Single-cell proteo-transcriptomic profiling reveals altered characteristics of stem and progenitor cells in patients receiving cytoreductive hydroxyurea in early-phase chronic myeloid leukemia

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Abstract

Hydroxyurea (HU) is frequently used in the early phase of chronic myeloid leukemia (CML) to achieve cytoreduction prior to tyrosine kinase inhibitor therapy. However, its impact on CML stem and progenitor cells (SPC) remains largely unknown. This study utilized targeted proteo-transcriptomic expression data on 596 genes and 51 surface proteins in 60,000 $CD14^{-}CD34^{+}$ cells from chronic phase CML patients to determine effects of short-term HU treatment (4-19 days) on CML SPC. Peripheral blood and bone marrow samples were obtained from 17 CML patients eligible for short-term HU treatment (3 patients before and after HU, 7 patients before HU and 7 patients after HU) and subjected to single-cell CITE-sequencing and/or flow cytometry analysis. The analysis revealed enhanced frequencies of hemoglobin-expressing (*HBA1, HBA2, HBB*) erythroid progenitor cells in blood and bone marrow following HU treatment. In addition, there was an accumulation of cell subsets with S/G2/M phase-related gene and protein expression, likely representing cells arrested in, or progressing slowly through, the cell cycle. The increased frequency of cells in S/G2/M phase after HU was observed already among the most immature leukemic stem cells (LSC), and patients with a large fraction of LSC in the S/G2/M phase showed poor responsiveness to tyrosine kinase inhibitor treatment. We conclude that short-term HU treatment entails differentiation of erythroid progenitor cells and alters the characteristics of LSC in CML. The results imply that studies of LSC and progenitor populations in CML should take effects of initial HU therapy into account.

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm characterized by a balanced t(9;22) translocation leading to formation of the Philadelphia chromosome and the *BCR::ABL1* fusion oncogene.[1,2] BCR::ABL1 conveys constitutive tyrosine kinase activity that translates into expansion and accumulation of malignant cells of different maturation stages in blood and bone marrow.[2] Imatinib as well as second and third-generation tyrosine kinase inhibitors (TKI) competitively block BCR::ABL1 kinase activity and have drastically improved survival in CML.[3,4]

Prior to the introduction of TKI therapy, CML patients received treatment with the chemotherapeutics hydroxyurea (HU) and busulfan, the cytokine interferon-α or allogeneic stem cell transplants.[5,6] While these treatments are no longer first-line options, many CML patients receive a short-term course of HU to reduce hyperleukocytosis

while awaiting definitive diagnosis, as advised by European LeukemiaNet (ELN) recommendations.[7] Although frequently used, recently published data suggest that HU pretreatment does not add value in terms of meeting ELN CML treatment response milestones in the TKI era, and that early-phase HU should be restricted to patients with symptomatic hyperleukocytosis or splenomegaly.[8]

HU inhibits ribonucleoside diphosphate reductase, thus limiting the available deoxyribonucleotides to target rapidly proliferating cells.[9] In addition to direct effects on cell cycle proliferation, HU has been shown to generate nitric oxide (NO) radicals.[10-12] While the role of NO in the antileukemic effects of HU is unclear, NO-induced elevation of cyclic GMP reportedly promotes γ-globin and fetal hemoglobin production and contributes to the favorable effects of HU in sickle-cell anemia.[10,13] *In vitro* studies suggest that NO may exert pro-proliferative, anti-proliferative, or differentiating effects on hematopoietic stem and progenitor cells (SPC) depending on concentration.[14]

CML is maintained and propagated by a rare population of *BCR::ABL1*-expressing leukemic stem cells (LSC).[15,16] These cells are considered partly resistant to TKI, which is why many patients require lifelong treatment with ensuing toxicity and risk of the development of resistance to TKI.[17] Considerable efforts have been devoted to developing strategies to target the therapy-resistant LSC population.[18] In many studies, CML patients have received HU prior to TKI, but to our knowledge no studies have evaluated to what extent HU affects the phenotype of the LSC and progenitors. In the present study, we aimed to address this issue through targeted proteo-transcriptomic single-cell profiling of >60,000 CML SPC from peripheral blood and bone marrow (BM) samples obtained before and after HU treatment. Our results revealed (i) enhanced erythroid maturation and (ii) increased fractions of cells in S/G2/M phase among CML SPC after HU treatment.

Methods

Samples from patients with chronic myeloid leukemia

Peripheral blood and/or BM (from the posterior iliac crest) diagnostic samples were collected from 17 chronic phase CML patients eligible for short-term treatment with HU (4-19 days) at Sahlgrenska University Hospital (Gothenburg, Sweden) and Uddevalla Hospital (Uddevalla, Sweden). From seven patients only pre-HU samples were collected, from seven patients only post-HU samples were collected, and from three patients samples were collected both before and after HU treatment. The characteristics of the patients and samples, as well as HU dosage information, are summarized in *Online Supplementary Table S1*. The study was approved by the Regional Ethics Review Board in Gothenburg (approval number 011-17) and conducted according to the principles of the Declaration of Helsinki. All patients gave written informed consent prior to sample collection.

Isolation and phenotypic analyses of peripheral blood and bone marrow mononuclear cells

Patients' peripheral blood and BM mononuclear cells (MNC) were isolated by density gradient centrifugation on Lymphoprep (STEMCELL Technologies). Samples were enriched for SPC using fluorescence activated cell sorting (FACS) of MNC, CD15 depletion, or $CD34^+$ cell selection using MACS Microbeads (Miltenyi Biotec) and were thereafter cryopreserved. Cells were subjected to immunophenotypic analysis and cell cycle analysis as detailed in the *Online Supplementary Methods*.

CITE-sequencing library preparation

Peripheral blood MNC obtained before and after HU treatment and BM MNC obtained after HU treatment from two patients (sCML14 and sCML23) were subjected to single-cell proteo-transcriptomic CITE (cellular indexing of transcriptomes and epitopes)-sequencing analysis, during which the expression of 596 genes and 51 surface proteins was assessed (*Online Supplementary Tables S2* and *S3*). Samples were barcoded and pooled, after which cells were labeled with AbSeq, fluorescent and unconjugated antibodies (*Online Supplementary Tables S3* and *S4*), and $CD14^-CD34^+$ MNC FACS-sorted. CITE-sequencing libraries were generated from the isolated $CD14^-CD34^+$ cells using the BD Rhapsody Single-Cell Analysis System (BD Biosciences) as described in Nilsson *et al.*[19] Further details regarding the CITE-sequencing library preparation and sequencing are provided in the *Online Supplementary Methods*.

Data analysis

Fastq files were processed using the BD Rhapsody Targeted Analysis Pipeline (v. 1.10.1; BD Biosciences) on the Seven Bridges Genomics platform (*https://www.sevenbridges.com*). Low-quality cells were removed from the UMI count files using genes expressed *versus* library size dot plots in SeqGeq (v. 1.8.0; BD Biosciences).[19] The mRNA and AbSeq RSEC UMI count files were analyzed in Rstudio (v. 2022.07.1+554; R [v. 4.2.1]) using the Seurat package (v. 4.2.0)[20] and in Python (v. 3.1.0) using the pySCENIC package (v. 0.12.1) as described in the *Online Supplementary Methods*.

Analyses were also performed on a previously published dataset of $CD14^-CD34^+$ CML BM cells that were subjected to CITE-sequencing using the same set of genes and surface proteins. Details on the generation of the uniform manifold approximation and projections (UMAP) in this analysis can be found in the original publication by Nilsson *et al.*[19]

Statistics

All statistical analyses were performed in GraphPad Prism (v. 9.5.1) and Rstudio (v. 2022.07.1+554; R [v. 4.2.1]). Differences in cell type proportions were evaluated in GraphPad Prism using the unpaired Mann-Whitney test. Differentially

expressed genes and proteins were assessed by the Wilcoxon rank sum test in R using Seurat's FindMarkers and FindAllMarkers functions.

