GCSF-R Expression in Myelodysplastic and Myeloproliferative Disorders and Blast Dysmaturation in CML

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Key Words: Flow cytometry; GCSF-R; CD114; Myelodysplastic syndrome; Chronic myelogenous leukemia

ABSTRACT

Objectives: To characterize granulocyte colony-stimulating factor receptor (CD114) expression in normal (n = 20), myelodysplastic (n = 34), and chronic myelogenous leukemia (CML; n = 5) bone marrow by flow cytometry.

Methods: Clinical bone marrow samples were analyzed using CD33/CD114/CD34/CD117/CD45. CD114 density (mean fluorescence intensity) and cellular distribution were evaluated on early blasts (CD33–), late blasts (CD33+), promyelocytes, and granulocytes.

Results: Normal CD114 acquisition occurred on early blasts, peaked on promyelocytes, and decreased on granulocytes. Forty percent of CD34+ blasts expressed CD114 and one-third were early blasts. In myelodysplastic syndromes, altered CD114 distribution was more informative than density changes. In CML, CD114 density was significantly decreased on early blasts and expression was essentially limited to late blasts. We observed a specific blast dysmaturation pattern in CML involving CD33, CD34, and CD114 that was 83% sensitive and 100% specific in initial diagnosis.

Conclusions: CD114 provides useful additional detail in phenotypic assessment of hematopoietic precursor maturation.

Myeloproliferative disorders and myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal stem cell hematologic disorders that can be diagnostically challenging.1-6 Ongoing advancements in flow cytometry instrumentation, reagents, and software have allowed more complex analysis of hematopoietic cell populations in normal and disease states.7-9 This includes application of flow cytometry in disorders such as chronic myeloproliferative and myelodysplastic disorders, for which analysis was once considered noncontributory.10-24 Several consensus documents have been published in the past several years in an attempt to standardize flow cytometry approaches to MDS diagnosis.25-28

CD34+ blast phenotypic abnormalities in MDS tend to be more stable and less subject to artifact than those observed on maturing myeloid series cells.16,17,19,24 We previously reported that abnormalities of blast CD117 (stem cell factor receptor) expression density occur with high frequency in myelodysplasia.17 Due to the functional relationship of cytokine and apoptosis receptors in the regulation of hematopoiesis, other growth factor receptors should provide additional detail for the assessment of blast maturation in these disorders.

We report here the pattern of granulocyte colony-stimulating factor receptor (GCSF-R) (CD114) expression in normal hematopoiesis and characterize maturation-related expression abnormalities in myelodysplastic and myeloproliferative disorders. We demonstrate a specific phenotypic maturation abnormality of chronic myelogenous leukemia (CML) myeloblasts that involves CD33, CD34, and CD114.
Materials and Methods

Patient Samples

Bone marrow specimens submitted to the Nebraska Medical Center’s clinical flow cytometry laboratory for diagnostic purposes were used as study samples. The samples represented either newly or previously diagnosed cases of MDS or CML. The normal control set consisted of samples submitted for evaluation of mild anemia or thrombocytopenia. These specimens were otherwise normal morphologically and phenotypically. The demographics of the study population are summarized in Table 1. The study was approved by the institutional review board at the University of Nebraska Medical Center.

Table 1

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CML, chronic myelogenous leukemia; CMML-2, chronic myelomonocytic leukemia; ET, essential thrombocythemia; MDS-NOS, myelodysplastic syndromes—not otherwise specified; MPD-NOS, myeloproliferative disorders—not otherwise specified; RAEB, refractory anemia with excess blasts; RCMLD, refractory cytopenia with multilineage dysplasia.

