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ORIGINAL ARTICLE

USB1 deficiency disrupts neutrophil maturation via RNA dysregulation independent of global pre-mRNA splicing

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Abstract

Background: U6 biogenesis 1 (USB1) gene mutations cause poikiloderma with neutropenia (PN), which is clinically characterized by skin hyperpigmentation, nail dysplasia, neutropenia, and an elevated risk of cancer. USB1 functions as an RNA exonuclease involved in RNA maturation and stability regulation, although its precise mechanism of action in the hematopoietic system remains unclear.

Methods: We established a myeloid cell-specific USB1 knockout mouse model (USB1fl/fl-Lyz2-cre) using CRISPR/Cas9. Using a combination of research methods, including Western blot, flow cytometry, messenger RNA (mRNA) sequencing, microRNA (miRNA) sequencing, and quantitative polymerase chain reaction (qPCR), we investigated the effects of USB1 deficiency on neutrophil development and differentiation, along with the underlying signaling molecular mechanisms.

Results: Experimental results indicated that USB1 deficiency in mouse myeloid cells not only leads to dysregulation of miRNA expression but also interferes with the developmental, differentiation, and maturation processes of neutrophils by affecting key genes, such as IL1a, Selp, and Kilt. This impact can be traced back to the stages of myeloid progenitor cells.

Conclusions: USB1 influences neutrophil maturation and myeloid progenitor cell differentiation by regulating the expression of specific miRNAs and mRNAs. This provides novel in vivo experimental evidence for understanding the pathogenesis of PN in patients.

KEYWORDS
microRNA, myeloid knockout mice, neutrophil maturation, USB1

1 | INTRODUCTION

Poikiloderma with neutropenia (PN) is caused by mutations in the human C16orf57 gene. This gene encodes USB1, a highly conserved protein involved in U6 small nuclear RNA (snRNA) biogenesis.[1–4] It is a rare autosomal recessive genetic skin disorder characterized by skin hypopigmentation, nail dystrophy, short stature, pulmonary disease, and neutropenia.[5,6] Individuals with this condition have a pronounced tendency to develop malignancies, with a notably increased risk of acute myeloid leukemia and myelodysplastic syndrome.[7]

USB1 is a 3′ to 5′ RNA exonuclease whose core function is to regulate RNA maturation and stability. It was initially discovered to act during the maturation of U6 snRNA, a key component of the spliceosome responsible for excising introns from precursor mRNA (pre-mRNA).[8–10] USB1 precisely trims excess uridine residues from the 3′ terminus of U6 snRNA and catalyzes the formation of a terminal 2′,3′-cyclophosphate group. This facilitates the binding of Lsm2-8 proteins, ultimately forming functional U6 snRNA-nucleoprotein complexes.[11] Mutations in this protein cause abnormal myelopoietic function in patients with PN, impairing neutrophil production and function.[12] Furthermore, previous studies have indicated that zebrafish models lacking USB1 exhibit splicing defects and reduced expression of abnormal transcripts.[13] This not only leads to developmental abnormalities but also induces neutropenia, consistent with the clinical features of the human hereditary disorder PN.[14] In contrast, Hilcenko et al.[6] found that lymphoblastoid cells derived from PN patients exerted only a limited effect on intracellular U6 snRNA homeostasis and overall pre-mRNA splicing efficiency. Moreover, Jeong et al.[5] discovered that the primary mechanism by which USB1 regulates hematopoietic function does not involve U6 snRNA or global splicing, but rather functions as a microRNA (miRNA) deadenylase.

Consequently, current research has primarily focused on how USB1 deficiency leads to defective splicing of precursor mRNAs and miRNA instability, subsequently triggering hematopoietic developmental abnormalities across multiple species, including zebrafish and yeast. Although USB1 gene mutations have been identified as the genetic cause of bone marrow failure syndrome, the precise molecular mechanisms by which they affect neutrophil development, differentiation, maturation, cell death, and homeostasis, as well as the specific pathways that induce physiological dysfunction, require further investigation. To investigate USB1's physiological role, we employed CRISPR/Cas9 technology to establish a myeloid cell USB1 knockout mouse model. This model aims to elucidate the molecular mechanisms of USB1 in neutrophil development, differentiation, and maturation, providing a theoretical foundation for future clinical studies.

2 | MATERIALS AND METHODS

2.1 | Mice

$USB1^{fl/fl}$ and Lyz2-Cre+ mice were developed in collaboration with the Shanghai Southern Model Organism Technology Co., Ltd. $USB1^{fl/fl}$ mice contained two LoxP sites flanking the third exon, and all mice had a C57BL/6J background. All experimental mice were 16 weeks old.

2.2 | Western blot

Bone marrow (BM), liver, thymus, and kidney tissue samples were collected from mice and homogenized using RIPA lysis buffer (P0013B; Bristol-Myers Squibb). The lysates were separated by electrophoresis, with 40 μg of protein loaded per lane. Proteins were subsequently transferred onto polyvinylidene fluoride (PVDF) membranes for Western blot analysis. After treatment with the blocking solution, the required primary antibody and species-matched secondary antibody (925-32211, 926-68072; LI-COR) were applied sequentially. Detection and imaging were performed using the Odyssey CLx system, with quantitative analysis of band intensities performed via Image Studio software (version 5.2).