Results

Hydroxyurea alters the proportions of stem and progenitor cells in blood and bone marrow from patients with chronic myeloid leukemia

Peripheral blood and BM samples obtained from 17 newly diagnosed CML patients before and after HU treatment were analyzed for SPC frequencies using flow cytometry (Figure 1A). The analysis revealed a non-significant reduction of the frequency of CD34$^+$ cells following HU treatment (Figure 1B), possibly reflecting reduced proliferation of CML SPC. In addition, the frequency of immature CD34$^+$CD38$^-$ cells among CD34$^+$ SPC in BM was significantly lower after treatment, with a similar but non-significant trend in blood (Figure 1C). Results from paired samples obtained before and after HU treatment from three patients supported these findings (*Online Supplementary Figure S1*).

Proteo-transcriptomic CITE-sequencing analysis of chronic myeloid leukemia stem and progenitor cells

To further elucidate the effects of HU on the CD34$^+$ CML SPC compartment, paired blood and BM samples from two patients (obtained before and 7 or 9 days after HU treatment) were subjected to proteo-transcriptomic CITE-sequencing analysis of 596 genes and 51 proteins (Figure 2). Twenty-six thousand single CD14$^-$CD34$^+$ cells were successfully sequenced with sequencing saturations of 93% and 97% for mRNA and AbSeq libraries, respectively. After quality control, 21,044 cells remained for analysis.

Hydroxyurea treatment induces erythroid maturation among chronic myeloid leukemia stem and progenitor cells

Following integration of mRNA expression data by patient, dimensionality reduction and clustering of cells from all six

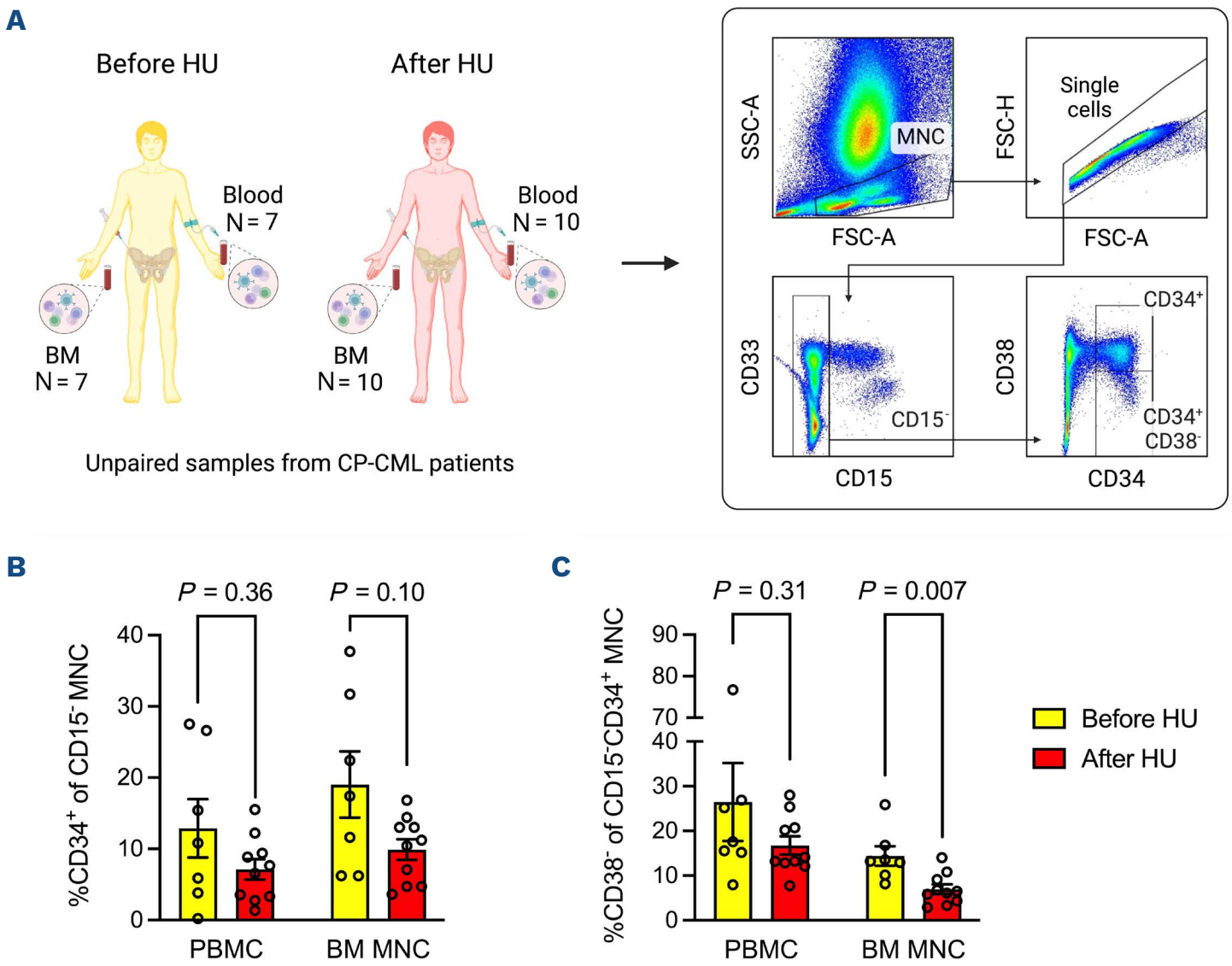

Figure 1. Hydroxyurea alters chronic myeloid leukemia stem and progenitor cell frequencies in blood and bone marrow. (A) Samples and representative gating strategy used for flow cytometry analysis of the effects of hydroxyurea (HU) on stem and progenitor cells in blood and bone marrow from patients with chronic phase chronic myeloid leukemia (CML). (B, C) Effect of HU treatment on the frequency of (B) CD34$^+$ and (C) CD34$^+$CD38$^-$ cells in CML patients' blood and bone marrow samples obtained before (N=7) or a median of 9 days (range, 4-19) after HU treatment (N=10). Statistics by the Mann-Whitney test. Error bars represent the standard error of mean. BM: bone marrow; CP-CML: chronic phase chronic myeloid leukemia; SSC: side scatter; FSC: forward scatter; MNC: mononuclear cells; PBMC: peripheral blood mononuclear cells.

analyzed samples (blood before and blood/BM after HU) were performed (Figure 3A). The obtained UMAP did not contain patient-specific clusters (*Online Supplementary Figure S2A*) and the confined protein expression of known cell type markers (*Online Supplementary Figure S2B*) supported the validity of the mRNA-based clustering. Clusters were manually annotated based on expression patterns of known marker genes (Figure 3B, *Online Supplementary Table S5*), and proteins (Figure 3C, *Online Supplementary Table S6*). Cells annotated as LSC were $CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}$ and expressed the CML LSC markers CD25 and CD26. Myeloid progenitors (MP) were characterized by expression of *SPINK2, CSF3R, CEBPA*, CD45RA and CD371. While all megakaryocytic/erythroid progenitor clusters (MEP, MKP, EP) expressed the lineage-specific transcription factor *GATA1*, megakaryocytic progenitors (MKP) were further defined by expression of *MPL, VWF* and CD9, and erythroid progenitor (EP) clusters by CD35. Megakaryocytic/erythroid progenitors (MEP) expressed *GATA1*, but lacked concurrent committed MKP/EP marker expression. The most mature EP cluster (EP-II) showed a distinct expression of *HBB, AHSP* and CD235a, and eosinophil/basophil/mast cell progenitors (EBMP) were characterized by expression of *HDC* and *GATA2*. To assess proportional shifts within the SPC compartment as a result of HU treatment, cells from blood and BM samples obtained before and after treatment were separately highlighted on the UMAP (Figure 3D). Although all nine clusters were represented in all samples, post-treatment samples showed apparent cell density increases in EP-II and cycling erythroid progenitor (EP-Cy) clusters and concomitant decreases in the EP-I cluster. BM samples obtained after HU treatment showed a similar pattern of cell cluster distribution as the paired blood samples, although the fraction of cells in the EP-II cluster was even more pronounced in the BM samples. The EP-Cy and EP-II clusters displayed increased expression of an array of checkpoint-related genes such as *CCNE1* (EP-Cy, EP-II), *CHEK2* (EP-Cy), *CDKN2C* (EP-Cy), *FANCI* (EP-Cy) and *TRIP13* (EP-Cy) (*Online Supplementary Table S5*), with cells in the EP-II cluster additionally defined by upregulation of the hemoglobin subunits *HBA1, HBA2*, and *HBB* (Figure 3E). Based on this finding, we additionally employed SCENIC analysis focusing on *GATA1*, a key transcription factor for erythropoiesis.[21] The *GATA1* regulon was identified based on expressional patterns in the paired blood samples from both patients. Many genes within this regulon showed significant upregulation following HU treatment, including *HBB, HBA1, E2F2* and *TFRC* (*Online Supplementary Table S7*).