Specimen Processing

Bone marrow aspirates were collected in sodium heparin and processed according to established procedures. Mononuclear cells were isolated from the bone marrow samples by Ficoll-Hypaque density gradient centrifugation (Accu-Prep; Accurate Chemical, Westbury, NY). Contaminating RBCs were removed by hypotonic lysis with 0.9% sodium chloride. Isolated cells were then washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 1 mM HEPES buffer, 1-glutamine, penicillin, streptomycin, and 10% calf serum. Cells were counted by trypan blue dye exclusion, aliquoted at 0.5 × 10⁶ cells per tube, and washed once in 1 mL cold PBS. The cells were resuspended in 100 μL PBS with 0.5% bovine serum albumin (PBS/BSA) and incubated with 5 μL CD33-fluorescein isothiocyanate (FITC), 10 μL CD114-phycocerythrin (PE), 10 μL CD34-PE–Texas red (ECD), 10 μL CD117-PE cyanin 5.1 (PC5), and 10 μL CD45-PE cyanin 7 (PC7) for 30 minutes at 4°C. All antibodies were obtained from Beckman Coulter (Brea, CA) except for CD114-PE, which was obtained from BD Pharmingen (San Jose, CA). After incubation, the cells were washed with 1 mL PBS and the cell pellets resuspended in 1 mL PBS/BSA for immediate analysis.

Data Acquisition

All analyses were performed on a single, designated Beckman Coulter FC500 flow cytometer. Instrument photomultiplier tube and amplifier settings were established and quality controlled by the clinical cytometry staff on a daily basis. Background noise was determined using isotype fluorescent control antibodies (Beckman Coulter) with negative populations set in the first decade of the log scale. Compensation settings for CD114 analysis were established independent of clinical quality control using CD34-FITC, CD34-PE, CD34-ECD, CD34-PC5 and CD45-PC7 (Beckman Coulter). For each sample, 50,000 CD45-positive events were collected.

Data Analysis

CXP and Kaluza software (Beckman Coulter) were used to analyze CD114 expression patterns on myeloid cell populations. CD114 expression density as mean fluorescence intensity (MFI) on myeloid cell populations was performed using a logicle (linear-log) 4-log plot display. Myeloid cell populations (maturation stages) were defined as follows: early blasts, CD34+, CD117+, and CD33–; late blasts, CD34+, CD117+, and CD33+; promyelocytes, CD34–, CD117+, and CD33+; and granulocytes, CD34+, CD117–, and CD33+; and high side light scatter. CD114 MFI was determined for each of these populations on a CD114 logicle display. An antigen mapping/color precedence method of analysis was used as previously
described. Populations of interest were defined and gated using Boolean population gates as defined above. Group mean MFI values and standard deviations were calculated for each myeloid maturation stage for the normal, CD33+ MDS, and CML groups.

The distribution of CD114 expression on CD34+ blasts was evaluated for each case. Blasts were gated as CD117 and CD34 dual-positive events, and the relative number of CD114-positive blasts was determined on a CD45 by CD114 display. The CD114-positive blasts were then gated, and the relative distribution between CD33– (early) and CD33+ (late) blasts was recorded. In 9 (26%) of 34 MDS cases, there was no measurable CD33 acquisition by the blasts. These cases were evaluated for the total percentage of myeloblasts expressing CD114 but were excluded from the maturation stage density measurements and CD114+ blast subset distribution calculations. All data were compared for statistical significance (P < .05) using 1-way analysis of variance with the Tukey post hoc test and independent t test. SPSS statistical software for Windows (version 21, SPSS, Armonk, NY) was used for this purpose.

Following characterization of the CML CD33/CD34 blast pattern, the information was communicated to the 3 clinical flow cytometry service pathologists in an informal fashion. One year following dissemination of the information, we retrospectively collected performance data to assess sensitivity and specificity in new diagnosis and remission assessments.