2.3 | Flow cytometry analysis

The spleen and thymus tissues were ground using glass slides to obtain tissue suspensions. The BM was ground using a grinding rod and washed to obtain a BM suspension. Peripheral blood was collected in a tube containing ethylenediaminetetraacetic acid (EDTA). The following antibody cocktail was employed for staining immune cell populations in BM, thymus, spleen, and peripheral blood: staining was performed using a mouse antibody cocktail comprising APC-CD8, PE-CD45, PE/Cy7-B220, Percp/Cy5.5-CD3, APC/Cy7-CD11b, and FITC-CD4. Analysis of B-cell developmental stages: the B-cell developmental stages were analyzed using a combination of FITC-IgD, PE-CD43, PE-Cy7-B220, and APC-IgM antibodies. Hematopoietic stem cell and lineage differentiation analysis BM cells were first labeled with a biotinylated mouse lineage antibody cocktail, followed by secondary staining with APC/Cy7-streptavidin conjugated to multiple progenitor/stem cell antibodies, including PE-FIt3, PE-CD16/32, PE/Cy7-Sca1, APC-cKit, FITC-CD34, and Percp/Cy5.5-CD127. Neutrophil maturation and differentiation analyses: after CD16/32 antibody-mediated purification, BM cells underwent multicolor antibody staining using combinations of FITC-CD90/B220/NK1.1, BV421-Gr-1, Percp/Cy5.5-CD115/CD3, APC-c-Kit, PE-CXCR2, APC/Cy7-CD11b, and PE/Cy7-CXCR4. Peripheral blood cell counts in mice were analyzed at ILAS (China). The details of all antibodies used are provided in Table S1.

2.4 | RNA-seq and miRNA-seq library preparation and analysis

Myeloid progenitor cells were isolated from the BM using the CD117 (cKIT) Positive Selection Kit (18757; StemCell). Shanghai Meiji Biotechnology Co., Ltd. constructed RNA and miRNA sequencing libraries. Transcriptome sequencing yielded 41.75 Gb of clean data, with each sample exceeding 6.34 Gb of clean data and a Q30 base percentage of above 94.51%. In total, 26 844 genes were detected, comprising 79 408 transcripts. miRNA sequencing yielded 76.12 M raw reads, with each sample exceeding 11.55 M raw reads and a Q30 base percentage above 96.52%. Intergroup differential analysis was performed using DESeq2 software.

For transcriptome sequencing, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses employed a hypergeometric distribution algorithm to identify function categories significantly enriched within gene sets. Enrichment was considered significant when the Benjamini–Hochberg-corrected *p*-value (*p*adjust) was <0.05. The protein–protein interaction (PPI) network of the target gene was analyzed using STRING database. Variable splicing analysis was performed using the rMATS software. For miRNA sequencing, StarBase, miRWalk, and miRTarBase were used to predict miRNA target genes, followed by functional annotation within the KEGG database. R language scripts were used to perform KEGG analyses of the miRNA target genes within the gene sets. Calculations were conducted using Fisher's exact test, with significant enrichment deemed present when the *p*-value (*p*adjust) was ≤0.05.

2.5 | Real-time PCR

c-Kit$^+$ BM suspensions were sorted and centrifuged, and RNA was extracted using TRIzol reagent (15596026CN, Thermo Fisher Scientific), chloroform, isopropanol, and other reagents. Reverse transcription was performed using the PrimeScript Kit (RR036A; Takara). Experimental reactions were conducted using a PCR instrument and TB Green Reagent Kit (RR820A; TaKaRa). The sequences of all qPCR primers are listed in Table S2.

2.6 | Statistical analysis

FlowJo software was used to analyze the flow cytometry (FC) data, and GraphPad Prism software (version 8) was used for statistical data processing. Continuous data are presented as the mean ± standard deviation (SD). Intergroup differences were considered statistically significant at $^*p < 0.05$, denoted as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Before comparative analysis, data distribution was assessed for normality (Shapiro–Wilk test) and homogeneity of variance (*F*-test). Based on these assessment results, a paired two-tailed *t*-test was employed for data meeting parametric testing criteria; otherwise, the nonparametric Mann–Whitney *U* test was applied.

3 | RESULTS

3.1 | USB1 myeloid knockout mice were successfully generated using the CRISPR/Cas9 system

Patients with PN present with severe, noncyclic neutropenia. Therefore, to investigate the role of USB1 deficiency in myeloid cells, we generated a myeloid cell-specific USB1 knockout mouse model (USB1$^{fl/fl}$-Lyz2) using the Cre-LoxP system (Figure 1A). Subsequently, we confirmed the genotyping at the DNA level and verified the knockout efficiency using Western blot. USB1 expression in the BM