Utilizing Seurat's CellCycleScoring function, EP-Cy were defined by the high proportion of S/G2/M phase cells within the cluster (Figure 4A). Their cycling nature was also supported by the cluster differential expression analysis (*Online Supplementary Table S5*), in which many of the upregulated genes were associated with cell division. Further exploration of the clusters displaying consistent proportional changes across patients revealed that the proportionally decreased EP-I population primarily comprised cells in G0/G1 phase, whereas the increasing EP-Cy and EP-II clusters mainly included S/G2/M phase cells (Figure 4A). The shift towards S/G2/M phase following HU treatment was also seen among

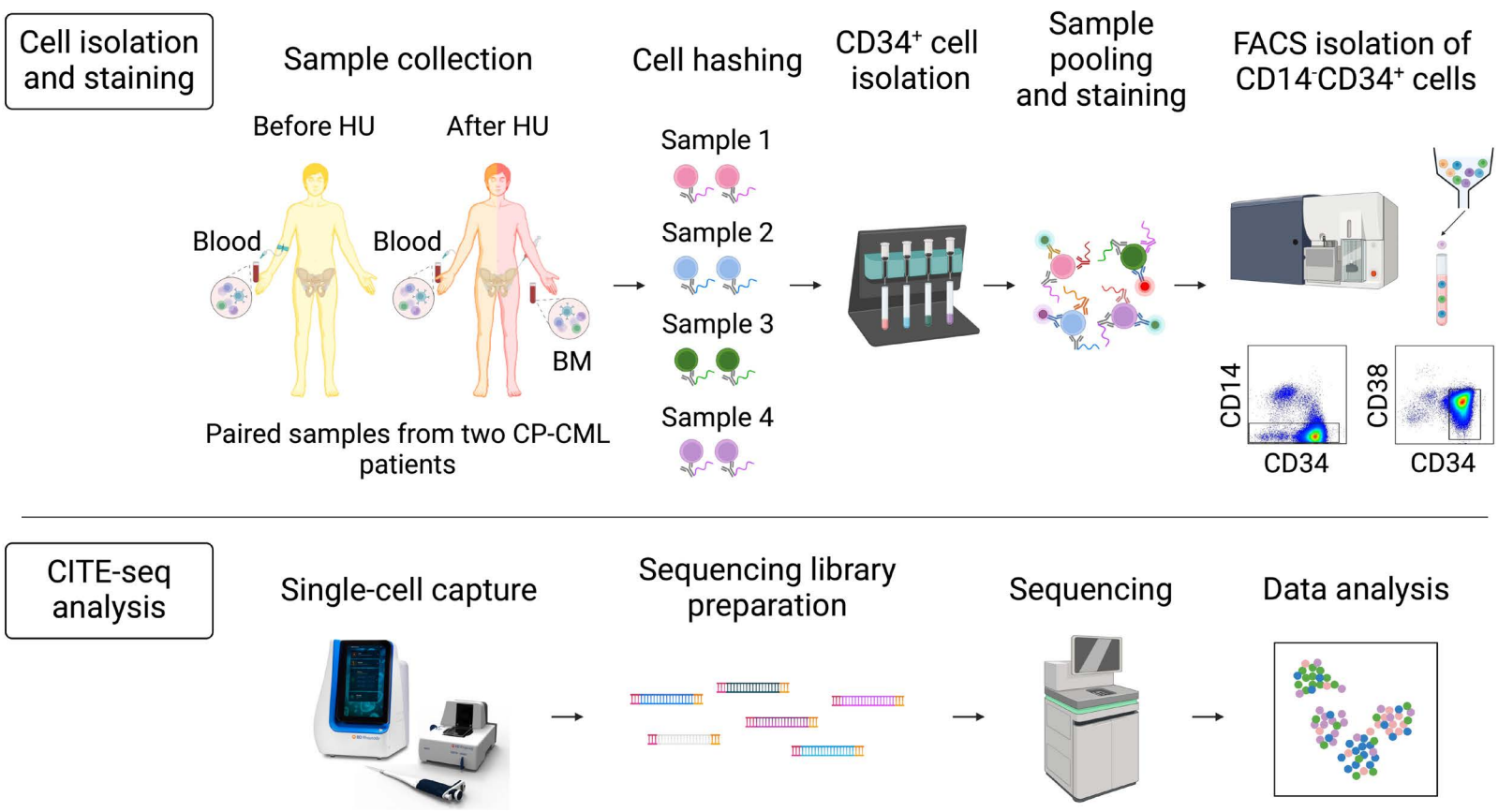

Figure 2. Workflow for proteo-transcriptomic CITE-sequencing analysis of $CD14^{-}CD34^{+}$ stem and progenitor cells from chronic phase chronic myeloid leukemia patients. HU: hydroxyurea; BM: bone marrow; CP-CML: chronic phase chronic myeloid leukemia; FACS: fluorescence-activated cell sorting: CITE-seq: cellular indexing of transcriptomes and epitopes by sequencing.

all SPC, disregarding cluster identity (Figure 4B). Furthermore, the expression pattern of the *CHEK2* gene (known to be upregulated following cell cycle arrest[22]) suggested that cells in the EP-Cy and EP-II clusters may be arrested in the cell cycle (Figure 4C).

To further address aspects of cell cycling in blood samples obtained before and after HU treatment, we performed flow cytometry analysis using DAPI and Ki67 staining of $CD14^{-}CD34^{+}$ cells (Figure 4D). In agreement with the CITE-sequencing findings, a larger fraction of $CD14^{-}CD34^{+}$ cells appeared in the S/G2/M cell cycle phases after HU treatment (Figure 4E). Furthermore, an increased frequency