Results

The patient demographics for the MDS and CML patient samples are shown in Table 1. The sex and age distributions of the 20 patient samples comprising the control group were not significantly different from the combined neoplastic groups. The 20 control group samples were evaluated for CD114 expression density (MFI) on early blasts, late blasts, promyelocytes, and granulocytes, with group mean MFI and SD values calculated for each maturation stage. CD114 expression was initiated on early blasts with density increasing through the late blast stage (Table 2 and Image 1A). Peak expression density occurred at the promyelocyte stage (Table 2 and Image 1D), and a moderate decrease in CD114 density was observed on the maturing, post-promyelocyte granulocytes (Table 2 and Image 1D). The CD114 expression pattern mirrored CD33 (Image 1A) and was accompanied by peak CD117 density at the late blast stage (Image 1C). The mean ± SD MFI values for the control group were 0.69 ± 0.15, 2.45 ± 0.46, 3.79 ± 0.64, and 1.16 ± 0.22 for early blasts, late blasts, promyelocytes, and granulocytes, respectively (Table 2).

Nine of the 34 MDS cases evaluated showed no detectable CD33 expression (CD33– MDS) on the blast population and were excluded from early/late subset distribution and maturation stage density measurements. In the CD33-expressing MDS group (CD33+ MDS), mean CD114 MFI values for the defined myeloid maturation stages (Table 2) were statistically different from the control and CML groups only for the granulocyte stage (P = .004 and P = .03, respectively), which exhibited a higher mean density. In the CML group, we observed significantly decreased CD114 density in the CD33– early blasts (Table 2) in comparison to both control and MDS groups (P = .02 and P = .001, respectively) but not in the later maturation stages.

In the control group, 41.6% ± 6.2% of the CD34+ blasts expressed CD114. For the combined MDS group as well as the CD33+ MDS and CD33– MDS subgroups, there were no significant differences in the percentage of blasts expressing CD114 (Table 3). In the CML group, the mean percentage of blasts expressing CD114 (19.0% ± 7.5%) was approximately 50% of the control, but this did not reach statistical significance (P = .07). This change was, however, significantly different in comparison to the combined MDS group and the CD33– MDS subgroup (P = .04 and P = .03, respectively).

In the control group, 32.4% ± 15.2% of CD114+ blasts were CD33– (early), and 67.6% ± 15.2% were CD33+ (late) (Table 4). There were no significant differences in the mean distributions for the CD33+ MDS subgroup, but variance was much higher, as evidenced by an increase in the standard deviation. While performing analysis, we noted significant pattern differences in the distribution of blast CD114 expression in individual MDS cases that were not apparent in the group.
mean values. Examples of normal Image 2Bl and abnormal Image 2Cl and Image 2Dl patterns of CD114 distribution in individual MDS cases are illustrated in comparison with a normal control Image 2Al. The MDS group cases were partitioned based on early vs late subset distribution, but there was no association with the World Health Organization (WHO) MDS subtypes (not shown).

The CML group CD114+ blast subset distributions were statistically different in comparison to both the control group and the CD33+ MDS subgroup (P = .01 and P = .01, respectively) (Table 4). In the CML cases, CD114 expression was essentially absent on the early blasts (3.5% ± 4.0% and found exclusively on the late blasts (96.5% ± 4.0%). Most notably, in the CML cases, a distinct, abnormal pattern of blast differentiation Image 3Cl and Image 3Dl was apparent by comparison with the normal control pattern Image 3Al and Image 3Bl. The blasts exhibited a shift on the CD33 axis, with the late blasts showing peak density for both CD33 and CD34. Expression of CD114 was observed on this late blast population and was associated with retention of CD33 and loss of CD34 (arrow, Image 3C). An additional 6 historic cases of CML at presentation were evaluated, and the same dysmaturation pattern involving CD33 and CD34 was observed. Three of these additional cases are shown Image 4Bl, Image 4Cl, and Image 4Dl compared with normal historic bone marrow Image 4Al. In 1 case, a remission bone marrow was available for analysis, and reversion of the abnormal CD33/CD34 blast pattern was demonstrated following Gleevec therapy Image 5Al and Image 5Bl.