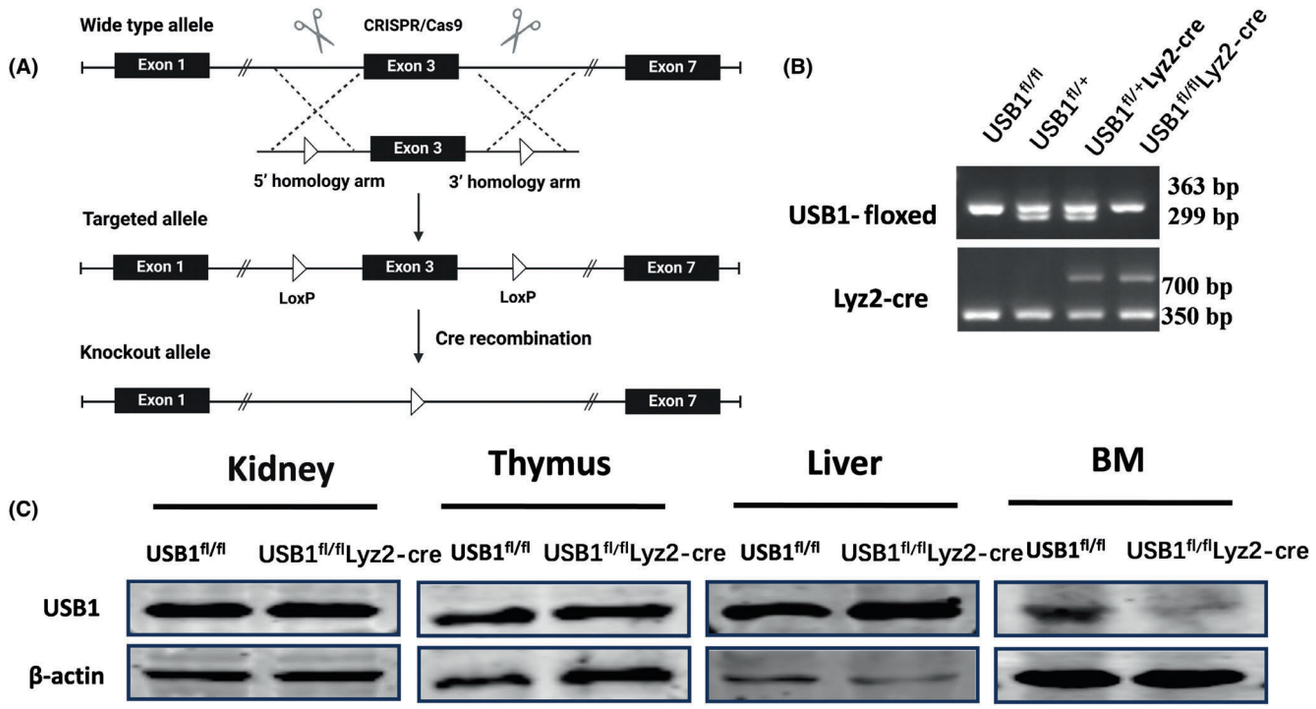

FIGURE 1 A myeloid-specific USB1 knockout mouse model has been successfully established. (A) Construction of a mouse model with myeloid cell-specific USB1 deficiency using the Cre-loxP system. Figure was created using BioRender. (B) Gel electrophoresis for genotyping. (C) Western blot (WB) experiments validated the expression of USB1 protein in the bone marrow (BM) and other mouse tissues.

was significantly reduced at the protein level compared to that in other major organs (Figure 1B,C).

3.2 | USB1 deficiency results in a reduced proportion of mature neutrophils accompanied by compensatory proliferation of B cells

To assess the impact of USB1 myeloid cell-specific knockout on the differentiation hierarchy, we employed the USB1$^{fl/fl}$-Lyz2 mouse model to evaluate its effects on terminally differentiated cells in vivo. Peripheral blood analysis revealed a markedly reduced proportion of neutrophils in USB1$^{fl/fl}$-Lyz2 mice, whereas the proportions of monocytes, eosinophils, and basophils exhibited a downward trend, although this trend was not statistically significant (Figure 2A). FC analysis revealed markedly reduced myeloid cell proportions in the peripheral blood and BM of USB1$^{fl/fl}$-Lyz2 mice, along with increased B-cell and T-lymphocyte proportions in the BM and peripheral blood, respectively. Furthermore, no significant alterations were observed in the proportion of terminally differentiated cells in the spleen (Figure 2B). T cells originate as precursor T cells, primarily produced in the BM (or fetal liver), which then enter the thymus and undergo multiple highly regulated stages of development until maturation.[15,16] In this study, although an increased proportion of T lymphocytes was observed in the peripheral blood, FC analysis of the thymus revealed no significant alterations in the proportion of T cells at any developmental stage (Figure 2C). Subsequent analysis of neutrophil subsets revealed a reduced overall proportion of BM neutrophils in USB1$^{fl/fl}$-Lyz2 mice, characterized by increased immature neutrophils and decreased mature neutrophils (Figure 3A). This suggests that USB1 plays a crucial role in the regulation of neutrophil maturation. Notably, despite achieving myeloid cell USB1 gene knockout in this model, we observed an elevated proportion of B cells at different developmental stages within the lymphoid lineage following further subpopulation analysis of B cells in the BM (Figure 3B). Based on this, we hypothesized that myeloid-specific USB1 deficiency may lead to a differentiation bias in hematopoietic progenitor cells, directing them toward the lymphoid lineage (B-cell lineage).

3.3 | USB1 deficiency in myeloid cells results in the suppression of differentiation in their upstream myeloid progenitor cells

We observed a reduction in the proportion of myeloid cells following USB1 myeloid knockout, which was consistent with the anticipated outcome. However, we also noted an increase in the proportion of B cells. Consequently, we need to further investigate whether USB1 knockout in myeloid cells induces a differentiation bias or impairment in upstream myeloid progenitor cells. Therefore, we devised a FC gating strategy for the upstream progenitor cells. Results indicate that the proportion of hematopoietic stem cells (HSCs) and their downstream common lymphoid progenitor cells (CLP) showed no significant alteration (Figure 3C). Therefore, USB1 myeloid knockout did not result in a bias toward lymphoid differentiation of hematopoietic progenitor cells, ruling out the possibility that it induces B-cell expansion by influencing the fate decision of early progenitor cells. USB1 deficiency may affect the later stages of B-cell differentiation. Furthermore, the experiments demonstrated a significant reduction in the proportion of myeloid progenitor cells (MP), common myeloid progenitor cells (CMP), and granulocyte-macrophage progenitor cells (GMP) (Figure 3C). Yue et al.[17] also established a mouse model with HAX1 gene-specific deletion in myeloid cells and similarly observed reduced differentiation capacity in myeloid progenitor cells and inhibition of GMP differentiation. Additionally, Richardson et al.[18] employed Lyz2-Cre to generate an ERK myeloid cell knockout mouse model and observed an increased proportion of neutrophils and upstream myeloid progenitor cells. Similarly, our research indicates that USB1-specific knockout in myeloid cells may suppress the differentiation capacity of upstream myeloid progenitor cells, ultimately leading to a reduced neutrophil proportion. Despite a reduced proportion of megakaryocyte–erythrocyte progenitor cells (MEPs) detected by FC (Figure 3C), peripheral blood analysis revealed that platelet and red blood cell counts remained within the normal range (Figure 2A).

