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The Corepressor Rcor1 Is Essential for Normal Myeloerythroid Lineage Differentiation

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Abstract

Based on its physical interactions with histone-modifying enzymes, the transcriptional corepressor Rcor1 has been implicated in the epigenetic regulation blood cell development. Previously, we have demonstrated that Rcor1 is essential for the maturation of definitive erythroid cells and fetal survival. To determine the functional role of *Rcor1* in steady-state hematopoiesis in the adult, we used a conditional knockout approach. Here, we show that the loss of Rcor1 expression results in the rapid onset of severe anemia due to a complete, cell autonomous block in the maturation of committed erythroid progenitors. By contrast, both the frequency of megakaryocyte progenitors and their capacity to produce platelets were normal. Although the frequency of common lymphoid progenitors and T cells was not altered, B cells were significantly reduced and showed increased apoptosis. However, Rcor1-deficient bone marrow sustained normal levels of B-cells following transplantation, indicating a non-cell autonomous requirement for Rcor1 in B-cell survival. Evaluation of the myelomonocytic lineage revealed an absence of mature neutrophils and a significant increase in the absolute number of monocytic cells. Rcor1-deficient monocytes were less apoptotic and showed ~100-fold more colony-forming activity than their normal counterparts, but did not give rise to leukemia. Moreover, $Rcor1^{-/-}$ monocytes exhibited extensive, cytokine-dependent self-renewal and overexpressed genes associated with hematopoietic stem/progenitor cell expansion including *Gata2*, *Meis1*, and *Hoxa9*. Taken together, these data demonstrate that Rcor1 is essential for the normal differentiation of myeloerythroid progenitors and for appropriately regulating self-renewal activity in the monocyte lineage.

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Author Contributions

H.Y. and D.C.G.: conceived and designed the experiments, collected data, analyzed and interpreted data, and wrote the manuscript; G.F.: analyzed and interpreted data; G.M. and W.H.F.: conceived and designed the experiments, analyzed and interpreted data, and wrote the manuscript. H.Y. and D.C.G. contributed equally to this work.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

Keywords

Corepressor proteins; Epigenetics; Hematopoiesis; Erythropoiesis; Monocytes; Neutrophils

Introduction

Transcriptional corepressors are critical components of gene expression regulatory machinery. Their primary function is to serve as a platform to couple different histone modification activities that regulate gene expression. Through diverse transcription factor and cofactor interactions, tightly regulated gene expression patterns can be achieved by a limited number of proteins. Among the best characterized transcriptional corepressors is Rcor1 (CoREST) [1], a core component of a chromatin-modifying repressor complex that includes histone deacetylase 1/2 (HDAC1/2) [2–4] and the histone 3 lysine 4 (H3K4) demethylase Kdm1a (also known as LSD1) [5, 6]. HDAC1/2 removes acetyl groups from histone tails whereas Kdm1a removes monomethylation/dimethylation marks from H3K4 [7]. Although much attention has been focused on understanding the biochemical functions of Rcor1, its functional role in hematopoiesis has only recently been explored.

Rcor1 is expressed in hematopoietic stem cells, progenitor cells, and their differentiated progeny [8, 9]. Biochemical studies have identified several DNA binding transcription factors that physically interact with the Rcor1/Kdm1complex in different hematopoietic cell types. For example, Gfi1 and Gfi1b [10] have been shown to regulate key aspects of hematopoietic differentiation in vivo. The zinc finger protein Gfi1 is critical for granulocyte differentiation [11–13] and also regulates the production of common lymphoid progenitors as well as B-cell and T-cell differentiation [11, 12, 14–17]. The Gfi1 homolog, Gfi1b, is necessary for both erythroid and megakaryocytic differentiation [18, 19]. Although these interactions suggest potential roles for Rcor1 in multilineage differentiation, it is not yet known which hematopoietic cell lineages are functionally dependent on Rcor1 activity.

To directly assess Rcor1 function throughout the hematopoietic system and to bypass the embryonic lethality in the whole-animal Rcor1 knockout model [20], we generated an *Mx1-Cre*-driven *Rcor1* knockout mouse. The loss of Rcor1 in adult hematopoietic cells leads to a complex phenotype that includes a complete block in erythroid and neutrophil differentiation but a sparing of the megakaryocyte lineage. These deficiencies are accompanied by an increase in monocytic cells that display abnormal self-renewal and reduced apoptosis.

Materials and Methods

Mice

Generation of *Mx1-Cre; Rcor1*$^{flox/-}$ mice (B6.CD45.2) and genotyping was performed as previously described [20]. Adult mice were injected every other day (three injections total) with ~175 μg of double stranded polyinosinic/polycytidylic acid (poly(I:C); 0.5 mg/ml, GE Healthcare Bio-Sciences, Pittsburgh, PA, http://www.gelifesciences.com). B6.CD45.1, B6.CD45.2/B6 Thy1.2 CD45.1 hybrids, or double congenic B6.CD45.1 Hbbd mice (8–12

weeks old) were used as transplant recipients. 1–3 × 10^6 control or *Rcor1*$^{-/-}$ unfractionated bone marrow (BM) was injected i.v. into irradiated (500–700 cGy) recipient mice. Sorted Lin$^-$Sca1$^-$c-kit$^+$ (LSK) cells (1,500–2,500 cells) from untreated or poly(I:C) treated *Mx1-Cre; Rcor1*$^{flox/-}$ and control donors were cotransplanted with competitor BM (1–2 × 10^5 cells) from B6.CD45.1 or B6.CD45.1 EGFP$^+$ (TgN(act-EGFP)OsbY01) donors into lethally irradiated recipient mice (1,000 cGy). Recipients were maintained on antibiotic-supplemented water [21]. All animal procedures were performed in accordance with the Institutional Animal Use and Care Committee at OHSU.

Flow Cytometry Analysis and Fluorescence-Activated Cell Sorting

Single-cell suspensions from BM, spleen, and peripheral blood were prepared as previously described [22]. Immunophenotyping of myeloerythroid progenitors [23], common myeloid progenitors (CMP), granulocyte-monocyte progenitors (GMP), megakaryocyte-erythroid progenitors (MEP) [24], and common lymphoid progenitors (CLP) [25] was performed on an LSRII, Canto, fluorescence-activated cell sorting (FACS) Calibur or Fortessa cytometer (BD, Franklin Lakes, NJ, http://www.bdbiosciences.com). Cell sorting was performed using a BD Influx or a BD Vantage sorter. Both dead cells (propidium iodine$^+$) and doublets were excluded. Data were analyzed with FlowJo software (Ashland, OR, http://www.flowjo.com/). Antibodies (and clones) used in this study included: CD71 (R17217), Mac1 (M1/70), Gr1 (RB6–8C5), B220 (RA3–6B2), IgM (eB121–15F9), CD3 (145–2C11), and c-kit (2B8) (eBioscience, San Diego, CA, http://www.ebioscience.com); CD19 (1D3), CD4 (H29.19), CD5 (53–7.3), CD8 (53.6.7), CD16/32 (2.4G2), CD34 (RAM34), and CD115 (AFS98) (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com); TER119, Sca1 (D7), CD150 (TC15–12F12.2), CD105(MJ7/18), CD127/IL-7Rα (A7R34), and CD41 (MWReg30) (Biolegend, San Diego, CA, http://www.biolegend.com/). For apoptosis analysis, an Annexin-V Apoptosis Detection Kit I (BD Biosciences) was used with 7-AAD. For analysis of CMP/GMP/MEP, the lineage panel included B220, CD3, CD4, CD8, Gr1, Ter119, CD19, IgM, and CD127. For analysis of myeloerythroid progenitors, the lineage panel included B220, CD4, CD8, Mac1, and Gr1. To sort megakaryocyte progenitors (MkP), Ter119 was also included within this lineage mixture. For CLP analysis and LSK sorting, the lineage panel included B220, CD3, CD4, CD5, CD8, Mac1, Gr1, and Ter119.