Following identification of the CML blast pattern, 6 new cases of CML were diagnosed by our hematopathology service for which bone marrow specimens were submitted for morphology, flow cytometry, and molecular detection of p210 BCR-Abl. Flow cytometry, which is interpreted by

![Image 1](image1.png)

**Table 3** Percentage of CD34+ Blasts Expressing CD114

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<td>Control (n = 20)</td>
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<td>CD33+ MDS (n = 25)</td>
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<td>CD33– MDS (n = 9)</td>
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<td>Combined MDS (n = 34)</td>
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<td>CML (n = 5)</td>
<td>19.0 ± 7.5b</td>
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CML, chronic myelogenous leukemia; MDS, myelodysplastic syndromes.

* Values are presented as mean ± SD.

**Table 4** Percentage of CD114+ Blasts That Are Early vs Late

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<th>Late (CD33+)</th>
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<td>Control (n = 20)</td>
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<td>CD33+ MDS (n = 25)</td>
<td>32.7 ± 24.1</td>
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<td>CML (n = 5)</td>
<td>3.5 ± 4.0b</td>
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CML, chronic myelogenous leukemia; MDS, myelodysplastic syndromes.

* Values are presented as mean ± SD.

b Statistically significant difference in comparison to the other 2 groups.
an independent pathologist, is reported at least 24 hours in advance of morphologic and molecular studies. Five of the 6 new cases of CML were identified by the interpreting flow cytometrist as having a blast maturation pattern consistent with or suggestive of CML. The sixth case was identified as having an abnormal blast phenotype and maturation profile consistent with a myelodysplastic/myeloproliferative disorder and not specifically CML. This case was ultimately called atypical CML by the independent morphologist.

The hematopathology service also received 17 remission assessment bone marrow specimens in patients with CML on antikinase therapy. Three of the 17 samples were interpreted

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**Image 2** Representative histograms demonstrating CD114+ blast subset distribution abnormalities in select myelodysplastic syndromes (MDS) cases (B-D). Normal bone marrow (A) is shown for comparison. Histograms are gated for CD117+ events, and blast CD114 expression is evident as red events. The antigen map is shown below the image with order of color precedence listed from top to bottom. In normal bone marrow (A), CD114 expression is initiated on the early blasts and accompanies CD34 downregulation and CD33 upregulation as the myeloblasts (red) mature to promyelocytes (green). In normal bone marrow, approximately one-third of the CD114-positive myeloblasts are early blasts (CD33–), and two-thirds are late blasts (CD33+). B, MDS case with a normal relative CD114+ blast subset distribution. C, MDS case illustrating late CD114 acquisition. The CD114+ blasts are 6% CD33– and 94% CD33+. D, MDS case with early CD114 acquisition. The CD114+ blasts are 67% CD33– and 33% CD33+.

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**Image 3** Representative histograms demonstrating the characteristic blast maturation abnormality observed in chronic myelogenous leukemia (CML) (C, D). Two normal bone marrow samples (A, B) are shown for comparison. Histograms are gated for CD117+ events, and the antigen map is shown below the image with order of color precedence from top to bottom. Blast CD114 expression is visualized as red events. In contrast to the normal maturation pattern seen in histograms A and B, the blast CD33 and CD34 profile in CML appears inverted, and the blasts exhibit peak density for CD33 and CD34 on the late blast subset. CD114 acquisition is observed primarily on the CD33- and CD34-bright blast population (arrowhead) and is accompanied by loss of CD34 expression.
as positive for the CML blast profile by flow cytometry and were also called positive by morphology. In 14 samples, the blast maturation profile was interpreted as normal by the flow cytometrist, and the bone marrow was also described as normal microscopically by the morphologist. One of those 14 samples had 15% p210 BCR-Abl-positive cells by quantitative real-time reverse polymerase chain reaction (rtPCR), and the case was ultimately signed out by the morphologist as minimally involved based on the molecular findings. Two of the 14 samples had p210 BCR-Abl-positive cells between 1% and 2% by quantitative rtPCR, and the remaining 11 samples were all less than 0.3%.

In that period, the hematopathology service evaluated 30 additional bone marrow specimens with a clinical history of leukocytosis (nonlymphoid). In none of the cases was the blast maturation profile described by the flow cytometrist as characteristic, indicative, or suggestive of CML, and none were ultimately diagnosed as CML by morphology or molecular analysis.