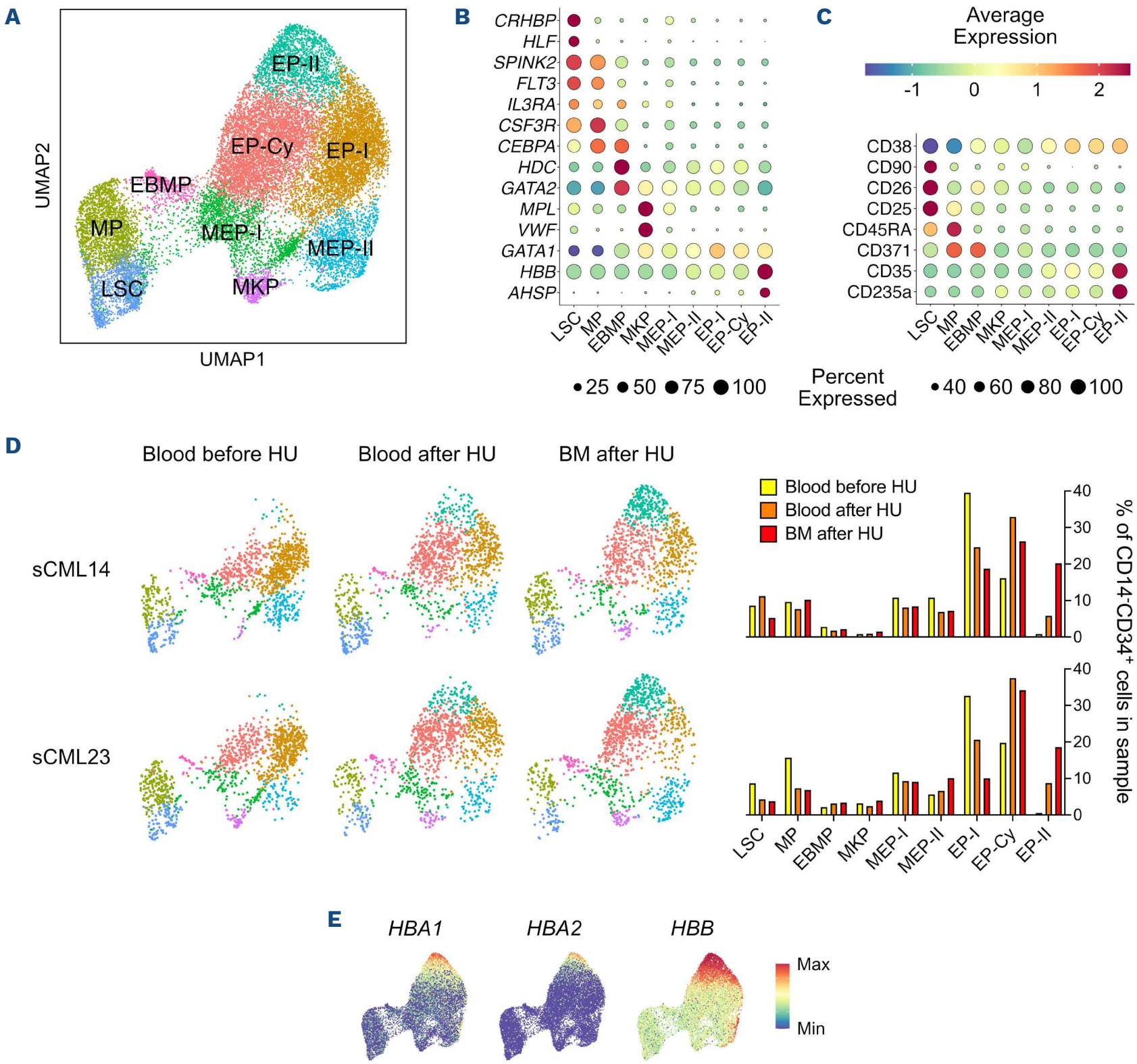

Figure 3. Hydroxyurea stimulates erythroid maturation among chronic myeloid leukemia stem and progenitor cells. Analysis of $CD14^{-}CD34^{+}$ CITE-sequencing data from two patients with chronic phase chronic myeloid leukemia (sCML14 and sCML23) with peripheral blood samples collected before and after hydroxyurea (HU) treatment and paired bone marrow samples obtained after treatment. "After HU" samples were collected after 7 and 9 days of treatment, respectively. (A) Uniform manifold approximation and projection (UMAP) representation of an mRNA-based clustering analysis of cells from all six samples (21,044 cells) after integration by patient. (B, C) Scaled expression of selected marker genes (B) and proteins (C) used for annotation of each UMAP cluster. Colors represent average expression level and dot size represents the percentage of cells expressing the specific marker within the cluster. (D) Distribution of cells from each analyzed sample across clusters. In the left panel, all cell populations are subsampled to 1,286 cells. (E) Feature plots of expression of the genes *HBA1, HBA2* and *HBB* among cells in the UMAP. Red indicates maximum expression and blue minimum expression. MP: myeloid progenitors; LSC: leukemic stem cells; EBMP: eosinophil/basophil/mast cell progenitors; EP: erythroid progenitors; EP-Cy: cycling erythroid progenitors: MEP: megakaryocytic/erythroid progenitors; MKP: megakaryocytic progenitors; BM: bone marrow.

of 2n Ki67^{hi} cells was observed in samples following HU treatment (Figure 4F). Cells with this phenotype are often denoted G1 cells, but at the transcriptional level there was no increase in G1 cells following HU. We therefore speculate that this subset represents cells with an S phase transcriptional profile that fail to synthesize new DNA due to HU. In combination, CITE-sequencing and flow cytometry findings thus implied HU-induced effects, including erythroid maturation and a shift towards S/G2/M phase, among CML SPC.

To validate these findings, we utilized a previously published dataset of CML SPC that had undergone CITE-sequencing.[19] This cohort included samples from five patients prior to HU administration and seven patients who had previously received HU (Figure 5A). CML patients who had never received HU in this dataset (likely harboring less proliferative malignant clones) were excluded from the analysis to avoid bias. Clusters were annotated using a similar approach to that described above (*Online Supplementary Figure S3A, B*). Also in this cohort, SPC from HU-treated patients had a significantly higher fraction of cells in the hemoglobin subunit-expressing erythroid cluster (EP-IV) (Figure 5A-C). The treated patients additionally showed a higher proportion of cells in the EP-Cy-II cluster which displayed increased expression of S/G2/M and checkpoint-related markers, including *CHEK2* (Figure 5B-D).

The shift towards S/G2/M-related gene expression after hydroxyurea treatment is present already among the most immature chronic myeloid leukemia cells

To achieve higher resolution for analysis of the effects of HU on the most immature CML stem cell population in the paired CITE-sequencing data, CD34 and CD38 protein expression data were utilized for flow cytometry-like gating