Hemoglobin Electrophoresis

Performed as previously described [26].

In Vitro Colony-Forming Assays

For colony-forming unit (CFU) assays, cells were plated in duplicate or triplicate in 35 mm dishes in mouse methylcellulose complete medium (HSC007, R&D Systems, Minneapolis, MN, https://www.rndsystems.com) or in cytokine-free methylcellulose medium (M3234, Stem Cell Technologies, Vancouver, Canada, http://www.stemcell.com). Colonies were scored 7–10 days after plating. For serial replating assays, pooled day 8 colonies were washed in Dulbecco's modified Eagle's medium containing 10% serum and 1 × 10^4 cells were replated in fresh methylcellulose medium. For megakaryocyte (Mk) progenitor assays, 1 × 10^5 unfractionated BM were cultured in Mega-CultC (04950, Stem Cell Technologies)

supplemented with mTPO (50 ng/ml) and mIL3 (10 ng/ml). Cultures were dehydrated, fixed, stained, and CFU-Mk were scored.

Morphological Analysis and Immunofluorescence Microscopy

BM touch preparations, peripheral blood, cytospun sorted cells, and colonies were stained with May-Grunwald and Giemsa stains. Tibias were fixed, decalcified, and cryopreserved as previously described [22] and vWF expression was detected with anti-vWF (A0082, Dako, http://www.dako.com), goat anti-rabbit cyanine 3, and 4′,6-diamidino-2-phenylindole. Imaging was performed with a Zeiss (Thornwood, NY, http://www.zeiss.com) Axiovert S-100, an AxioCam HRc camera and a Zeiss plan-neofluar ×20/0.50 lens, a Zeiss plan-neofluar ×40/1.30 lens, or a Zeiss plan-neofluar ×63/1.25 oil lens.

Quantitative RT-PCR

Sorted Mac1^{+}Gr1^{lo} cells from induced *Mx1-Cre; Rcor1*$^{flox/–}$ mice and controls treated with poly(I:C) and from primary recipients of *Rcor1*$^{–/–}$ and control BM were isolated by FACS. RNA was extracted using TRIzol and treated with DNase. Reverse transcription reactions were performed using Superscript III (Invitrogen, Grand Island, NY, http://www.lifetechnologies.com). Quantitative RT-PCR (qPCR) was performed with an Applied Biosystems PRISM 7900HT Fast Real-Time PCR system using SYBR green PCR master mix (Grand Island, NY, http://www.lifetechnologies.com). Relative abundance of each cDNA was determined according to the standard curve and normalized to 18S RNA levels. The primers used are listed below: *18S*$_{fwd}$, 5′-CTCAACACGGGAAACCTCAC-3′; *18S*$_{rev}$, 5′-CGCTCCACCAACTAAGAACG-3′; *Gata2*$_{fwd}$, 5′-CGCCTGTGGCCTCTACTACAA-3′; *Gata2*$_{rev}$, 5′-TTTCTTGCTCTTCTTGGATTTGCT-3′; *Hoxa9*$_{fwd}$, 5′-AACAATGCCGAGAATGAGAGC-3′; *Hoxa9*$_{rev}$, 5′-CGAGTGGAGCGAGCATGTAG-3′; *Meis1*$_{fwd}$, 5′-AAGATACAGGACTTACCATCCTTCA-3′; *Meis1*$_{rev}$, 5′-GTCTATCATGGGCTGCACTATTCT-3′.

Statistical Analysis

Data were analyzed using Microsoft Excel or Prism 6. A two-tailed, unpaired Student's *t* test was used and a *p*-value of <.05 was considered significant.

Results

Deletion of *Rcor1* in Adult Hematopoietic Cells Produces a Lethal Anemia and Increases Myelomonocytic Cells

To assess Rcor1 function in steady-state, adult hematopoiesis, we generated mice carrying the *Mx1-Cre* transgene and a single functional *Rcor1* allele in which exon 4 was flanked by loxP sites (*Mx1-Cre; Rcor1*$^{flox/–}$). Administration of poly(I:C) was used to induce Cre expression in interferon-responsive cells [27], and 2 weeks following Cre induction, genotype analysis confirmed the absence of the *Rcor1*^{flox} allele in the BM (Fig. 1A, 1B). Hereafter, we refer to these poly(I:C) treated, Rcor1-deficient mice as *Rcor1*$^{–/–}$. Age-matched *Rcor1*$^{flox/–}$ and *Mx1-Cre; Rcor1*$^{+/flox}$ mice were used as controls.

Typically, 80% of the *Rcor1*$^{-/-}$ mice died within 2–3 weeks following poly(I:C) treatment (Fig. 1C), a time course consistent with acute BM failure. Analysis of the peripheral blood revealed a rapidly progressive anemia in *Rcor1*$^{-/-}$ mice suggestive of a near complete block in red cell production (Fig. 1D). By contrast, platelet counts increased transiently and then quickly returned to normal levels (Fig. 1E). The total number of circulating white blood cells was persistently elevated due exclusively to an increase in circulating myelomonocytic cells (Fig. 1F) and infiltrates of these cells were found in the liver (Fig. 1G).

As the *Mx1* promoter has been reported to be active in BM stroma [28] it was important to determine whether the *Rcor1*$^{-/-}$ phenotype was due primarily to a hematopoietic cell-intrinsic defect. Chimeric mice in which *Mx1-Cre; Rcor1*$^{flox/-}$ cells contributed to >90% of hematopoietic cells were generated by the transplantation of unfractionated BM (Fig. 1H, 1I). Two weeks after Cre induction, these BM-restricted *Rcor1*$^{-/-}$ mice developed marked anemia, increased circulating myelomonocytic cells, and a transient increase in platelet counts that was indistinguishable from the non-BM restricted *Rcor1*$^{-/-}$ mice (Fig. 1D–1F). Together, these data demonstrate that Rcor1 expression is required in a hematopoietic cell-intrinsic manner to maintain the steady-state production of cells of both the myeloid and erythroid lineages.

Rcor1 Is Required for the Maturation of Committed Erythroid Progenitors

To identify the specific stages of adult erythroid cell maturation that were disrupted by the loss of Rcor1, progenitors expressing CD71 and Ter119 were evaluated [29]. These markers distinguish proerythroblasts (CD71^{hi}Ter119^{lo}), maturing erythroblasts (CD71$^{hi/int}$Ter119^{hi}), and reticulocytes (CD71^{-}Ter119^{hi}) in normal BM; however, this normal expression pattern was completely disrupted in *Rcor1*$^{-/-}$ BM and spleen (Fig. 2A). In order to resolve this, we identified phenotypically defined erythroid progenitors in the lineage-Sca1^{-}Kit^{+} (LS^{neg}K) myeloerythroid progenitor compartment as described by Weissman and colleagues [23]. This analysis revealed a normal frequency of bipotential megakaryocyte/erythroid progenitors (Pre-MegE) and a marked accumulation of Pre-CFU-E and CFU-E progenitors indicating that the loss of *Rcor1* does not prevent erythroid progenitor specification (Fig. 2B). To functionally test the maturation of *Rcor1*$^{-/-}$ erythroid progenitors, LS^{neg}K cells were plated in cytokine supplemented methylcellulose. Rcor1-deficient BM showed a 90% reduction in the formation of burst forming unit-erythroid (Fig. 2C) colonies comprised of maturing RBCs that develop from Pre-MegE and Pre-CFU-E. These findings indicate that *Rcor1*$^{-/-}$ deficiency blocks the terminal differentiation of erythroid-committed progenitor cells.