3.4 | USB1 deficiency affects mRNA and miRNA levels in myeloid progenitor cells

Previous studies have indicated that USB1 mutant cells do not exhibit global alterations in precursor mRNA splicing. However, USB1 may influence hematopoietic processes by modifying other RNAs.[5] To further investigate how USB1 affects neutrophil development and maturation and to trace disease mechanisms back to their developmental origins, we sought to elucidate the molecular mechanisms underlying differentiation defects in the early stages. Therefore, we selected myeloid progenitor cells, whose proportion was significantly reduced in USB1 myeloid-deficient mice, for RNA and microRNA sequencing. We isolated c-kit$^+$ cells from mouse BM. The results demonstrated that the purity of c-kit$^+$ cells reached 82.1%, with the proportion of the MP cell population accounting for 68.62% (Figure 4A). Therefore, this method can effectively yield MP cells. Furthermore, we observed that USB1 gene expression in c-kit$^+$ cells of knockout mice was significantly downregulated but not entirely absent (Figure 4B). microRNA sequencing revealed significant alterations in miRNA levels within myeloid progenitor cells, with 36 miRNAs exhibiting altered expression (28 downregulated and 9 upregulated; $p < 0.05$). We identified specific miRNAs (miR-9-5p/3p, miR-188-5p, miR-335-3p, and miR-144/451a) exhibiting significantly altered expression levels (Figure 4C). Previous studies have demonstrated that miR-9-5p is involved in the activation of neutrophil NF-κB and JAK2/STAT3 signaling pathways.[19] Separately, miR-188-5p and miR-335-3p contribute to the regulation of acute myeloid leukemia.[20] Furthermore, miR-144/451a plays a critical role in erythrocyte development and maturation.[21] KEGG annotation and enrichment analysis revealed that miRNAs exhibiting significant differential

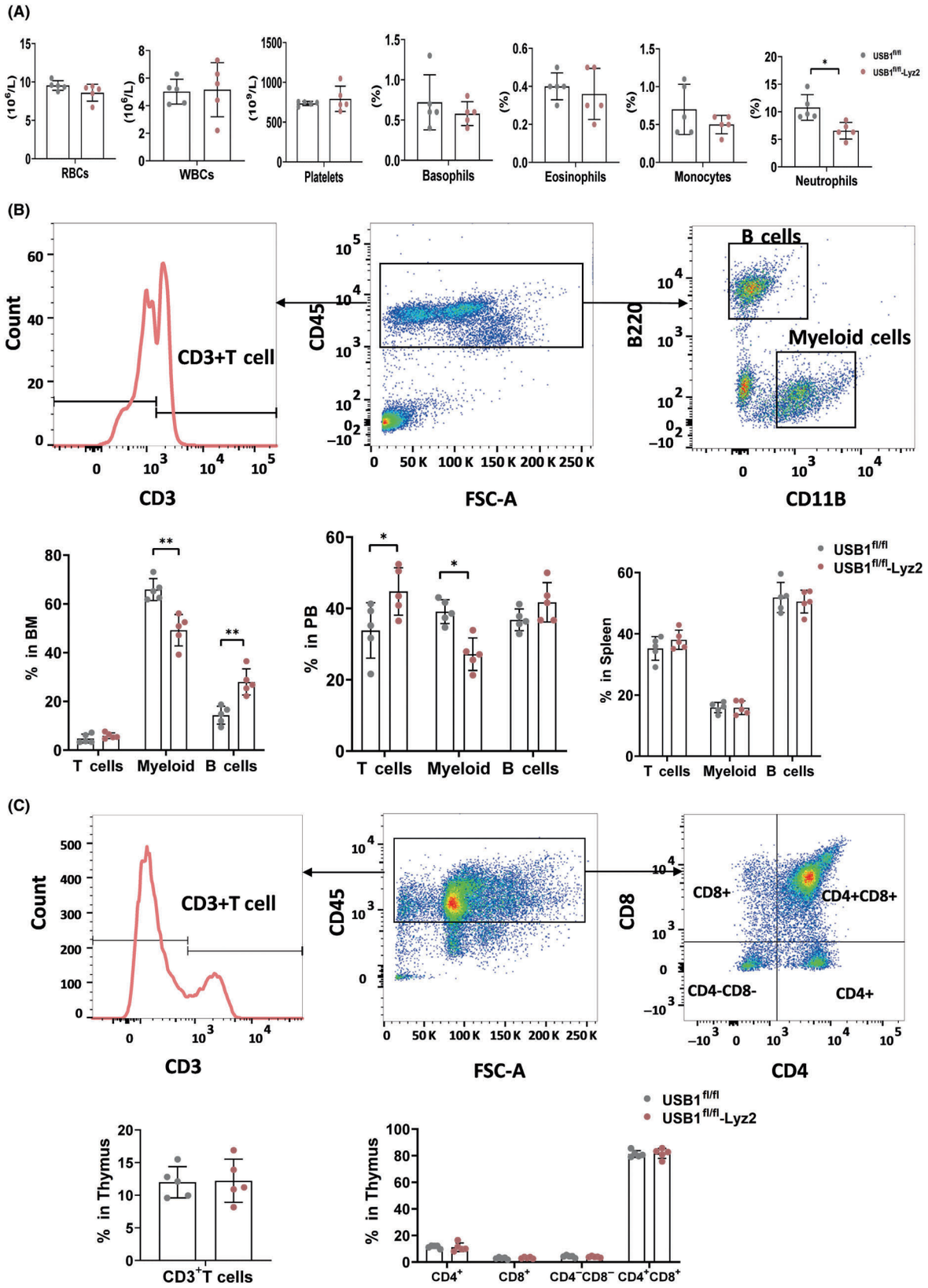

FIGURE 2 USB1 deficiency in myeloid cells leads to changes in the proportion of terminally differentiated cells. (A) Peripheral blood mononuclear cells, eosinophils, basophils, and neutrophils ratio in complete blood count ($N = 5$). (B) Flow cytometry (FC) analysis of the proportion of immune cell populations in mouse bone marrow (BM), peripheral blood, and spleen ($N = 5$). (C) FC analysis of the proportion of T cells at different stages of development in the thymus. The trials used mice aged 16 weeks ($N = 5$). Data are presented as mean ± standard deviation (SD). $^{*}p < 0.05$; $^{**}p < 0.01$.