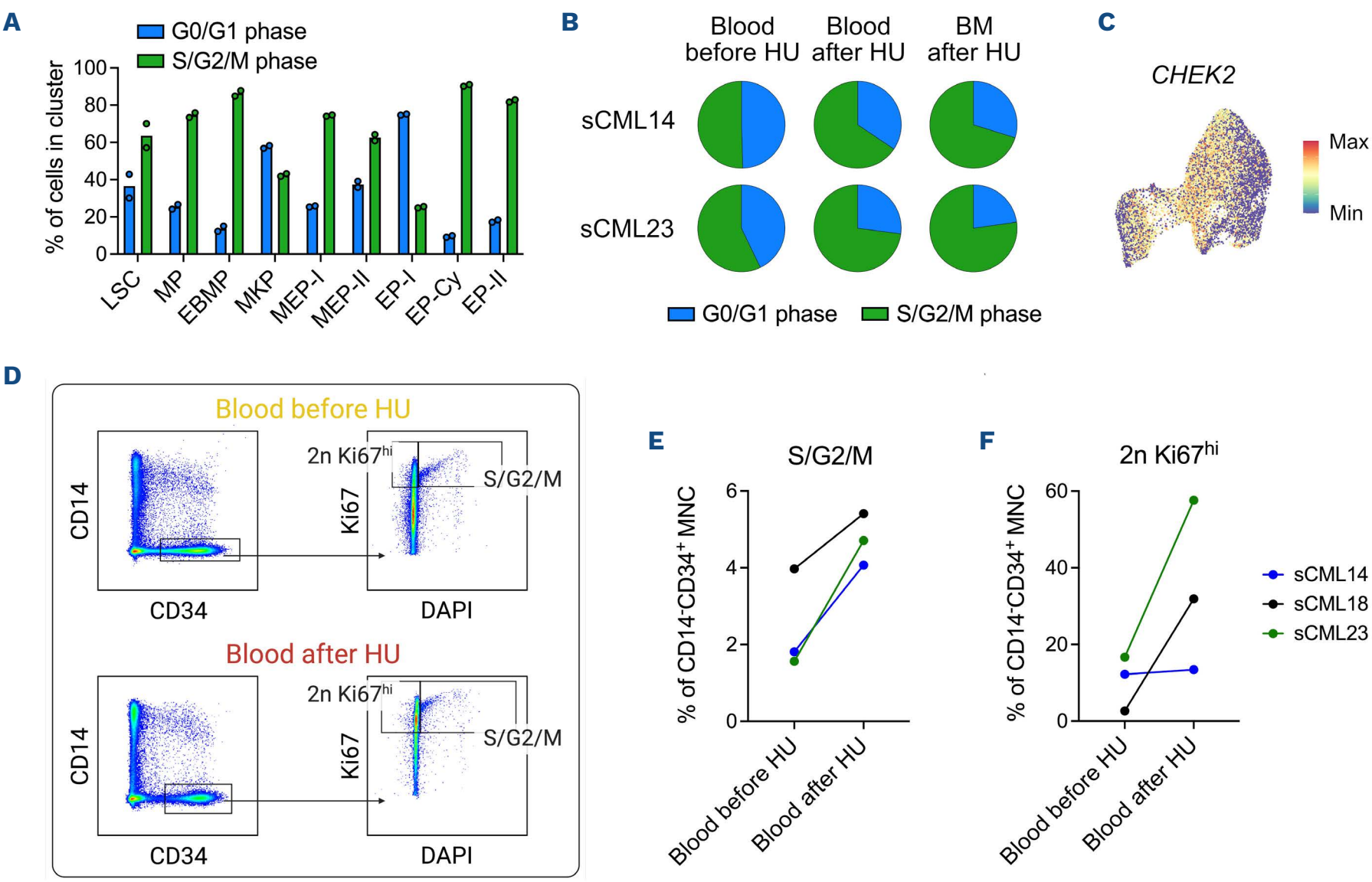

Figure 4. Hydroxyurea induces transcriptional and proteomic features associated with S/G2/M phases of the cell cycle among chronic myeloid leukemia stem and progenitor cells. (A-C) Graphs show results of an analysis of aspects of cell cycling in the paired CITE-sequencing dataset (2 patients; blood samples obtained before and after, and bone marrow samples obtained after, 7-9 days of hydroxyurea [HU] treatment). (A, B) Proportion of cells in G0/G1 *versus* S/G2/M phase (A) in each uniform manifold approximation and projection (UMAP) cluster and (B) among CD14$^-$CD34$^+$ cells in each sample. (C) Feature plot of *CHEK2* gene expression among cells in the UMAP. Red indicates maximum expression and blue minimum expression. (D-F) Graphs show results from flow cytometry cell cycle analysis of paired blood samples collected before and after HU treatment (3 patients). (D) Representative gating strategy. (E, F) Effect of HU treatment on frequencies of (E) cells in S/G2/M phase and (F) 2n Ki67^{hi} cells among chronic myeloid leukemia stem and progenitor cells. LSC: leukemic stem cells; MP: myeloid progenitors; EBMP: eosinophil/basophil/mast cell progenitors; MKP: megakaryocytic progenitors; MEP: megakaryocytic/erythroid progenitors; EP: erythroid progenitors; EP-Cy: cycling erythroid progenitors; BM: bone marrow; MNC: mononuclear cells.

(*Online Supplementary Figure S4A*). This identified 4,222 immature CD34⁺CD38⁻ cells that were included in subsequent analysis. The positions of the gated cells within the larger CD14⁻CD34⁺ UMAP confirmed that the selected cells mainly derived from the immature LSC and MP clusters, with additional contribution from MEP and MKP clusters (*Online Supplementary Figure S4B*). In line with the flow cytometry results shown in *Online Supplementary Figure S1B*, the CITE-sequencing data showed marginally reduced frequencies of CD38⁻ out of CD34⁺ cells following HU treatment (*Online Supplementary Figure S4C*).

Re-clustering of the selected cells yielded nine clusters (Figure 6A), without major patient-specific clustering (*Online Supplementary Figure S4D*). Based on expressional patterns (*Online Supplementary Figure S4E, F*, *Online Supplementary Tables S8* and *S9*), the UMAP largely divided into stem cells/multipotent progenitors (SC/MPP; e.g., characterized by CD90, *HLF*, CD25, CD26), cells with myeloid lineage commitment (MP; e.g. CD371, CD45RA, *SPINK2, CSF3R*, *CEBPA*) and cells showing signs of megakaryocytic/erythroid/eosinophil/basophil/mast cell lineage bias (MEP/EBMP; e.g., *GATA1, MPL*, CD35, *HDC*).

Assessment of the relative proportions of cells in G0/G1 *versus* S/G2/M phase across samples revealed an HU-induced shift towards S/G2/M phase-related gene expression also among the immature CD34⁺CD38⁻ cells (Figure 6B). The