To determine whether a normal hematopoietic microenvironment can attenuate this block in erythroid maturation, BM was isolated from CD45.2 *Rcor1*$^{-/-}$ mice with the Hbbd hemoglobin haplotype and transplanted into CD45.1 recipients that carry the Hbbs hemoglobin haplotype. Donor cell engraftment was monitored up to 9 weeks post-transplant, and all recipient mice appeared healthy with normal RBC indices (data not shown). Although >50% of circulating leukocytes were derived from *Rcor1*$^{-/-}$ donors, no *Rcor1*$^{-/-}$ donor-derived hemoglobin was detected in the peripheral blood (Fig. 2D). These data

demonstrate a cell-autonomous requirement for Rcor1 during the maturation of committed erythroid progenitor cells into mature red blood cells.

Thrombopoiesis Is Not Rcor1 Dependent

Although a previous in vitro study suggested that Rcor1 is essential for megakaryocytic maturation [10], platelet counts were not reduced following the deletion of *Rcor1* in vivo (Fig. 1E). Megakaryocytes showed both a normal morphology and frequency in $Rcor1^{-/-}$ BM (Fig. 3A, 3B). The frequency of immunophenotypically defined megakaryocyte progenitors (MkP: Lin^{-}, $Scal\text{-}1^{-}$, $c\text{-}kit^{+}$, $CD150^{+}$, $CD41^{+}$) [23] was indistinguishable from controls (Fig. 3C, 3D). In culture conditions supporting megakaryocyte differentiation from MkP, $Rcor1^{-/-}$ BM readily produced $acetylcholinesterase^{+}$ ($AchE^{+}$) megakaryocytic colonies (Fig. 3E). To exclude the possibility that $Rcor1^{flox/-}$ megakaryocytes persist after poly(I:C) treatment due to incomplete Cre-mediated recombination in vivo, MkP were sorted from BM (Fig. 3C) and genotyped. Only the $Rcor1^{-}$ allele was detected, indicating complete ablation of *Rcor1* in progenitors of the megakaryocytic lineage (Fig. 3F). Together, these data indicate that Rcor1 is not essential for megakaryocytic progenitor specification, megakaryocyte maturation, or platelet production.

Non-Cell Autonomous Suppression of B-Cell Survival in Rcor1-Deficient Mice

To determine whether Rcor1 is essential for normal lymphocyte production, we evaluated $B220^{+}$ B cells and $CD3^{+}$ T cells in *Rcor1*-deficient mice. Although B cells were reduced by greater than twofold in BM, spleen, and peripheral blood (PB), the number of T cells was not significantly altered (Fig. 4A, 4B). The frequency of CLP in the BM was not impacted by the loss of Rcor1 (Fig. 4C). However, apoptosis was increased 2.6-fold in Rcor1-deficient B cells compared to controls (Fig. 4D). Non-hematopoietic BM stromal cells have been shown to provide an important functional niche for B cells [30, 31]. To assess whether restricting the loss of Rcor1 to hematopoietic cells was sufficient to suppress B cells, we generated chimeric mice in which *Mx1-Cre;* $Rcor1^{flox/-}$ cells contributed to >90% of hematopoietic cells (Fig. 1H) and analyzed B cells 2 weeks after Cre induction (Fig. 4E). The loss of B cells observed in the hematopoietic-restricted knockout mice indicated a hematopoietic cell-intrinsic requirement for Rcor1 in supporting B-cell survival. Surprisingly, we found that in a transplant setting in which Rcor1-deficient donor cells comprise less than 50% of total BM cells (Fig. 4F, 4G), $Rcor1^{-/-}$ donor cells gave rise to B cells at the same frequency as control donor cells (Fig. 4H). Genotype analysis of peripheral blood isolated from host mice confirmed *Rcor1* deletion in donor cells (data not shown). Consistent with normal B-cell levels in this setting, no difference in B-cell apoptosis was detected in $Rcor1^{-/-}$ B-cells post-transplant (Fig. 4I). These results indicate that the significant loss of B cells observed when Rcor1-deficient hematopoietic cells comprise nearly all BM is primarily due to a non-cell autonomous effect that is mediated by $Rcor1^{-/-}$ cells in other hematopoietic lineages.

Rcor1 Deficiency Leads to an Absence of Mature Neutrophils and an Increase in Monocytic Cells

Evaluation of leukocytes in the blood and BM, the $Rcor1^{-/-}$ mice revealed the presence of many immature myelomonocytic cells and an almost complete absence of maturing/mature

neutrophils (Fig. 5A, 5B). Cells expressing the $Mac1^{+}Gr1^{hi}$ neutrophil phenotype were markedly decreased within the BM, spleen, and blood, and those $Mac1^{+}Gr1^{hi}$ cells that were detected were either eosinophils or monocytes (Fig. 5C–5F and Supporting Information Fig. S1A). More than 50% of the *Rcor1*$^{-/-}$ BM cells had a $Mac1^{+}Gr1^{lo}$ phenotype compared to 10% in control mice (Fig. 5C, 5D). Both the expansion of $Mac1^{+}Gr^{lo}$ cells and loss of $Mac1^{+}Gr1^{hi}$ cells were also observed in *Rcor1*$^{-/-}$ BM chimeric mice indicating this phenotype is due to a hematopoietic cell-autonomous defect (Fig. 1H and Supporting Information Fig. S1B). Normally, the $Mac1^{+}Gr1^{lo}$ cell population is primarily comprised of maturing granulocytes, with a few monocytes (Fig. 5E) [32–34]. By contrast, the $Mac1^{+}Gr1^{lo}$ cell population from *Rcor1*$^{-/-}$ BM was almost exclusively monocytes, with only a few immature granulocytes (Fig. 5F). Five functionally and developmentally distinct subpopulations of myelomonocytic cells (R1-R5) can be resolved by distinct patterns of CD48 and Gr-1 expression [34]. In the *Rcor1*$^{-/-}$ mice, although the maturing granulocytic cell population (R1) was essentially absent, granulocyte-committed precursor populations (R4, R5) were detected at a normal frequency, suggesting that neutrophil differentiation but not specification is Rcor1 dependent (Fig. 5G, 5H). The frequency of monocytic cells (R2) and mixed potential cells (R3) was increased 15-fold and 2.3-fold, respectively. Supporting this finding of expanded monopoiesis, most of the $Mac1^{+}$ cells in the Rcor1-deficient mice also coexpressed CD115, the CSF-1 receptor (Fig. 5I). Furthermore, *Rcor1*$^{-/-}$ cells displayed the identical myelomonocytic differentiation defects after transfer into wild-type hosts (Fig. 5J, 5K and Supporting Information Fig. S1C, S1D). Although in some mice the *Rcor1*$^{-/-}$ myelomonocytic cells slowly increased over time, progression to leukemia was not observed for up to 12 months.

Rcor1 Deficiency Leads to the Accumulation of Abnormal Monocytic Cells

To further evaluate monocytic cell expansion in the *Rcor1*$^{-/-}$ mice, the frequency of both phenotypically and functionally defined myeloid progenitor cells was assessed. Although the number of the $LS^{neg}K$ myeloerythroid progenitors was increased 14-fold in the *Rcor1*$^{-/-}$ BM (Fig. 6A), distinct populations of the downstream CMP and GMP could not be easily identified due to aberrant patterns of cell surface marker expression (Fig. 6B). Using the phenotypic analysis described by Pronk et al. [23] as an alternative approach, we found that some myeloid progenitors inappropriately expressed CD105 and that pre-GM (GMP precursors) were reduced by 66%, whereas GMPs were increased by ~60% (Supporting Information Fig. S2A–S2D).