Discussion

The GCSF-R mediates granulocyte proliferation and differentiation in both normal and malignant hematopoiesis. The gene is located on chromosome 1p32-35 (CSF3R gene) and belongs to class I of the cytokine (hematopoietic) receptor superfamily. Human GCSF-R is composed of 813 amino acids with a molecular mass of 130,000 to 150,000 Da. The molecule comprises an immunoglobulin-like domain, a cytokine receptor homology domain, 3 fibronectin type III-like domains, and transmembrane and cytoplasmic domains. The GCSF-R exerts biologic effects via ligand (granulocyte colony-stimulating factor [G-CSF])–induced homodimerization of the receptor. The G-CSF/GCSF-R complex mediates survival, proliferation, and maturation of neutrophils and regulates granulopoiesis under both steady-state and stress conditions. Cell signaling via GCSF-R is mediated primarily by the Jak/STAT pathway, but the Ras/Raf/MAP kinase and PKB/Akt pathways are also involved in signal transduction.
Mutations of the GCSF-R gene have been well-documented in congenital neutropenia as well as the associated predisposition to myelodysplasia and acute myeloid leukemia. A single-nucleotide polymorphism in the GCSF-R gene has also been linked to predisposition to high-risk MDS.

In the present study, we characterized maturation-related surface density and distribution of CD114 in normal hematopoiesis. Our interest was in providing further detail in blast maturation that would be useful in the phenotypic diagnosis of myelodysplastic and myeloproliferative disorders. Abnormalities in CD114 expression in leukemia cells, leukemic cell lines, and MDS have been reported by several groups.

The pattern of CD114 expression in normal hematopoiesis was similar to that reported in earlier studies using ligand binding detection methods. We found that CD114 expression is initiated on early blasts and increases in density through the late blast stage, with peak density occurring at the promyelocyte stage. We noted a subsequent decrease in CD114 density on the maturing granulocytes. Shinjo et al used fluorescently labeled G-CSF in flow cytometric analysis and, similar to our findings, reported the CD114 density pattern on myeloid precursors cells as follows: CD33–CD34+ (early blasts) < CD33+CD34+ (late blasts) < CD33+CD34– cells (promyelocytes). In contrast to our findings, they reported that CD114 expression density was highest on mature granulocytes. We measured CD114 expression density on ficolled bone marrow specimens containing only a small proportion of mature granulocytes. It is possible that CD114 density is higher on pure populations of fully mature granulocytes than on promyelocytes, but this seems unlikely given the observed density decrease on the post-promyelocyte stages that we observed. Shinjo et al also used ligand binding as a detection method, and it is possible that a change in receptor affinity could account for this discrepancy. Begley et al evaluated receptor expression via binding of 125I-labeled murine G-CSF. Similar to our findings, using direct receptor detection methods, they reported the highest CD114 density on human promyelocytes.

We observed similar mean CD114 density values for the defined myeloid maturation stages in the MDS groups in comparison with the control group. The MDS group stages, however, exhibited higher variance in expression density. Kimura and Sultana also reported more variation in CD114 density on the CD34+ blast populations in MDS. They observed that higher-grade MDS cases showed the highest CD114 density variation (either increased or decreased) in comparison with the control group. During case analysis, we observed individual MDS cases with significantly increased or decreased CD114 density in comparison with the control group. We partitioned the MDS cases based on CD114 density on the CD34+ blast population but observed no relationship to WHO MDS subtype (not shown).

In the CML cases, we observed significantly decreased mean CD114 density on early blasts in comparison with both the control and MDS groups but not on the later myeloid maturation stages. Lee et al performed flow cytometric analysis of peripheral blood samples in CML using an anti-CD114 antibody. They compared receptor density on mature granulocytes and monocytes in CML with a normal control group and reported a statistically significant lower mean CD114 receptor density on the MDS granulocytes but not on the monocytes. They did not, however, evaluate earlier stages of myeloid differentiation. We observed lower mean CD114 density at all differentiation stages in the CML group in comparison with the control and MDS groups. In contrast to Lee et al, when comparing the control and CML groups, the smallest difference we observed was for CD114 density on the granulocytes.