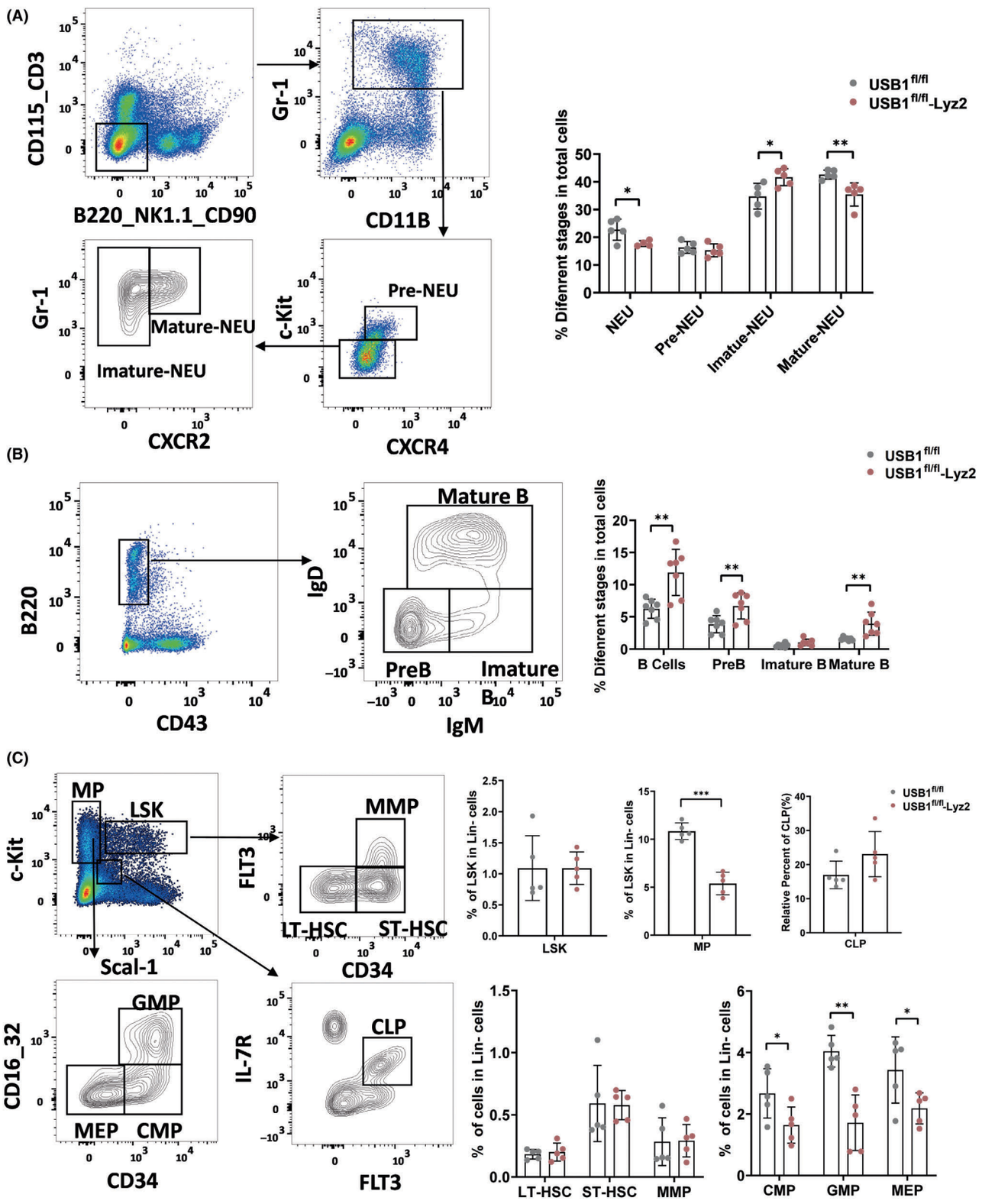

FIGURE 3 USB1 deficiency in myeloid cells leads to the inhibition of differentiation in their upstream myeloid progenitor cells. (A) Flow cytometry (FC) analysis of neutrophils at different developmental stages in the bone marrow (BM) of mice ($N=5$). (B) B-cell subpopulation analysis based on FC ($N=7$). (C) FC analysis of the proportion of hematopoietic stem cells (HSCs) and their downstream hematopoietic progenitor cells in mouse BM ($N=5$). Data are presented as mean ± standard deviation (SD). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. CLP, common lymphoid progenitors; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor cells; LSK, Lin−Sca1+c-Kit+; LT-HSC, long-term hematopoietic stem cells; MEP, megakaryocyte–erythroid progenitor cells; MP, myeloid progenitor; MPP, multipotent progenitor; NEU, neutrophil; ST-HSC, short-term hematopoietic stem cells.