To functionally assess myeloid cell progenitor activity in *Rcor1*$^{-/-}$ mice, the myeloid colony-forming activity of both unfractionated BM and sorted $LS^{neg}K$ cells was tested. *Rcor1*$^{-/-}$ BM formed significantly more myeloid colonies than controls on a per cell basis, indicative of increased progenitor activity (Fig. 6C). Specifically, colony-forming activity for monocytes/macrophages (CFU-M) was 16-fold higher in *Rcor1*$^{-/-}$ BM. Although mutant $LS^{neg}K$ cells were also CFU-M biased, they formed 50% fewer myeloid CFU than their normal counter parts (Fig. 6D), produced no detectable granulocytic colonies (CFU-G) and showed a 66% decrease in granulocyte/monocyte/macrophage colonies (CFU-GM). Thus, the majority of the total myelomonocytic CFU activity in the *Rcor1*$^{-/-}$ BM must have arisen from cells outside of the $LS^{neg}K$ progenitor cell phenotype (Fig. 6A).

As the Mac1^{+}Gr1^{lo} myeloid cells comprise a large proportion of the Lin^{+} BM cells in *Rcor1*$^{-/-}$ mice, we directly tested their functional activity. As expected, control Mac1^{+}Gr1^{lo} cells had minimal CFU potential, forming <1 colony per 5,000 cells. By contrast, *Rcor1*$^{-/-}$ Mac1^{+}Gr1^{lo} cells generated a mean of 92 CFU-M colonies per 5,000 cells (Fig. 6E).Morphological analysis of the colonies derived from these mutant Mac1^{+}Gr1^{lo} cells confirmed their monocytic identity and also revealed that these cells were capable of differentiating into macrophages (Fig. 6F). Remarkably, the Mac1^{+}Gr1^{lo} cells from Rcor1-deficient mice also possessed extensive serial replating activity and continually produced CFU-M for up to five serial passages in culture (Fig. 6G). Importantly, this robust CFU-M activity was dependent on the presence of cytokines, indicating these monocytic cells were not able to proliferate autonomously. Finally, analysis of apoptosis in Mac1^{+}Gr1^{lo} BM cells directly isolated from *Rcor1*$^{-/-}$ mice revealed a 60% reduction in the frequency of apoptotic cells (Fig. 6H). Together, these data indicate that the Mac1^{+}Gr1^{lo} cell subset in the *Rcor1*$^{-/-}$ mice is cytokine-dependent, monocyte lineage cells that are resistant to apoptosis, and possess extensive, abnormal self-renewal activity.

Transcription Factors that Regulate Stem/Progenitor Cell Proliferation Are Derepressed in Rcor1-Deficient Monocytic Cells

As we previously demonstrated that in fetal liver erythroid progenitors, Rcor1 suppresses hematopoietic stem/progenitor cell gene expression signatures [20], we evaluated the expression levels of *Gata2*, *Meis1*, and *Hoxa9* in Rcor1-deficient Mac1^{+}Gr1^{lo} cells. These transcription factors are known regulators of hematopoietic stem cell proliferation [35–38] and are normally expressed at high levels in hematopoietic stem cells relative to more differentiated myelomonocytic cells [39–42]. Moreover, their activity has been implicated in myeloid leukemia [43–46], and expression of *Gata2* and *Hoxa9* has been demonstrated to be elevated in patients with myelodysplastic syndrome (MDS) [47–50]. qPCR revealed large increases in the transcript levels (8- to >200-fold) of all three genes in Rcor1-deficient Mac1^{+}Gr1^{lo} cells relative to controls (Fig. 6I). Thus, *Gata2*, *Hoxa9*, and/or *Meis1* derepression may enhance the proliferative capacity and self-renewal activity of Rcor1-deficient monocytic cells. Future studies will elucidate whether Rcor1 directly regulates their expression in myeloid cells.

Discussion

This study provides the first in vivo functional analysis of the corepressor Rcor1 in adult hematopoiesis and identifies its critical roles in myeloerythroid lineage differentiation. The Rcor1/Kdm1a complex was previously shown to be a cofactor for Gfi1 or Gfi1b mediated transcriptional repression in hematopoietic cell lines [10]. A comparison of hematopoietic defects in our Rcor1 knockout models with those reported for Kdm1a, Gfi1, and Gfi1b knockout mice provides additional insights into the biological relevance of these biochemical interactions. For example, if Rcor1 works together with Kdm1a, Gfi1, or Gfi1b to regulate a specific hematopoietic lineage in vivo, then it would be predicted that the loss of any component of this complex would cause identical lineage-specific defects. Indeed, the erythroid defects observed in the *Rcor1*$^{-/-}$ mice largely phenocopy those observed in both Kdm1a-deficient and Gfi1b-deficient mice [19, 51]. Likewise, the ablation of Kdm1a, Gfi1,

or Rcor1 causes severe neutropenia in adult mice [11, 12, 51]. Thus, all available data support previous reports that Rcor1/Kdm1a/Gfi1b and Rcor1/Kdm1a/Gfi1 complexes regulate erythroid and granulocytic maturation, respectively.

Importantly, hematopoietic defects associated with Rcor1-, Kdma1-, Gfi1-, or Gfi1b-deficiency in vivo demonstrate several functional differences. One of the most discordant phenotypes is within the megakaryocyte lineage. While loss of Kdm1a or Gfi1b produces severe reduction in platelets counts [19, 51], the loss of Rcor1 causes a transient increase in platelets. Moreover, *Gfi1b*$^{-/-}$ cells derived from either fetal liver or adult BM cannot form AchE^{+} megakaryocytic colonies in culture [18, 19], whereas *Rcor1*$^{-/-}$ BM readily produces AchE^{+} CFU-Mk colonies. A second marked phenotypic difference among Rcor1, Kdm1a, and Gfi1 mutant mice is within the lymphoid progenitors. Whereas loss of Kdma1 or Gfi1 impairs the formation of CLP [51, 52], a normal CLP frequency was found in Rcor1-deficient mice. The maintenance of thrombopoiesis and CLP production in Rcor1-deficient mice might reflect functional redundancy among Rcor family members [53]; however, our findings also raise the possibility that Gfi1b, Gfi1, and Kdm1a may normally use a cofactor other than Rcor1 to maintain these two lineages.

Despite a striking loss of B cells in the Rcor1-deficient mice, our transplant studies demonstrate that this phenotype is attributable to a non-cell autonomous mechanism. Suppression of lymphopoiesis can be associated with emergency granulopoiesis [54]. Given that loss of *Rcor1* throughout the BM leads to a severe neutropenia, we suspect that the ensuing stress response contributes to B-cell loss. Thus, in a transplant setting where *Rcor1*$^{-/-}$ cells do not contribute to the majority of BM cells (Fig. 4G), we speculate that because the remaining host cells produce normal levels of neutrophils, *Rcor1*$^{-/-}$ B cells are not suppressed. Future studies will determine whether there is a cell-autonomous requirement for Rcor1 in B- and T-cell maturation and/or function.

Interestingly, the adult *Rcor1* knockout mice exhibited a subset of phenotypes consistent with several clinical features associated with MDS and myeloproliferative disorders [55, 56]. Specifically, the differentiation of two major erythromyelocytic lineages, erythrocytes and neutrophils, was completely blocked following *Rcor1* deletion and occurred concomitantly with monocytosis and monocytic liver infiltration. These features meet the World Health Organization criteria for chronic myelomonocytic leukemia (CMML) [57, 58]. Although we have not observed transformation to leukemia in *Rcor1*$^{-/-}$ BM recipient mice for more than a year following transplant, more time may be required to develop the necessary secondary mutations. The CMML-like disease in mice lacking Rcor1 expression in BM raises the possibility that disruption of RCOR1 expression and/or function may also contribute to CMML pathology in humans. *RCOR1* mutations or copy number alterations have not yet been reported in CMML or myeloid malignancies. Given that in the mouse model, *Rcor1* haploinsufficiency does not affect normal hematopoiesis, we anticipate that a complete or near complete loss of *RCOR1* expression is likely required to elicit phenotypic alterations in humans. Consistent with this possibility, *RCOR1* loss was recently reported in a subset of diffuse large B-cell lymphoma patients and is associated with a prognostically significant gene expression signature [59].