In our control group, we observed that approximately 40% of the CD34+ blasts were expressing CD114. Yue et al reported a similar distribution in normal bone marrow, with 39% of blasts positive for CD114 expression. In contrast, Xu et al reported a significantly lower percentage, with only 21% of CD34+ blasts expressing CD114. In the MDS groups, we observed a similar mean percentage of CD34+ blasts expressing CD114 in comparison with the control group but with a higher variance. Yue et al reported a lower percentage of CD114+ blasts in the MDS cases in comparison with their control group, while Xu et al reported a higher percentage. The reported differences were not statistically significant in either study.

Neither Xu et al nor Yue et al evaluated the distribution of CD114+ blasts between the CD33– and CD33+ blast subsets. We found no significant difference in the mean distribution of CD114+ blast subsets comparing the CD33+ MDS subgroup with the control group. We did, however, note that due to the higher variance in the MDS group, the observable patterns of CD114+ blast subset distributions were informative in a significant number of MDS cases.

In the CML group, we found that the percentage of CD34+ blasts expressing CD114 was approximately 50% of the control group, but this did not quite reach statistical significance. This reduced percentage in the CML group was, however, significantly different from the combined MDS group and the CD33– MDS subgroup. In addition, the CML cases exhibited delayed CD114 receptor acquisition, as evidenced by statistically significant differences in the distribution of CD114-expressing blasts between the early and late blast compartments in comparison with both the control group and the CD33+ MDS subgroup.

In CML, the BCR-Abl fusion protein is associated with inhibition of GCSF-R (CD114) expression in response to G-CSF stimulation. Several groups have demonstrated that BCR-Abl inhibits expression of the C/EBPα enhancer binding protein transcription factors C/EBPα and C/EBPε, which...
are responsible for upregulation of GCSF-R transcription. C/EBPα knockout mice display a complete differentiation arrest of the myeloid series, and C/EBPβ knockout mice show aberrant granulocyte differentiation due to the inhibition of CD114 expression.

We observed a decreased percentage of CD114-positive blasts, delayed CD114 receptor acquisition, and decreased mean receptor density in the 5 CML cases evaluated. We observed a unique blast maturation abnormality involving CD33, CD34, and CD114, which was corroborated in an additional 6 historic cases of CML. Furthermore, the abnormal CD33 and CD34 maturation profile reverted to a normal pattern following suppression of the BCR-Abl–positive clone with Gleevec therapy. These observations indicate that the blast maturation profile in CML is likely linked to the documented inhibition of GCSF-R expression by BCR-Abl. In practice, the CML blast pattern in bone marrow was 100% specific and 83% sensitive in the diagnosis of CML when cases of nonlymphoid leukocytosis were evaluated. Sensitivity in CML remission assessment was as good as morphology but clearly no substitute for quantitative RT-PCR.

Drawbacks of this study include the small number of cases evaluated for GCSF-R expression and the absence of low-grade MDS cases such as refractory anemia (RA). There was a selection bias to collect higher-grade cases with more evident phenotypic abnormalities in the study design. Low-grade MDS cases are difficult diagnostically, and our experience is that only a small percentage of RA cases have sufficient phenotypic abnormalities to be unambiguously identified as low-grade MDS, even by highly trained analysts. We suspect that significant abnormalities of GCSF-R expression in RA would, like other phenotypic abnormalities, be of low frequency, but that remains to be demonstrated.

High-resolution analysis of CD34+ blast maturation can significantly enhance flow cytometric detection of myelodysplastic and myeloproliferative disorders.12,16,17,19,24 The GCSF-R (CD114) has a characteristic maturation-related expression profile in granulopoiesis and can provide additional phenotypic detail in flow cytometric assessment of CD34+ precursor maturation.

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References