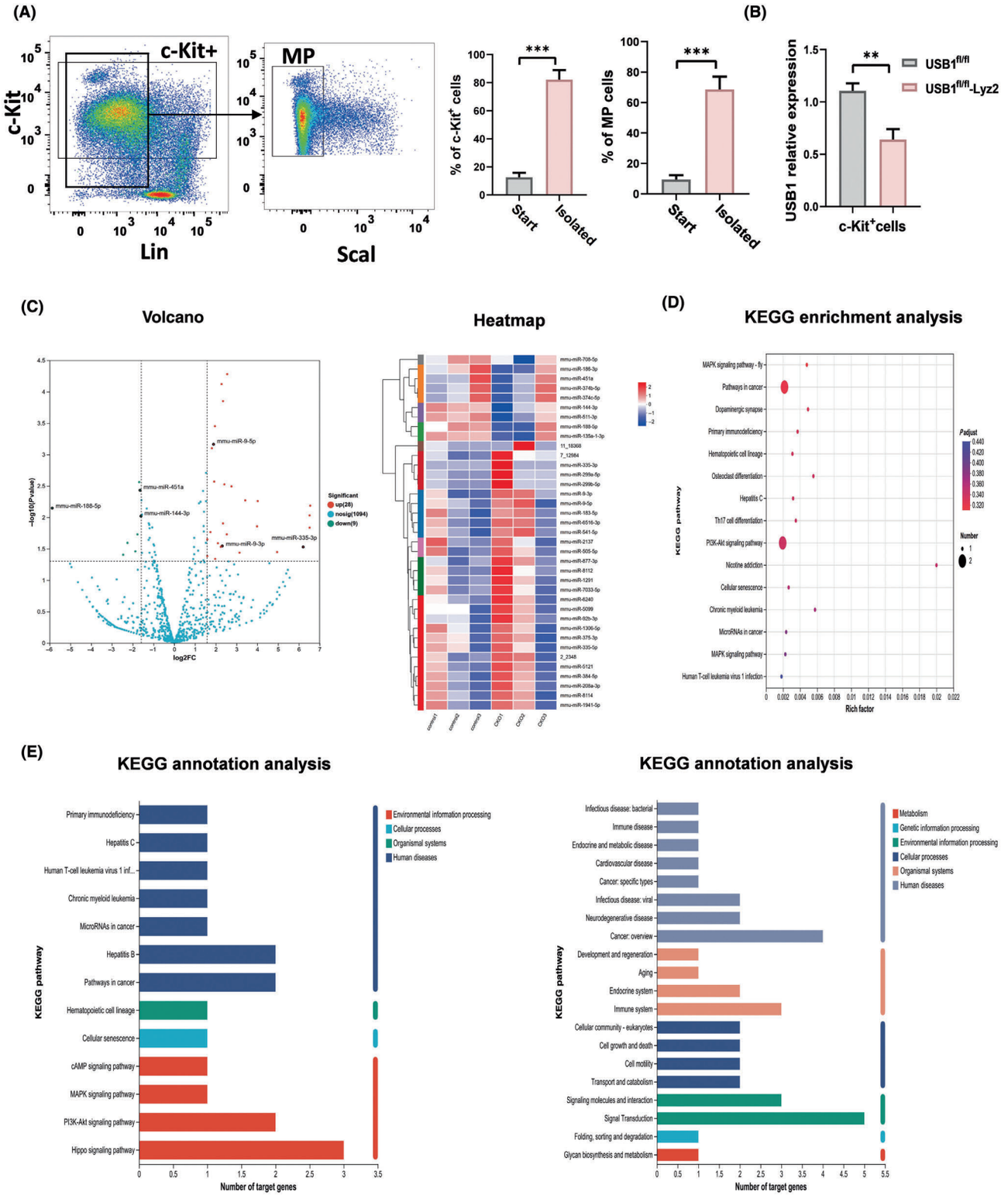

FIGURE 4 USB1 deficiency affects microRNA (miRNA) levels in myeloid progenitor cells. (A) Frequency of c-Kit$^+$ cells and their myeloid progenitor cells enriched by immunomagnetic beads. c-Kit$^+$ cells were isolated from mouse bone marrow (BM) and analyzed by flow cytometry (FC)for purity and subpopulations ($N = 3$). (B) Reverse transcription polymerase chain reaction (RT-PCR) analysis of USB1 messenger RNA (mRNA) levels in c-Kit-positive BM cells. Data are presented as mean ± standard deviation (SD). $^{**}p < 0.01$; $^{***}p < 0.001$. (C) Volcano plot (left) and clustered heatmap (right) of differentially upregulated and downregulated miRNAs ($N = 3$). (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of miRNAs in c-Kit-positive BM cells. (E) KEGG annotation analysis of miRNAs in c-Kit BM cells. Left: Secondary classification of pathways. Right: Three-level classification of pathways.

expression ($p < 0.05$) in USB1$^{fl/fl}$-lyz2 mice primarily impacted pathways associated with hematopoiesis and cancer progression, such as hematopoietic cell lineages and chronic myeloid leukemia, which are frequently implicated in PN (Figure 4D,E).

Differentially expressed genes (DEGs) between control (USB1$^{fl/fl}$) and CKO (USB1$^{fl/fl}$-lyz2) mice were analyzed using transcriptome sequencing. Compared to the USB1$^{fl/fl}$ group, the USB1$^{fl/fl}$-lyz2 group exhibited marked disparities between upregulated and downregulated genes, with 101 upregulated and 97 downregulated genes (Figure 5A). GO enrichment analysis revealed that the top 20 enriched gene sets predominantly involved immunoglobulin (Ig) production, immune response, immune system processes, B-cell receptor signaling pathways, and B-cell activation. These enriched gene sets largely participated in B-cell development, maturation, and immune response, with the genes primarily being members of the Ighv and Igkv families (Figure 5B). Research has indicated that cross-reactivity exists between neutrophils and B cells in the BM.[22] Therefore, we hypothesized that lineage plasticity exists between the B-cell and neutrophil lineages in the context of USB1 myeloid gene knockout-induced neutropenia. This finding qualitatively explains the significant enrichment of specific Ig genes in the transcriptome in the current study.

Transcriptome KEGG analysis revealed that DEGs were significantly enriched in pathways including "hematopoietic cell lineage," "necroptosis," "neutrophil extracellular trap formation," and "cellular senescence" (Figure 5C). IL5ra, Kilt, and IL1a are associated with "hematopoietic cell lineages," whereas processes such as "necrotic apoptosis" and "cellular senescence" involve genes, including IL1a, Camk2b, H2ac10, and Slc25a31. Furthermore, Selp, H2ac10, and Slc25a31 were highlighted in pathways associated with "neutrophil extracellular trap formation." We validated the enriched genes using reverse transcription polymerase chain reaction (RT-PCR), confirming consistency with the sequencing results (Figure 5D). Subsequently, to investigate the key downstream targets regulated by USB1 during neutrophil development and differentiation, we performed PPI analysis of these genes. The results indicated the existence of protein interactions between IL1a, Selp, and Kilt (Figure 5E). IL1a was enriched in "necrotic apoptosis," "cellular senescence," and "hematopoietic cell lineages." The pro-inflammatory cytokine Selp encodes P-selectin, a gene critical for early neutrophil recruitment that acts synergistically with E-selectin.[23] Kilt is indispensable for the self-renewal and differentiation of HSCs, promoting neutrophil maturation by enhancing their responsiveness to granulocyte-macrophage colony-stimulating factor (G-CSF).[24–26] Consequently, we hypothesized that USB1 deficiency may alter the expression of these genes, thereby influencing neutrophil development, differentiation, and maturation.