Conclusions

We have shown that the maturation of both erythroid cells and neutrophils is critically dependent on the transcription corepressor Rcor1. By contrast, monocytic cell survival and self-renewal are dramatically enhanced in the absence of Rcor1. These findings provide new insights into the complexity of the transcription regulatory networks that regulate normal hematopoiesis. As *Rcor1*$^{-/-}$ mice develop a neutropenia and monocytosis, it will be important to determine whether RCOR1 contributes to pathogenesis in a wide spectrum of human myeloid disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance Statement

In vitro interactions between histone modifying enzymes and the transcriptional co-repressor Rcor1 suggest it has an important role in the epigenetic regulation blood cell development. This study provides the first in vivo evidence that Rcor1 is essential for the normal production of mature red blood cells and neutrophils but not platelets. Rcor1-deficient monocytes are increased in number, have an enhanced capacity to self-renew, but remain cytokine dependent and do not give rise to leukemia. Rcor1 loss also leads to the aberrant expression of transcription factors that normally promote the replication of hematopoietic stem/progenitor cells. These findings demonstrate that Rcor1 is essential for red cell and neutrophil maturation while restricting the self-renewal potential of monocytes.

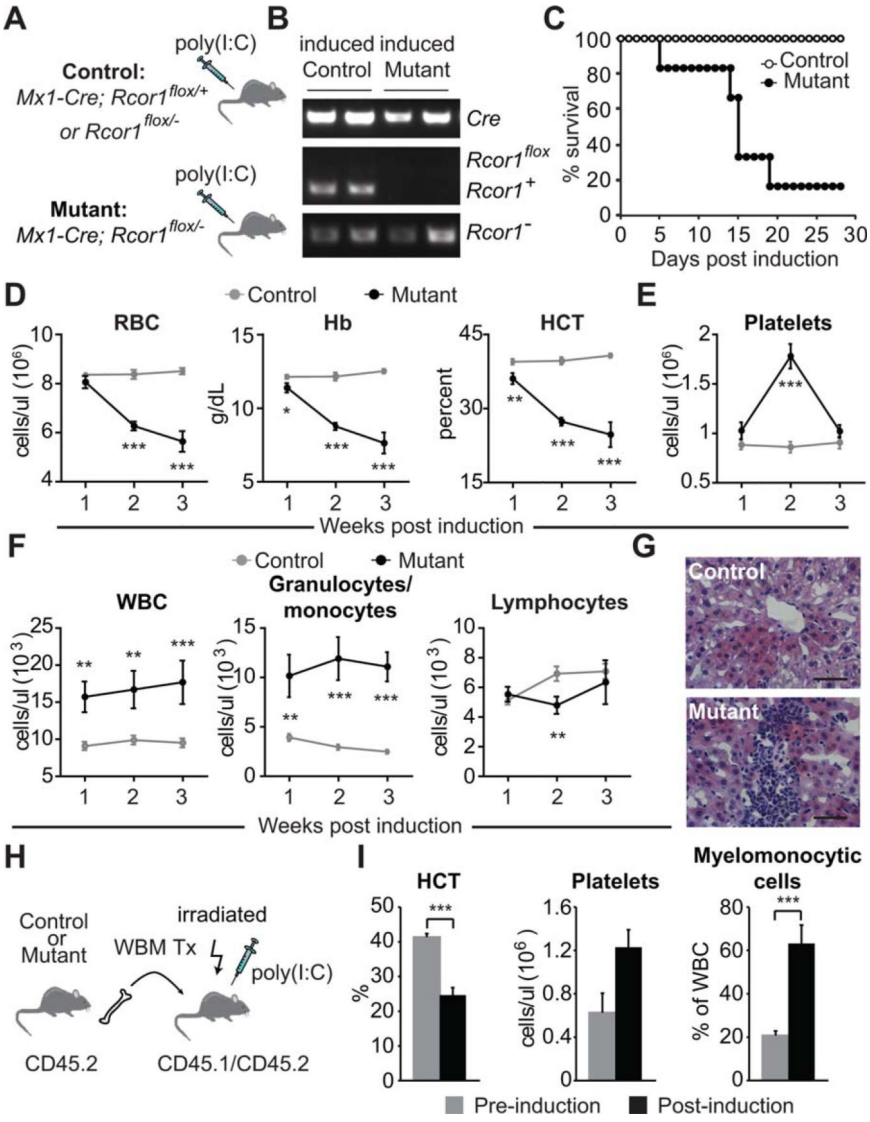

Figure 1.

Loss of *Rcor1* in adult mice causes a lethal anemia and the expansion of myelomonocytic cells. **(A):** Induction of Cre expression by injection of poly(I:C) in adult mice. **(B):** PCR genotype analysis of bone marrow (BM) cells 2 weeks after Cre induction. Only the $Rcor1^{-}$ allele was detected in the mutant BM. **(C):** Survival analysis after poly(I:C) treatment and *Rcor1* deletion ($n_{\text{mutant}} = 6$; $n_{\text{control}} = 10$). **(D):** Erythropoiesis in $Rcor1^{-/-}$ mice. Progressive reductions in circulating RBC number, serum Hb, and HCT were observed. **(E):** Rcor1-deficient mice had transiently elevated platelet counts after Cre induction. **(F):** Elevated circulating WBC in $Rcor1^{-/-}$ mice was the result of increased myelomonocytic cells. **(G):** Liver infiltration by myelomonocytic cells in $Rcor1^{-/-}$ mice. **(H):** Generation of BM-chimeric mice to exclusively delete *Rcor1* from hematopoietic cells. **(I):** Peripheral blood analysis of BM-chimeric mice before and 2 weeks after *Rcor1* deletion ($n = 3$). For (D–F), a minimum of seven mice for each genotype was tested at each time point. The average ±

SEM is shown; *, $p < .05$; **, $p < .01$; ***, $p < .001$. Scale bar = 50 μm. Abbreviations: Hb, hemoglobin; HCT, hematocrit; RBC, red blood cells;WBC, white blood cells.

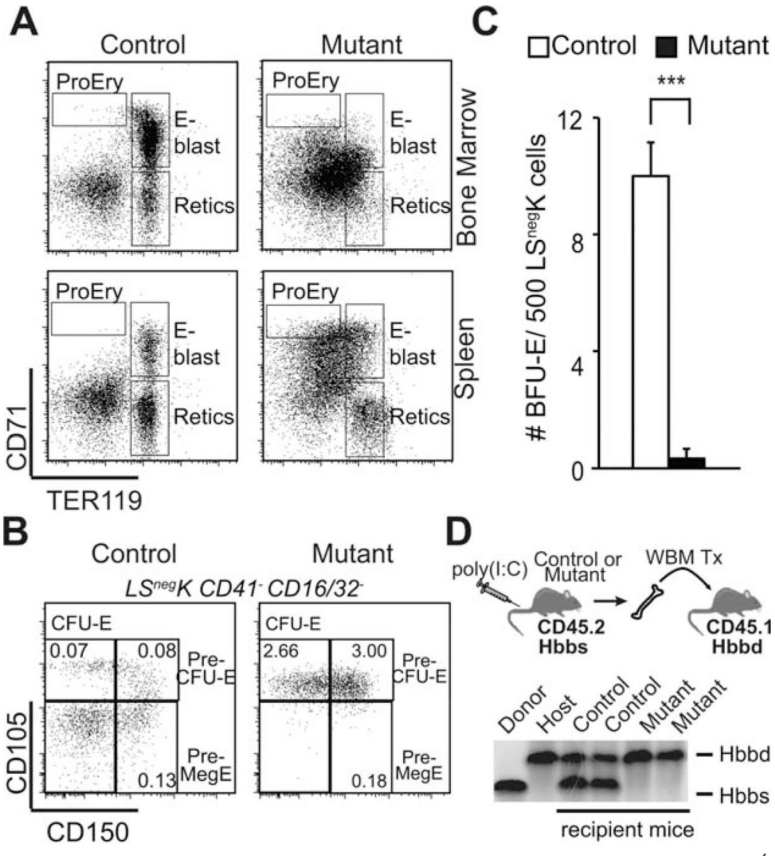

Figure 2.