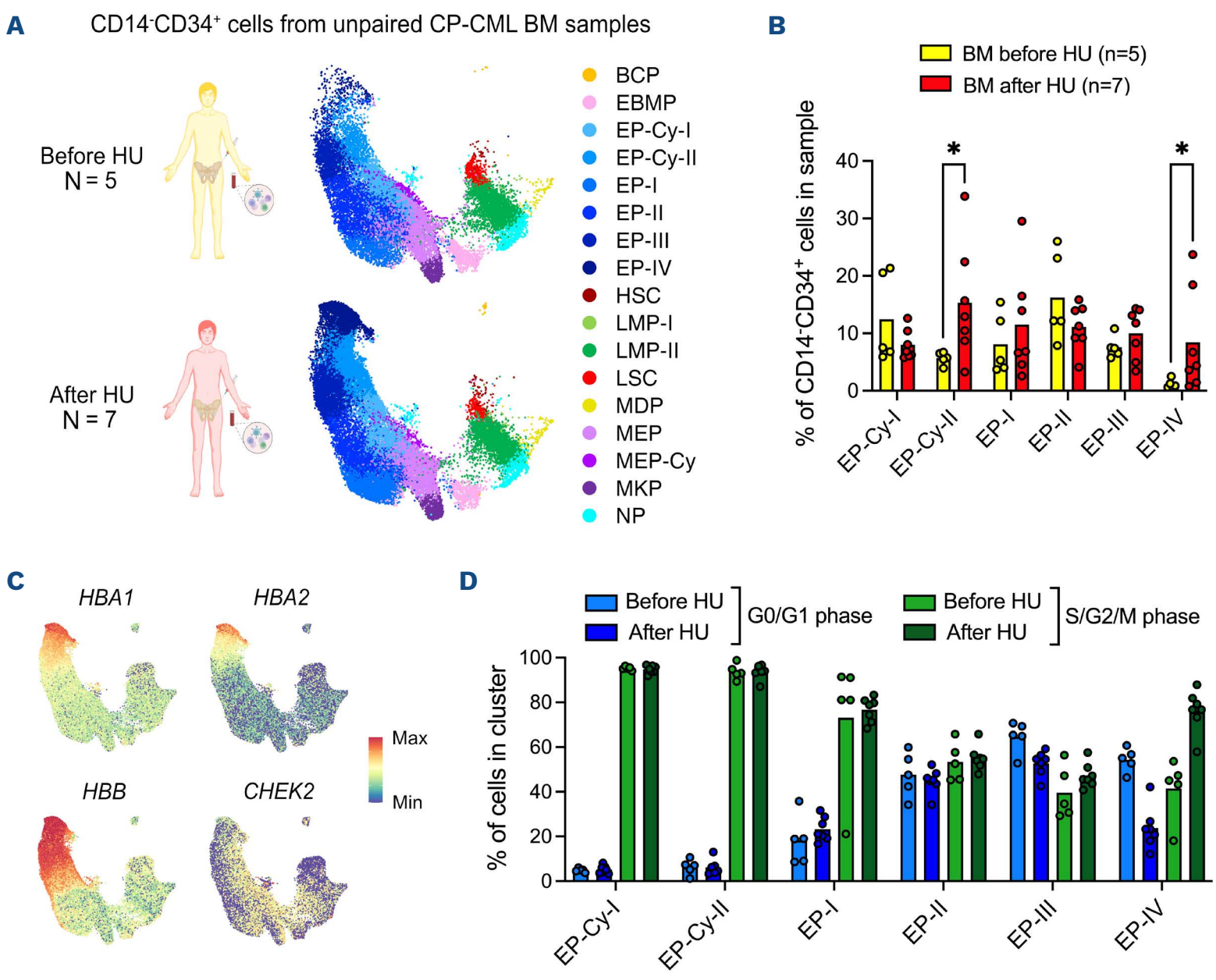

Figure 5. CITE-sequencing analysis of unpaired chronic myeloid leukemia bone marrow samples confirms enhanced frequencies of stem and progenitor cells with features of erythroid maturation and S/G2/M cell cycle phases after hydroxyurea treatment. Analysis of CITE-sequencing data from a previously published dataset of CD14⁻CD34⁺ bone marrow cells collected from patients with chronic phase chronic myeloid leukemia before (N=5; 17,968 cells) or after (N=7; 29,736 cells) a median of 9 days (range, 4-19) of hydroxyurea (HU) treatment (unpaired samples; Nilsson *et al.*[19]). (A) Visualization of the distribution of cells obtained before and after HU treatment across the CD14⁻CD34⁺ uniform manifold approximation and projection (UMAP) from Nilsson *et al.*[19] (B) Percentage of cells in each erythroid UMAP cluster in samples obtained before and after HU. Statistics by Mann-Whitney. **P*<0.05. (C) Feature plots of *HBA1, HBA2, HBB* and *CHEK2* gene expression. Red indicates maximum expression and blue minimum expression. (D) Proportion of cells in G0/G1 *versus* S/G2/M phase in each erythroid cluster. CP-CML: chronic phase chronic myeloid leukemia; BM: bone marrow; BCP: B-cell progenitors; EBMP: eosinophil/basophil/mast cell progenitors; EP-Cy: cycling erythroid progenitors; EP: erythroid progenitors; HSC: hematopoietic stem cells; LMP: lympho-myeloid progenitors; LSC: leukemic stem cells; MDP: monocyte/dendritic cell progenitors; MEP: megakaryocytic/erythroid progenitors; MEP-Cy: cycling megakaryocytic/erythroid progenitors; MKP: megakaryocytic progenitors; NP: neutrophil progenitors.

proportional increase in cells with S/G2/M phase-related gene expression observed after HU treatment thus seemingly occurs across differentiation stages and lineages, ranging from the most immature CD14$^-$CD34$^+$CD38$^-$ cells to more mature erythroid-committed progenitors.

Hydroxyurea reduces the frequency of chronic myeloid leukemia leukemic stem cells with quiescent characteristics

In the previous CITE-sequencing study by Nilsson *et al.*,[19] the CML LSC compartment (defined by high expression of CD90, CD26 and CD25 along with low expression of CD38 and CD45RA) separated into two clusters in a gene expression-based UMAP. One of the LSC subsets, denoted 'LSC-I', appeared quiescent with an expression pattern resembling that of TKI-resistant LSC.[23] The other LSC subset, denoted 'LSC-II', showed higher expression of S/G2/M phase markers.[19] To assess whether HU had an impact on the most immature LSC population in CML, we used the aforementioned dataset as a reference and performed Seurat-based cell label transfer to annotate the CD34$^+$CD38$^-$ cells in the current analysis of paired samples (Figure 7A). The identities of cells annotated as hematopoietic (HSC-I and -II) and leukemic (LSC-I and -II) stem cells (Figure 7B) as well as other cell types (*Online Supplementary Figure S5A*) largely corresponded to the previous lineage annotations (Figure 6A), supporting the validity of the cell label transfer. Expressional patterns for each annotated cell type are provided in *Online Supplementary Figure S5B, C*.

When assessing the impact of HU on stem cell subtype distribution, a proportional shift from the LSC-I to the LSC-II subpopulation was noted within the stem cell compartment after HU treatment (Figure 7C). The decreasing LSC-I population was dominated by G0/G1 cells, while the increasing LSC-II population predominantly consisted of cells in S/G2/M phase (Figure 7D). The actively cycling phenotype of the LSC-II population compared to that of LSC-I was supported by upregulation of a wide range of cell cycle-related genes (e.g., *CCNA2, BIRC5, E2F2, ZWINT, NDC80, TUBB*) in differential expression analysis (*Online Supplementary Figure S5D, Online Supplementary Table S10*). To validate these results, the effect of HU on stem cell distribution was also assessed in the previously published dataset,[19] in which BM samples were obtained from five patients prior to HU treatment and from seven other patients after administration of HU. In line with the results above, the stem cell compartment of patients who had received HU contained a significantly lower proportion of G0/G1-dominated LSC-I and a higher proportion of actively cycling (S/G2/M-dominated) LSC-II, while the proportion of HSC was unaltered (Figure 8A, *Online Supplementary Figure S6A*). Of note, the frequency of LSC-I and LSC-II among all CD34$^+$CD38$^-$ immature cells in this analysis was also significantly reduced and increased, respectively, after HU treatment (*Online Supplementary Figure S6B*). Among HU-treated patients, the proportion of LSC-II did not correlate with time on HU treatment (R^2=0.18, P=0.35).

The majority of patients in the combined study cohort responded well to TKI treatment, but two patients did not attain a complete cytogenetic response (0% Ph$^+$ cells)[24] after 3 months of TKI treatment and eventually required second- or third-line ponatinib treatment to achieve complete cytogenetic response. These insufficient responders, who were analyzed after HU treatment, showed the highest proportion of LSC confined to the LSC-II subtype among all analyzed patients (Figure 8B). Mutational analysis, according to clinical practice in Sweden, did not show the presence of *BCR::ABL1* mutations in the poorly responding patients.

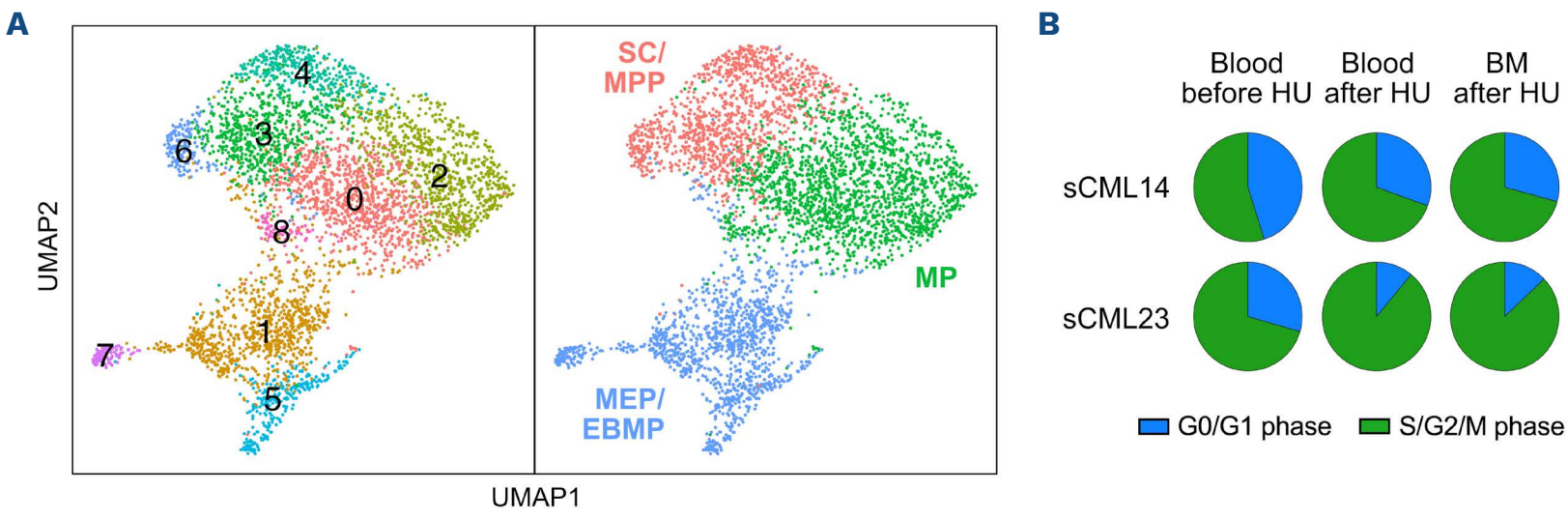

Figure 6. Hydroxyurea increases the fraction of cells in S/G2/M phase within the CD14$^-$CD34$^+$CD38$^-$ compartment in chronic myeloid leukemia. Analysis of the CD14$^-$CD34$^+$CD38$^-$ compartment in the paired CITE-sequencing dataset (2 patients; 4,222 cells; blood and bone marrow samples obtained before and/or after 7-9 days of hydroxyurea treatment). (A) Uniform manifold approximation and projection (UMAP) visualization of an mRNA expression-based clustering analysis of CD14$^-$CD34$^+$CD38$^-$ cells (left panel) and large-scale cluster annotation based on expressional patterns (right panel). (B) Proportion of CD14$^-$CD34$^+$CD38$^-$ cells in G0/G1 *versus* S/G2/M phase in each analyzed sample. UMAP: uniform manifold approximation and projection; SC/MPP: stem cells and multipotent progenitors; MEP/EBMP: megakaryocytic/erythroid and eosinophil/basophil/mast cell progenitors; MP: myeloid progenitors; HU: hydroxyurea; BM: bone marrow.