Moreover, a significant finding indicated that only 1% of genes exhibited global splicing alterations in the USB1$^{fl/fl}$-lyz2 group compared to the USB1$^{fl/fl}$ group (Figure 5F). Subsequently, we performed a Venn analysis of alternative splicing genes and DEGs, identifying only one gene that exhibited alternative splicing (Figure 5G). Therefore, only a small number of DGEs exhibited splicing abnormalities in USB1$^{fl/fl}$-lyz2 mice. Although Prakash Patil et al.[13] proposed in zebrafish models that USB1 influences neutrophil development by regulating tissue-specific alternative splicing, our mouse model studies indicate that the core mechanism by which USB1 regulates neutrophils does not operate through global splicing. This finding aligns with that of Jeong et al.,[5] who similarly detected no global splicing alterations in mRNA.

4 | DISCUSSION

PN is a rare autosomal recessive disorder characterized by cutaneous abnormalities, chronic neutropenia, and an increased risk of infections and malignancies.[3,27] Consequently, we established a myeloid-specific USB1 knockout mouse model. By investigating the functional impact of USB1 on neutrophils and the underlying molecular mechanisms, we revealed a critical role for USB1 in neutrophil maturation and differentiation. Furthermore, we demonstrated that differentiation defects could be traced back to the upstream myeloid progenitor cells. Although the most direct expected phenotype of USB1 deficiency is myeloid developmental defects, consistent with the neutropenia observed in human PN patients, we unexpectedly observed an elevated proportion of B cells in the BM of the conditional knockout mice. Subpopulation analysis of upstream progenitor cells ruled out the possibility of biased determination of the early hematopoietic stem cell fate. Transcriptome sequencing and GO enrichment analysis of DEGs revealed significant enrichment of B-cell receptor signaling pathways and Ig production. In a recent study, sequencing of neutrophils from COVID-19 patients similarly revealed enrichment of genes associated with Ig and B-cell lineages.[28] This study indicates that lineage plasticity exists between B-cell and neutrophil lineages during stress-induced hematopoiesis. Further studies have indicated that neutrophils can also provide costimulatory signals to B cells, inducing the production of IgG and IgA antibodies.[29] Consequently, the interaction between neutrophils and B cells may account for the increased proportion of B cells and GO enrichment associated with B cells. Although the precise driving mechanisms remain unclear, these findings provide novel insights into the relationship between B cells and neutrophil lineages.

Our study reveals a novel mechanism by which USB1 regulates the development of myeloid cells. We found that a set of key miRNAs, including miR-9-5p/3p, miR-188-5p, and miR-335-3p, exhibited significantly altered expression levels in the myeloid progenitor cells. These miRNAs are closely associated with neutrophil activation, leukemogenesis, and erythrocyte development.[19–21] Moreover, recent studies have indicated that in the majority of acute myeloid leukemia (AML) cases, the highly expressed miR-9 (miR-9-5p)/miR-9* (miR-9-3p) in myeloid progenitor cells inhibits neutrophil differentiation through the post-transcriptional regulation of ETS-related gene (ERG).[30] In our study, the abnormal expression of miR-9-5p/miR-9-3p may represent a key mechanism underlying both the suppression of myeloid progenitor cell differentiation and the reduced proportion of mature neutrophils. Consequently, USB1 may influence hematopoietic processes by regulating specific miRNAs, leading to abnormal myeloid differentiation.