Blocked erythroid lineage maturation in *Rcor1*$^{-/-}$ mice. **(A):** Flow cytometry analysis of developing red cells in the bone marrow (BM). Normal populations of maturing erythroid cells were not detected in mutant mice. **(B):** Flow cytometry analysis of BM progenitors by Pronk et al. [23]. Committed erythroid progenitors (CFU-E and Pre-CFU-E) were significantly expanded in *Rcor1*$^{-/-}$ mice ($n = 3$) relative to controls ($n = 3$), but bipotent erythroid and megakaryocyte progenitors (pre-MegE) were not. The frequency of each progenitor population in total BM is indicated. **(C):** Reduced erythroid colony activity in *Rcor1*$^{-/-}$ myeloerythroid progenitors (Lin^{-}Sca1^{-}c-kit^{+}, LS^{neg}K) (SEM is shown; ***, $p < .001$). Data from three independent experiments are shown. **(D):** Transplantation schema for assessing the RBC potential of *Rcor1*$^{-/-}$ BM cells in a wild-type hematopoietic microenvironment. Although PB donor cell analysis revealed a significant contribution of *Rcor1*$^{-/-}$ cells to circulating leukocytes in recipient mice 4 weeks post-transplant (not shown), hemoglobin derived from *Rcor1*$^{-/-}$ donor cells was below the level of detection. Abbreviations: BFU-E, burst forming unit-erythroid; CFU-E, colony-forming unit-erythroid; E-blast, erythroblasts; ProEry, proerythroblasts; Retics, reticulocytes.

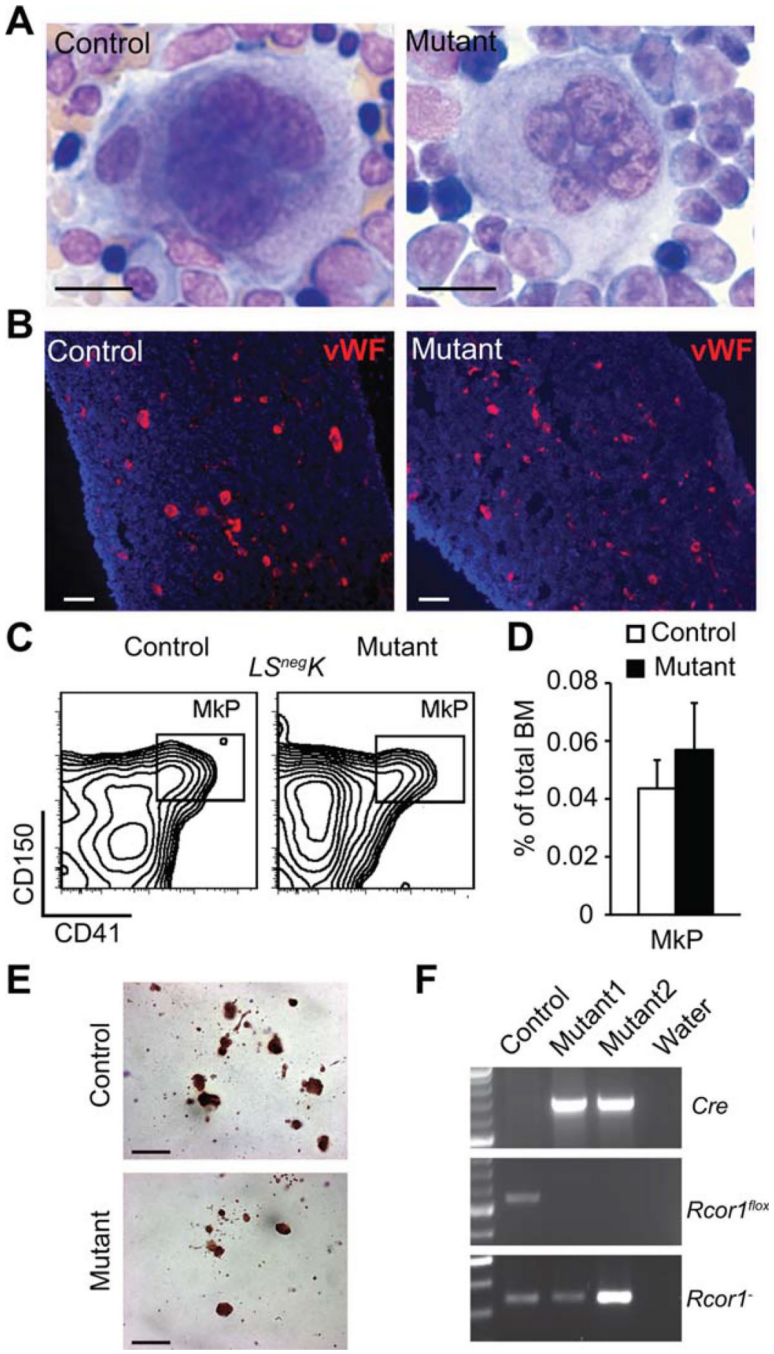

Figure 3.

Rcor1 is dispensable for thrombopoiesis. **(A):** May-Grunwald Giemsa stained bone marrow (BM) touch preparations revealed normal megakaryocyte morphology in *Rcor1*$^{-/-}$ mice. **(B):** vWF^{+} (red) megakaryocytes with a normal distribution in *Rcor1*$^{-/-}$ BM. Tibia sections were counterstained with 4′,6-diamidino-2-phenylindole (blue). **(C):** Flow cytometry analysis of MkP. **(D):** MkP frequency was not significantly altered in Rcor1-deficient BM ($n_{\text{mutant}} = 5$, $n_{\text{control}} = 3$). **(E):** Typical acetylcholinesterase^{+} CFU-Mk derived from control and mutant BM cells. **(F):** The *Rcor1*^{flox} allele was not detectable in sorted MkPs after Cre

induction. Scale bars = (A–B) 20 μm; (E) 100 μm. Abbreviation: MkP, megakaryocyte progenitors.