Discussion

Although many CML patients currently receive a course of cytoreductive treatment with HU prior to starting TKI therapy, this is to our knowledge the first study to report effects of this treatment on the SPC. As HU pre-treatment is indicated only in a subgroup of patients (those with high white blood cell counts or high platelet counts[7,25]), HU-related effects on the SPC compartment may affect conclusions of CML studies utilizing diagnostic samples from patients who have or have not received prior HU treatment. In this study, detailed proteo-transcriptomic CITE-sequencing analysis of SPC obtained from chronic phase CML patients before or after HU treatment indicated multiple HU-induced transcriptional changes within this compartment, including (i) increased erythroid maturation, and (ii) an increased proportion of cells in S/G2/M phase. Paired and unpaired comparisons of SPC from peripheral blood and BM samples implied increased frequencies of cells appearing in the most mature hemoglobin subunit-expressing erythroid clusters (EP-II/EP-IV) following HU treatment, which is in line with previous studies linking HU treatment to erythroid differentiation, in part via NO-induced *GATA1* activation, in other contexts.[13,26] A recent study by Krishnan *et al*. suggested that CML patients with a high degree of erythroid differentiation prior to TKI treatment were more likely to respond favorably to the TKI therapy; however, it did not take prior HU treatment into account.[27] In our

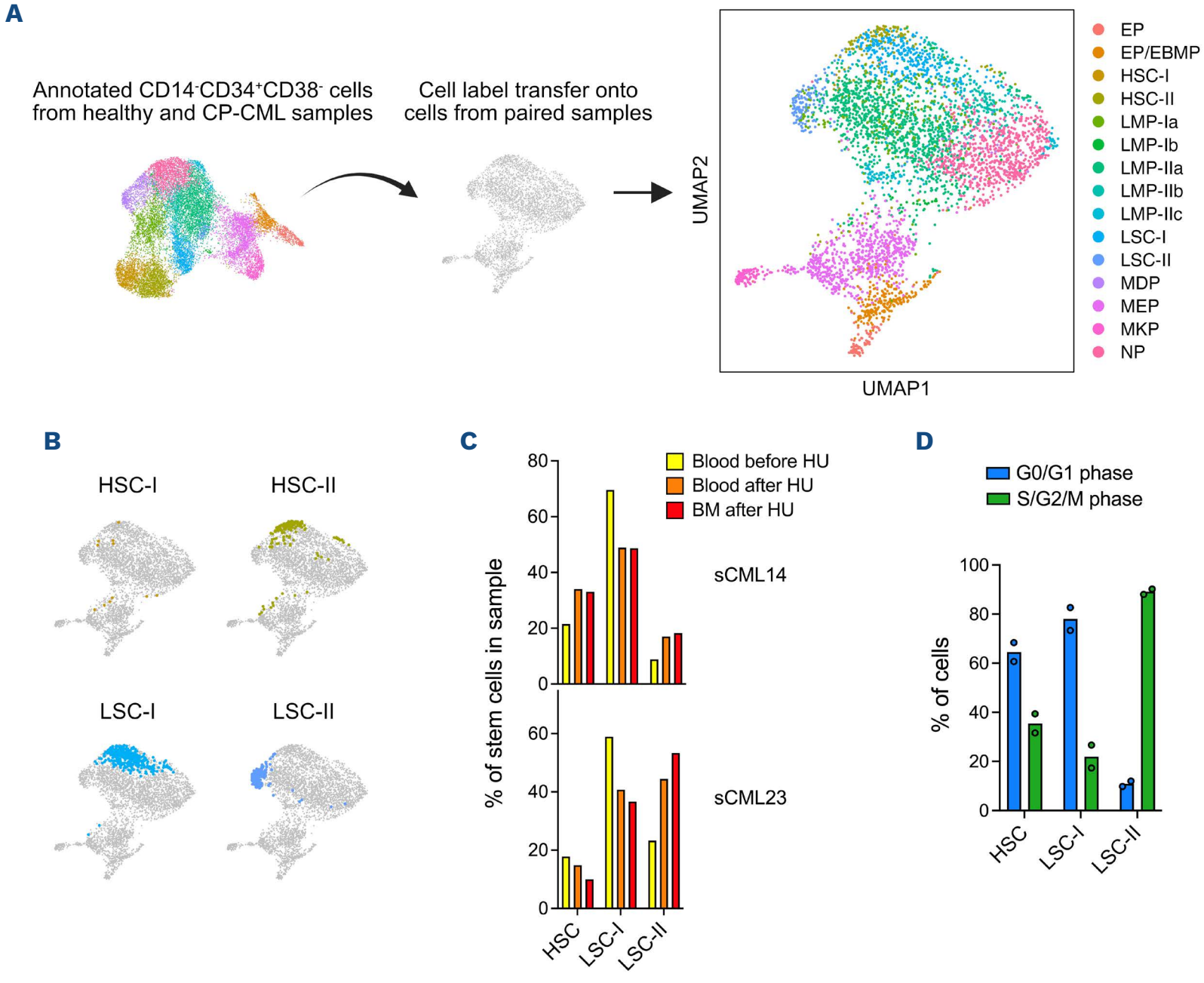

Figure 7. Hydroxyurea increases the fraction of cells in S/G2/M phase among the most immature leukemic stem cells. (A) Annotation of CD14-CD34+CD38- cells in the paired CITE-sequencing dataset based on cell label transfer from a previously published dataset of CD14-CD34+CD38- healthy/chronic phase chronic myeloid leukemia bone marrow cells (Nilsson *et al.*[19]). (B) Distribution of cells annotated as immature HSC-I, HSC-II, LSC-I, and LSC-II across the uniform manifold approximation and projection (UMAP). (C, D) Proportion of (C) stem cells annotated as HSC (HSC-I and HSC-II), LSC-I and LSC-II in each analyzed sample (after HU samples were collected after 7-9 days of hydroxyurea treatment), and (D) cells in G0/G1 *versus* S/G2/M phase in each stem cell subset. CP-CML: chronic phase chronic myeloid leukemia; BCP: B-cell progenitors; EP: erythroid progenitors; EBMP: eosinophil/basophil/mast cell progenitors; HSC: hematopoietic stem cells; LMP: lympho-myeloid progenitors; LSC: leukemic stem cells; MDP: monocyte/dendritic cell progenitors; MEP: megakaryocytic/erythroid progenitors; MKP: megakaryocytic progenitors; NP: neutrophil progenitors; HU: hydroxyurea.

