAGCGTAAACCTCGTTCCCA-3’; 12/15-Lox (forward) 5’—ACCACCGCGCATTTTand (reverse) 5’—AGC TTCCGGACCCAGCATTT; Nf1 (forward) 5’—GGTATGGACACAGGACCTTTGAT-3’ and (reverse) 5’—CTTCGCGTTACCATGAGCAC-3’; PU.1 (forward) CCGGATGTGCTTCCCTATC and (reverse) TCTACCCCTCCTCCTCATCGA; C/EBPz (forward) GACCATAG CCTTGT GTA CACTGATG and (reverse) TGGATCGATGTT GCTTCAAGT; Egr-1 (forward) CCATGAAACG CCCCCATATGCTT and (reverse): GCTCATCC GAGAGAGAAGG

Supplementary Figure E1. (A) Decreased numbers of blood monocytes in Alox15 mice. B6 and Alox15 blood cell populations were analyzed using Advia2120 hematology analyzer in the mouse mode (Siemens Healthcare Diagnostics, Deerfield, IL, USA); n = 6. (B—C) Increased percentage of neutrophils (Mac1+Gr1hi) and similar percentage of monocytes/macrophages (Mac1+Gr1I/C14/F4/80-) in the blood (B) and spleens (C) of Alox15 mice compared to B6; n = 5. (D) 12/15-lipoxygenase (12/15-LOX) is expressed during myeloid differentiation. B6 common myeloid progenitors (CMPs) (Lin−Sca1+Sca1+cKit+CD34+CD16/32lo) and granulocyte-macrophage progenitors (GMPs) (Lin−Sca1+Sca1+cKit+CD34+CD16/32hi) were enriched by cell sorting and subjected to real-time polymerase chain reaction analysis for 12/15-LOX normalized to actin gene expression levels (n = 4 independent experiments). (E) Splenocytes from Alox15 mice exhibit increased granulopoiesis. Splenocytes from B6 and Alox15 mice were plated in methylcellulose containing stem cell factor (SCF), interleukin (IL)-6, IL-3, and erythropoietin (EPO). Colonies were enumerated after 10 days. Shown are a summary of three experiments as ratio colony-forming unit granulocyte (CFU-G)/colony-forming unit macrophage (CFU-M) and numbers of each colony type. Error bars represent ± standard error of mean.
Supplementary Figure E2. Increased numbers of granulocyte-macrophage progenitors (GMPs) from Alox15 mice is not due to enhanced proliferation or survival. (A) For cell cycle analysis, bone marrow (BM) was stained at 1 x 10^6 cells/mL in 2% fetal calf serum (FCS) RPMI with 10 μg/mL Hoechst 33342 (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 45 minutes and 300 ng/mL pyronin Y for 45 additional minutes followed by prior to multicolor flow cytometric analysis. Shown are a representative flow cytometric plot gated on GMP and a summary of three independent experiments. (B) B6 and Alox15 BM were subjected to multicolor flow cytometry and Annexin-V/4',6-diamidino-2-phenylindole (DAPI) staining to determine levels of apoptosis defined as Annexin-V+DAPI ex vivo. Shown are a representative flow cytometric plot gated on GMP and a summary of three experiments. FMO = fluorescence minus one control. (C) B6 and Alox15 GMP were enriched by cell sorting and cultured in 25 ng/mL stem cell factor (SCF) in vitro for 48 hours. Cells were then stained with Annexin-V/TOPRO to determine apoptosis (Annexin-V+/TOPRO−) and dead cells (TOPRO+) (n = 5 in three independent experiments). Error bars represent ± standard error of mean.
Supplementary Figure E3. Treatment with ROS modulators are physiological. B6 BMM were cultured in the presence or absence of 10 mM Tiron and Alox15 BMM were cultured in the presence or absence of 100 uM BSO overnight. (A) ROS levels were measured by CM-H₂DCFDA and shown as mean fluorescent intensity (MFI) relative to B6. (B) Apoptosis was measured by percentage of Topro⁺ cells using flow cytometric analysis. n = 3 independent experiments. (C) No differences in total interferon regulatory factor-8 (IRF-8) expression protein expression in treated and untreated B6 and Alox15 BMM. Shown are a representative immunoblot and a summary of three independent experiments. Error bars represent ± standard error of mean.
Supplementary Figure E4. Addition of 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) or an inhibitor of 5-lipoxygenase (5-LOX) activity, MK-886, in methylcellulose assays have no effect on myeloid cell development. B6 and Alox15 bone marrow (BM) cells were plated in methylcellulose containing interleukin (IL)-3, IL-6, and stem cell factor (SCF) in the presence or absence of (A) 1 μM 12(S)-HETE or (B) 1.25 μM MK-886. Shown is ratio of colony-forming unit granulocyte (CFU-G)/colony-forming unit macrophage (CFU-M). n = 6 in three independent experiments. Error bars represent ± standard error of mean.