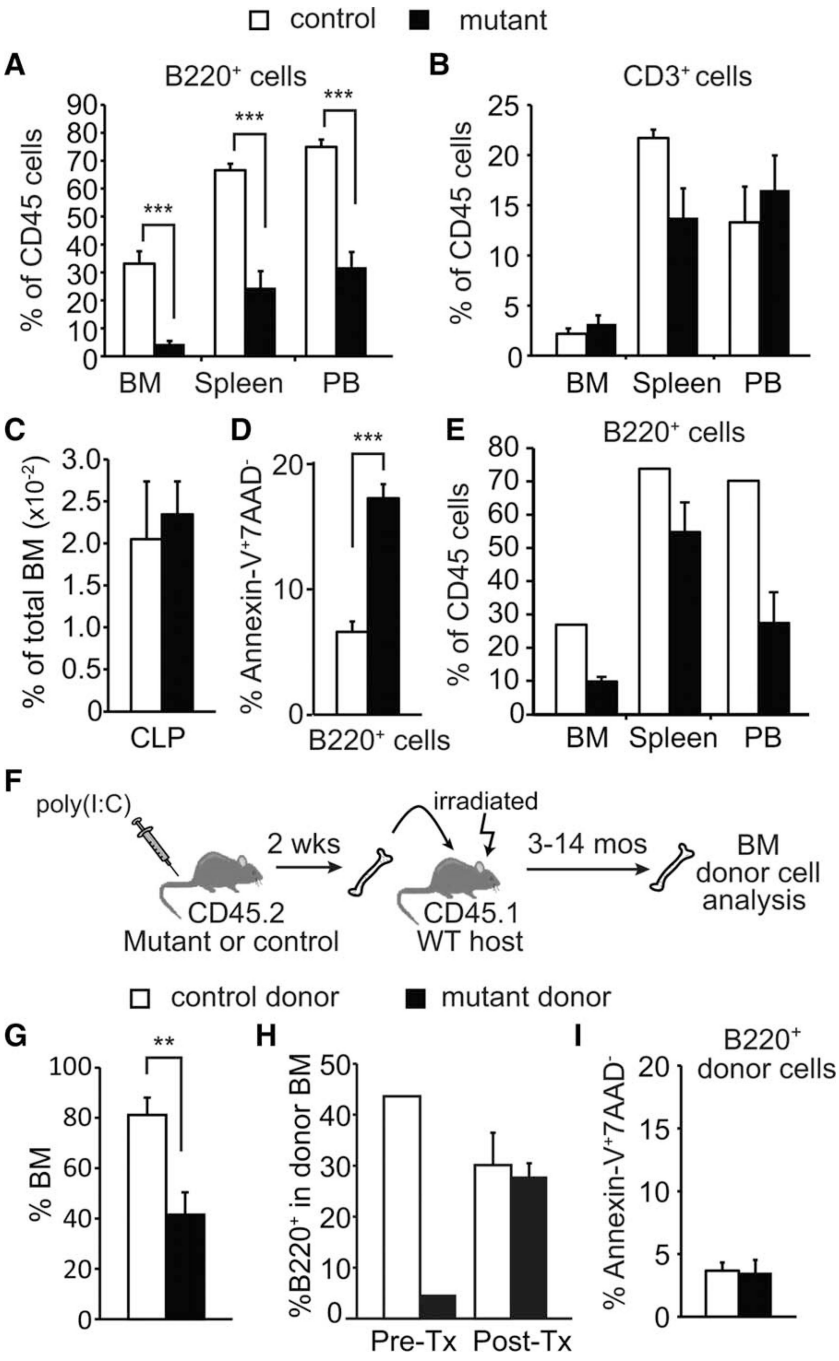

Figure 4.

Non-cell autonomous suppression of B-cell survival in Rcor1-deficient mice. **(A, B):** Deletion of *Rcor1* leads to a decreased number of B220$^+$ B cells in bone marrow, spleen, and peripheral blood, but does not significantly impact CD3$^+$ T cells (n_{mutant} = 6, $n_{control}$ = 5). **(C):** Normal frequency of CLP in *Rcor1*-deficient BM (n_{mutant} = 5, $n_{control}$ = 4). **(D):** Increased apoptosis in BM B cells following *Rcor1* deletion (n_{mutant} = 4, $n_{control}$ = 4). **(E):** Transplantation of *Mx1-Cre;Rcor1*$^{-/flox}$ BM into WT hosts followed by Cre induction (Fig. 1H for details) shows a reduced frequency of B cells in the BM, spleen, and PB after 2

weeks ($n_{\text{mutant cell recipients}} = 3$, $n_{\text{control cell recipients}} = 2$). **(F):** BM transplant schema. Donor WBM or sorted $Lin^{-}Sca1^{+}c\text{-}kit^{+}$ cells were transplanted into sublethally irradiated WT hosts. **(G):** Percent donor cell contribution in BM after transplant ($n_{\text{mutant cell recipients}} = 10$, $n_{\text{control cell recipients}} = 10$). **(H):** Representative donor cell contribution to B cells. Donor BM was analyzed for B-cell content prior to transplant (Pre-Tx) and 10 months post-transplant (Post-Tx). B cells derived from *Rcor1*$^{-/-}$ donor cells corrected to control levels following engraftment into WT hosts. **(I):** Apoptosis is not increased in *Rcor1*$^{-/-}$ donor $B220^{+}$ B cells post-transplant. Pooled data from three donors for each genotype are shown ($n_{\text{mutant cell recipients}} = 5$, $n_{\text{control cell recipients}} = 4$). SEM is shown; **, $p < .01$; ***, $p < .001$. Abbreviations: BM, bone marrow; CLP, common lymphoid progenitors; PB, peripheral blood; WBM, whole bone marrow.

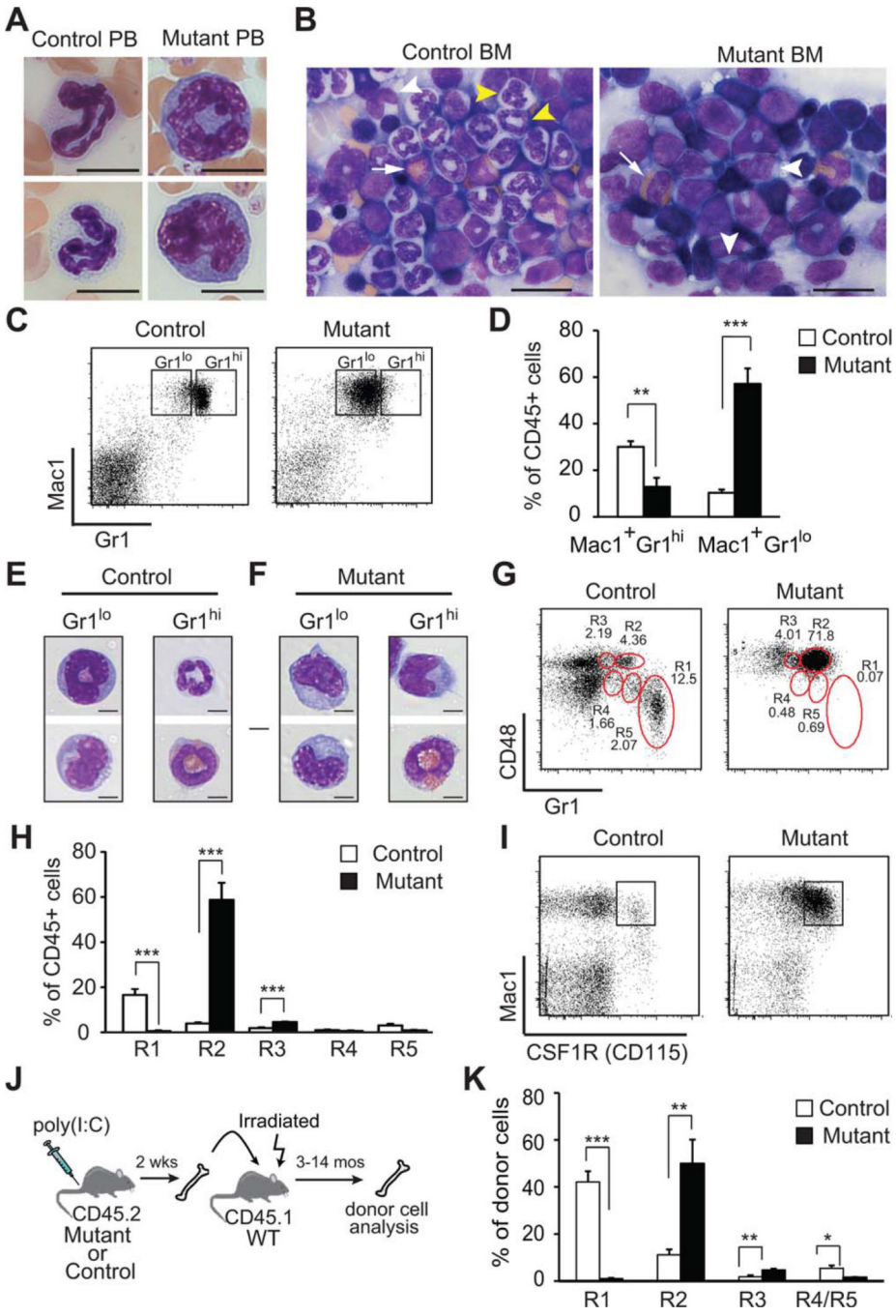

Figure 5.