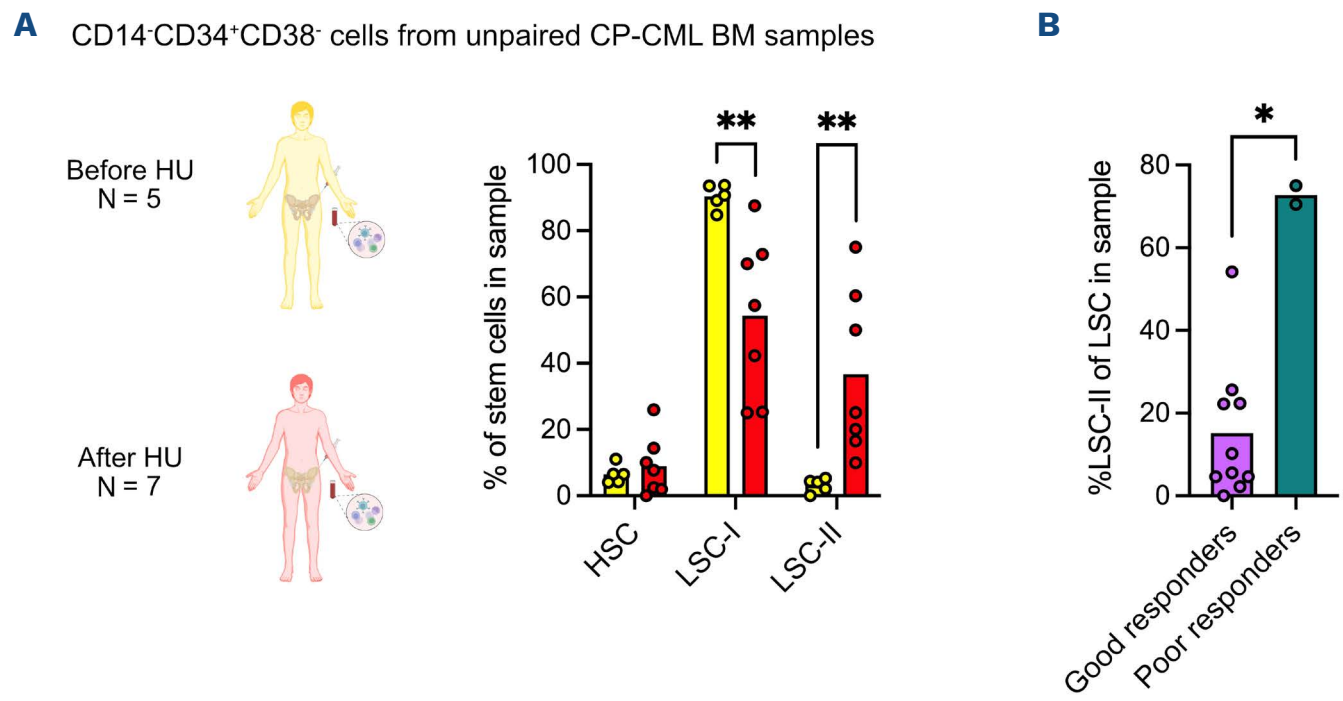

Figure 8. Induction of LSC-II may associate with poor response to tyrosine kinase inhibitor therapy. (A) Proportions of hematopoietic stem cells and leukemic stem cells (LSC-I and LSC-II) in the previously published dataset of unpaired CD14$^-$CD34$^+$CD38$^-$ bone marrow cells obtained before (N=5) or after a median of 9 days (range, 4-19) of hydroxyurea treatment (N=7) (Nilsson *et al.*[19]). (B) Frequency of LSC-II within the diagnostic LSC compartment for chronic phase chronic myeloid leukemia patients who did (poor responders, N=2) or did not (good responders, N=10) require second- or third-line ponatinib treatment to achieve a complete cytogenetic response. Statistics by the Mann-Whitney test. *P<0.05, **P<0.01. CP-CML: chronic phase chronic myeloid leukemia; BM: bone marrow; HU: hydroxyurea; HSC: hematopoietic stem cells.

limited cohort of patients, we did not observe any impact of erythroid progenitor cell frequencies on TKI responses. However, post-HU SPC comprised a higher proportion of erythroid progenitors than the paired samples obtained before treatment (blood pre- and post-HU proportions for patient sCML14, 56.5% and 63.4%, respectively, and for patient sCML23, 53.0% and 66.9%, respectively), highlighting the need to take HU status into account in such analyses. SPC from post-HU samples contained an increased fraction of cells in S/G2/M phase as compared to those obtained before HU treatment. This shift was observed among relatively mature erythroid progenitors as well among the most immature CML cells in both paired and unpaired samples. While an increased proportion of cells in S/G2/M phase is typically reflective of cells in active proliferation, it may in this instance derive instead from an HU-induced reduction in the availability of deoxyribonucleotides and consequent accumulation of cells in S phase.[28,29] Accordingly, following HU treatment we observed a larger fraction of 2n Ki67^{hi} cells suggesting transcriptional commitment to S phase but failure to replicate DNA. These assumptions are also in line with the findings of a study by Behbehani *et al.*[30] showing that although incorporation of 5-iodo-2´-deoxyuridine into primary acute myeloid leukemia cells was strongly reduced after *in vivo* HU treatment, the fraction of cells with signs of active cycling was increased rather than decreased. The reduced proportions of SPC observed in CML blood and BM following HU treatment, as well as the elevated expression of checkpoint-related genes (*CCNE1, CDKN2C, FANCI* and *TRIP13*) in the expanding cell populations, speak in favor of this mechanism.

At the LSC level, a consistent and significant shift towards stem cells expressing S/G2/M phase markers (LSC-II) was observed after HU treatment, while the fraction of the more quiescent LSC-I subset was reduced. Interestingly, the patients with the highest proportion of LSC-II cells after HU treatment did not respond optimally to initial TKI treatment and required second- or third-line ponatinib treatment to attain complete cytogenetic response. As the total amount of LSC (LSC-I + LSC-II) was unaltered by HU treatment (*data not shown*), we speculate that LSC-I and LSC-II are subgroups within the LSC population that differ in cell cycle stage. Upon HU treatment, the LSC may remain in the S/G2/M phase to a greater extent due to slower or halted progression through the cell cycle, reflected by enhanced proportions of LSC-II cells in the post-HU samples. Having a larger proportion of LSC in S/G2/M phase upon HU treatment may thus identify patients with more proliferative LSC clones, which may explain their slower and/or less straightforward normalization of hematopoiesis upon TKI treatment.

While no randomized trials assessing long-term outcomes of early-phase HU treatment have been performed, previous studies suggest that HU treatment for more than 6 months prior to TKI therapy is associated with a significantly lower rate of major molecular response[31] and that patients with HU pre-treatment may have poorer responses to TKI.[8] Also, combined HU/TKI treatment reportedly does not provide additional clinical benefit over TKI treatment alone.[32,33] However, since HU is mainly given to patients with greater disease burden, it is difficult to draw conclusions regarding its effects on disease outcome in the absence of randomized comparisons. Our results showed an increased proportion of LSC-II

following HU treatment and a non-optimal response to TKI in the two patients with the highest LSC-II content. Although the small sample size precludes conclusions regarding the role of LSC-II in TKI responsiveness, these findings point towards analysis of LSC-II/LSC-I ratios in a larger number of HU-treated patients to elucidate long-term effects on CML outcome.

In conclusion, the results of this study imply that short-term HU therapy enhances erythroid progenitor cell differentiation and alters the characteristics of CML LSC. Hence, HU status should be considered in future analyses addressing the SPC compartment in CML.

Disclosures

No conflicts of interest to disclose.

Contributions

HK and MSN designed, performed and analyzed experiments. MSN designed and performed the bioinformatic analysis with input from HK. TSB performed the SCENIC analysis. LW provided the material from the chronic myeloid leukemia patients. HK and MSN isolated mononuclear cells from patients' samples. AM, FBT, KH, PJ and LW conceived, designed and supervised the study. HK, MSN, KH, FBT and AM wrote the manuscript. All authors approved the manuscript prior to submission.

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Data-sharing statement

CITE-sequencing expression data are available online in a Zenodo repository (10.5281/zenodo.12580559). R codes are available upon request. For fastq files, please contact anna.martner@gu.se.

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