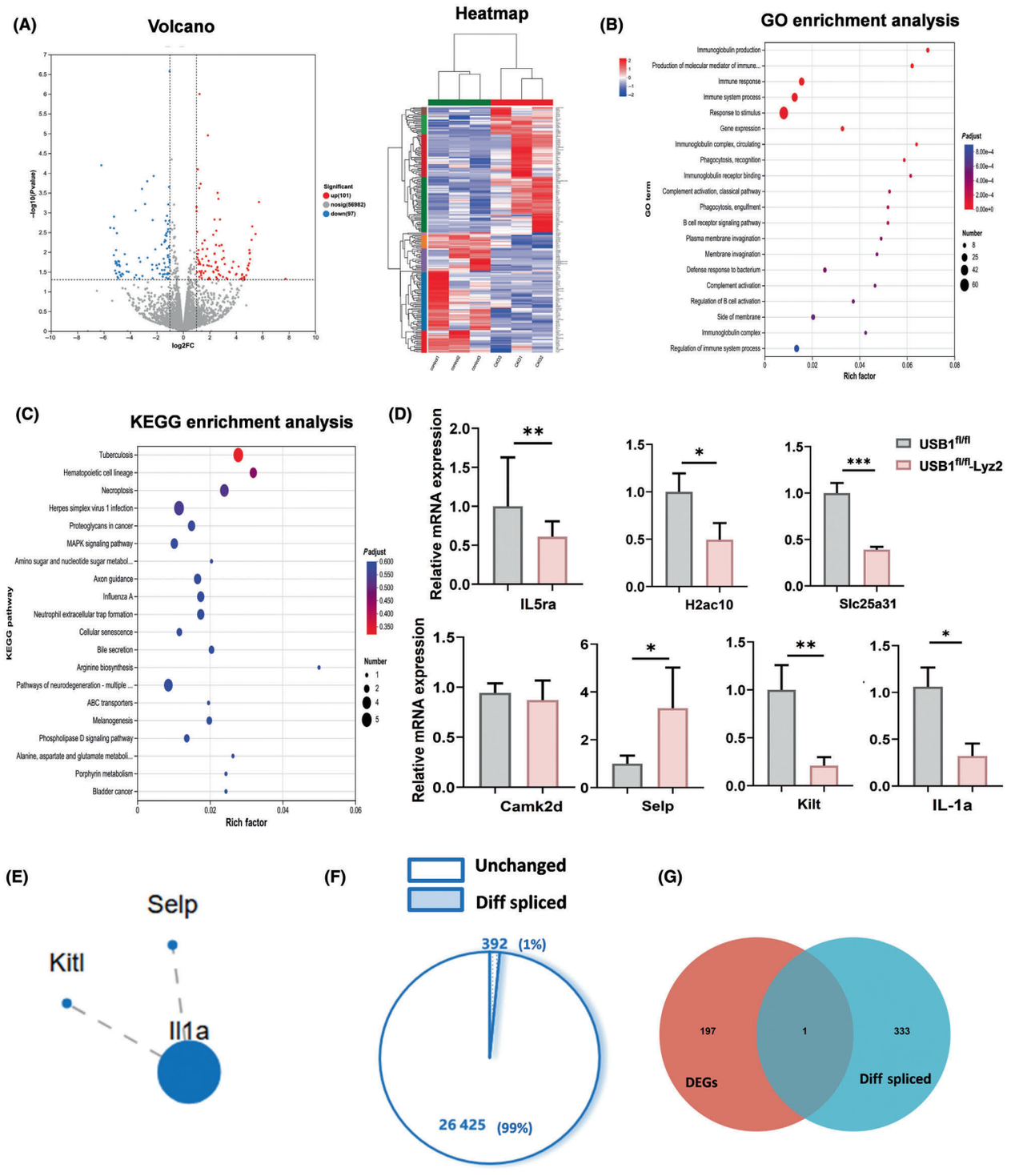

FIGURE 5 USB1 deficiency affects messenger RNA (mRNA) levels in myeloid progenitor cells. (A) Volcano plot (left) and clustered heatmap (right) of genes showing differential upregulation and downregulation ($N = 3$). (B) Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in c-Kit-positive bone marrow (BM) cells. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs in c-Kit-positive BM cells. (D) Reverse transcription polymerase chain reaction (RT-PCR) analysis of IL5A, H2ac10, Scl25a31, CAMK2D1, Selp, Kilt, and IL1a mRNA levels in c-Kit-positive BM cells ($N = 3$). Data are presented as mean ± standard deviation (SD). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (E) Protein–protein interaction (PPI) analysis of genes enriched in the "hematopoietic cell lineages," "neutrophil extracellular trap formation," "necroptotic apoptosis," and "senescence" pathways. (F) Pie chart illustrating splicing alterations in USB1fl/fl-Lyz2 mice compared to USB1fl/fl mice. The numerical annotations indicate the total number of local splicing variants and the proportion of differentially spliced genes relative to the unchanged genes. (G) Venn diagram illustrating the distribution of DGEs and alternative splicing genes between USB1fl/fl and USB1fl/fl-Lyz2 mice.

At the transcriptomic level, KEGG enrichment analysis of DEGs primarily revealed associations with the "hematopoietic cell lineage," "necroptosis," "neutrophil extracellular trap formation," and "cellular senescence" pathways. Among the genes involved in these pathways, protein interactions exist among IL1a, Kitl, and Selp, which may form a synergistic regulatory network that exerts multifaceted effects on myeloid progenitor cells. Kitl enhances neutrophil responsiveness to G-CSF, thereby stimulating maturation.[24–26] USB1 deficiency may mediate impaired neutrophil USB1 maturation by downregulating Kitl expression, which is a key mechanism of developmental arrest. Furthermore, studies have indicated that P-selectin promotes the formation of extracellular traps in neutrophils in mouse models.[31] We hypothesized that USB1 deficiency leads to upregulation of P-selectin, potentially exacerbating neutrophil activation and NETosis, thereby constituting a significant factor in neutropenia. IL-1a has primarily been studied as an alarmin secreted by nonhematopoietic cells during cell death.[32] However, research on the secretion of IL-1a by myeloid cells is limited. Recent studies have indicated that neutrophils are a major source of IL-1a and play pivotal roles in neutrophil recruitment.[33] A key finding was that, compared to the control group ($USB1^{fl/fl}$), no global splicing alterations were detected in the USB1 cKO ($USB1^{fl/fl}$-lyz2) transcriptome, and only a small number of DEGs exhibited splicing abnormalities. This is consistent with the findings of Jeong et al.,[5] further indicating that USB1 deficiency regulates gene expression via a pathway that is independent of precursor mRNA splicing. Therefore, the core mechanism by which USB1 regulates neutrophil development, differentiation, and maturation may not depend on global splicing alterations.

In this study, we demonstrated that USB1 myeloid cell knockout leads to a reduction in the proportion of mature neutrophils, a phenomenon attributable to the abnormal differentiation of myeloid progenitor cells. Furthermore, USB1 influences cellular development, differentiation, and maturation by regulating the expression of several miRNAs and mRNAs. However, our study has yet to elucidate the precise signaling pathways through which USB1 regulates the expression of these RNAs, including miRNAs and mRNAs. Furthermore, the upstream and downstream relationships of these RNAs require further investigation. This warrants further investigation in our subsequent research. Furthermore, USB1 gene knockout in myeloid cells affects altered RNA expression and differentiation abnormalities in upstream myeloid progenitor cells. Consequently, the impact of USB1 on upstream hematopoietic stem cells warrants further investigation. Establishing a hematopoietic-specific USB1 knockout mouse model for subsequent experiments is crucial. Investigating USB1's influence on hematopoietic processes from the apex of the hematopoietic hierarchy will deepen our understanding of the associated clinical mechanisms.

AUTHOR CONTRIBUTIONS

Hang Li: Conceptualization; data curation; formal analysis; writing – original draft. **Guiying Shi:** Data curation; formal analysis. **Jiaming Tang:** Data curation; formal analysis. **Yiying Huang:** Data curation; formal analysis. **Jie Wang:** Data curation; formal analysis. **Xuepei Lei:** Conceptualization. **Lin Bai:** Conceptualization; project administration; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

Lin Bai is an editorial board member of Animal Models and Experimental Medicine (*AMEM*) and a coauthor of this article. To minimize bias, she was excluded from all editorial decisions related to the acceptance of this article for publication.

DATA AVAILABILITY STATEMENT

Primary data are available upon reasonable requests.

ETHICS STATEMENT

All the animal experiments were approved by the Ethics Committee of the Institute of Medical Laboratory Animals, Peking Union Medical College (approval no.: BL23002).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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