Rcor1-deficiency produces a loss of neutrophils and increased numbers of monocytes. May-Grunwald Giemsa stained **(A)** blood smears and **(B)** BM touch preparations. Maturing and mature neutrophils in PB and the BM (yellow arrow heads) were absent from *Rcor1*$^{-/-}$ mice, whereas monocytes (white arrow head) and eosinophils (white arrow) were present. **(C):** Mac1 and Gr1 expression on bone marrow cells. **(D):** A significant reduction in the proportion of Mac1^{+}Gr1^{hi} cells and a significant increase in the proportion of Mac1^{+}Gr1^{lo} cells were observed in Rcor1-deficient BM (n_{mutant} = 9, $n_{control}$ = 9). **(E, F):** Morphology of

sorted myelomonocytic cells from control (E) and Rcor1-deficient (F) BM. Most *Rcor1*$^{-/-}$ cells were monocytes. **(G):** Phenotype of myelomonocytic cells mice based on CD48 and Gr1 expression patterns. **(H):** Although Rcor1-deficient mice lacked mature granulocytes (R1), they retained granulocytic precursor cells (R4, R5) and had significantly increased monocytic (R2) and bipotential (R3) cell populations relative to control mice, (n_{mutant} = 4, $n_{control}$ = 4). **(I):** Consistent with their monocytic phenotype, most *Rcor1*$^{-/-}$ Mac1^{+} cells coexpressed CSF1R. (J-K) *Rcor1*$^{-/-}$ donor cells maintained their abnormal monocytic phenotype after transplant into wild-type hosts (n_{mutant} = 5, $n_{control}$ = 5). SEM is shown; **, $p < .01$; ***, $p < .001$. Scale bars = 10 μm (A), 20 μm (B), and 5 μm (E, F). Abbreviations: BM, bone marrow; PB, peripheral blood.

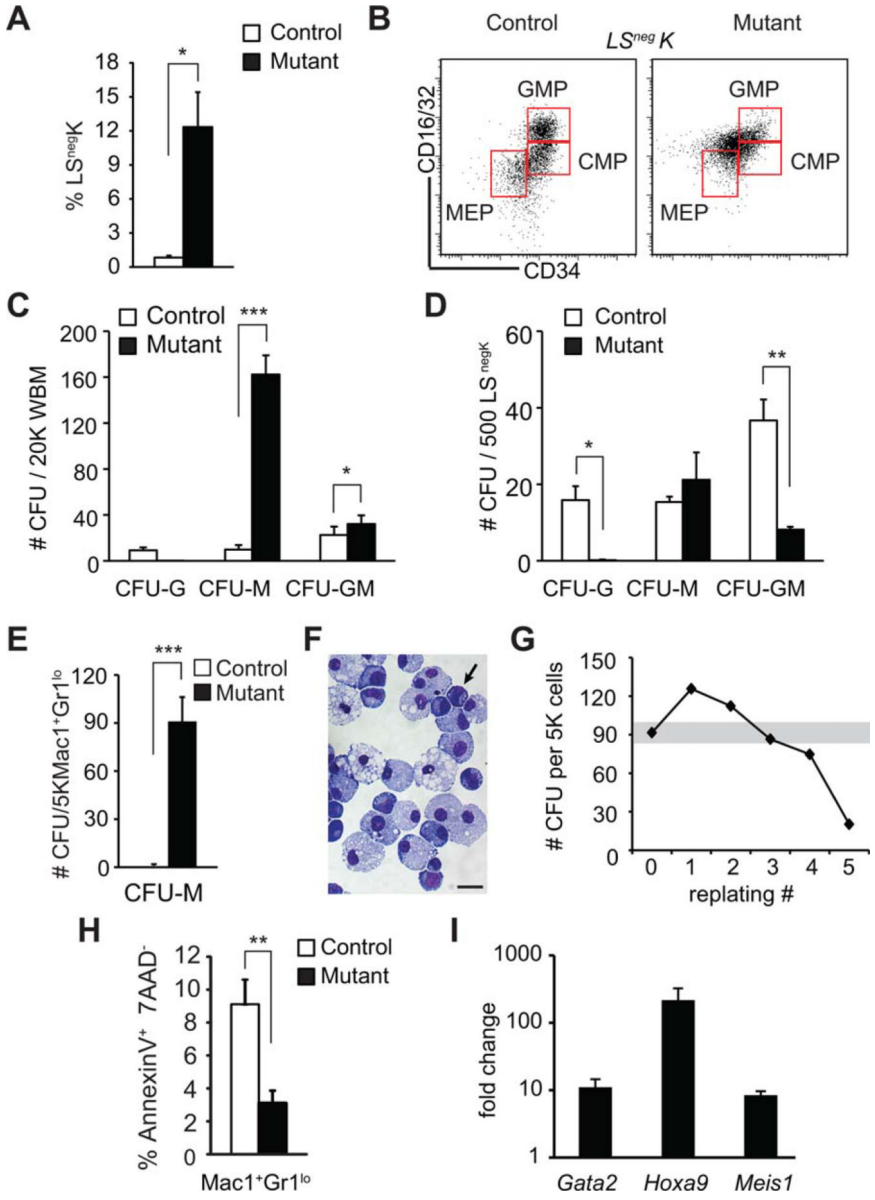

Figure 6.

Enhanced self-renewal activity in Rcor1-deficient monocytic cells. **(A):** Expansion of Lin^{-}Sca1^{-}ckit^{+} (LS^{neg}K) myeloerythroid progenitor cells in *Rcor1*$^{-/-}$ bone marrow (BM) (n_{mutant} = 5, $n_{control}$ = 4). **(B):** Aberrant CMP/GMP/MEP progenitor [24] fluorescence-activated cell sorting profile in mutant mice. **(C, D):** Myelomonocytic progenitor colony-forming activity in: (C) unfractionated BM; (D) sorted LS^{neg}K cells; **(E)** sorted Mac1^{+}Gr1^{lo} cells. Pooled results from three independent experiments are shown. **(F):** May-Grunwald Giemsa stained cells from pooled *Rcor1*$^{-/-}$ colonies shown in (E). Immature cells similar to the Mac1^{+}Gr1^{lo} cells directly isolated from mutant mice persist in culture (arrow). **(G):** CFU activity following serial replating every 8 days. Mac1^{+}Gr1^{lo} cells from *Rcor1*$^{-/-}$ mutants produced stable levels of CFU-activity through four of five serial replatings. **(H):** Decreased apoptosis in Mac1^{+}Gr1^{lo} cells in *Rcor1*$^{-/-}$ BM (n_{mutant} = 5, $n_{control}$ = 4). **(I):** *Gata2*, *Hoxa9*,

and *Meis1* are derepressed in Rcor1-deficient $Mac1^{+}Gr1^{lo}$ cells. Pooled results from three to five qRT-PCR analyses are shown. SEM is indicated, *, $p < .05$; **, $p < .01$; ***, $p < .001$. Scale bar = 10 μm. Abbreviations: CFU, colony-forming unit; CMP, common myeloid progenitors; GMP, granulocyte-monocyte progenitors; MEP, megakaryocyte-erythroid progenitors.