Characteristic repartition of monocyte subsets as a diagnostic signature of chronic myelomonocytic leukemia

Running head: Flow cytometry signature of CMML

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Increase in the classical monocyte subset over 94% of circulating monocytes discriminates CMML from other monocytoses with high specificity

This characteristic increase in classical monocytes disappears in CMML patients who respond to hypomethylating agents.
Abstract

Chronic myelomonocytic leukemia (CMML) is a myelodysplastic syndrome/myeloproliferative neoplasm whose diagnosis is currently based on the elevation of peripheral blood monocytes to more than $1.1 \times 10^9$/L, measured over at least 3 months. Diagnosis can be ambiguous, e.g. with pre-fibrotic myelofibrosis or reactive monocytosis. We set up a multi-parameter flow cytometry assay to distinguish CD14+/CD16- classical from CD14+/CD16+ intermediate and CD14low/CD16+ non-classical monocyte subsets in peripheral blood mononucleated cells and in total blood samples. Compared to healthy donors and patients with a reactive monocytosis or another hematological malignancy, CMML patients demonstrate a characteristic increase in the fraction of CD14+/CD16- cells (cut-off value, 94.0%). The associated specificity and sensitivity were 95.1% and 90.6% in the learning set (175 samples), 94.1% and 91.9% in the validation set (307 samples), respectively. The accumulation of classical monocytes, which demonstrate a distinct gene expression pattern, is independent of the mutational background. Importantly, this increase disappears in patients who respond to hypomethylating agents. We conclude that an increase in the fraction of classical monocytes over 94.0% of total monocytes is a highly sensitive and specific diagnostic marker that rapidly and accurately distinguishes CMML from confounding diagnoses.
Introduction

According to the World Health Organization (WHO) classification, the diagnosis of chronic myelomonocytic leukemia (CMML), which is the most frequent myelodysplastic syndrome/myeloproliferative neoplasm (MDS/MPN), is based on the elevation of peripheral blood monocytes to more than 1.10⁹/L, measured over at least 3 months.¹² Bone marrow cell dysplasia, which enforces the diagnosis when present, is not an absolute requirement.¹ Some patients with reactive monocytosis can fulfill this criterion whereas patients with MDS and a low white blood cell (WBC) count but as much as 80% of monocytes do not.

The Nomenclature Committee of the International Union of Immunological Societies has approved a nomenclature that subdivides monocytes into three subsets.³ This subdivision was validated by gene expression profiling.⁴–⁶ Specifically, the expression of CD14, a receptor for bacterial lipopolysaccharides, and CD16, which is the low affinity receptor for IgG (Fcγ-III receptor), distinguishes CD14⁺/CD16⁻ (classical) from CD14⁺/CD16⁺ (intermediate) and CD14low/CD16⁺ (non-classical) human monocytes.³ Classical monocytes constitute the major population of human monocytes (~85%) in healthy conditions.³ These subsets differ in their chemokine receptor expression and phagocytic activity,³,⁵,⁷ i.e. CD14⁺/CD16⁻ monocytes express high levels of CCR2 and low levels of CX3CR1 whereas CD16⁺ monocytes express high levels of CX3CR1 and low levels of CCR2.⁸–¹⁰ Akin to lymphocytes, these subsets may be endowed with specific functions.⁴–⁶,¹¹,¹²

Here, we demonstrate that an increase in the fraction of classical CD14⁺/CD16⁻ monocytes at the expense of intermediate and non-classical monocytes is a highly specific marker that rapidly, accurately, and simply distinguishes CMML from
confounding diagnoses. Interestingly, the monocyte subset repartition is normalized in patients who respond to hypomethylating agents.
Material and methods

Patients and samples

Peripheral blood samples were collected on EDTA after informed consent according to the Declaration of Helsinki. A learning cohort included patients with a CMML diagnosis according to the WHO criteria (n=53), age-matched healthy donors (n=39), young healthy blood donors (n=26), patients with reactive monocytosis (n=33), or non-CMML hematological malignancies (n=24), including myeloproliferative neoplasms (MPN, n=12), myelodysplastic syndrome (MDS, n=7), juvenile myelomonocytic leukemia (n=1) and lymphoid malignancies (n=4). CMML patients were enrolled between 2012 and 2013 in a non-interventional study initiated by the Groupe Francophone des Myélodysplasies (GFM) and approved by the ethical committee of Cochin Hospital, according to current regulations and ethical concerns. A validation cohort included CMML patients enrolled between 2013 and 2015 (n=86), age-matched healthy donors (n=68), patients with reactive monocytosis (n=74), or a diagnosis of MDS (n=65) or MPN (n=12) or MPN/MDS (1 atypical CML, 1 juvenile myelomonocytic leukemia) referred to as “non-CMML”. Detailed characteristics of these groups are in table 1. Cytogenetic risk was classified according to the Spanish CMML classification.13 Gene mutations were screened as described.14

Multi-fluorochrome staining and analysis of monocytes subsets

Flow cytometry analysis of monocyte subsets was performed following consensus recommendations.15,16 Peripheral blood mononuclear cells (PBMC) were sorted17 and labeled with antibodies (Supplementary table1). At least 60,000 events in the CD14+/CD16− gate (Supplementary Figure 1) were analyzed by flow cytometry (LSRII, BD Biosciences). Whole peripheral blood (200μL) cells were labeled with antibodies (Supplementary table1) following a lyse/no wash procedure (Versalyse, Beckman
Coulter). At least 50,000 events in the CD14+/CD16- gate (Supplementary Figure 2) were analyzed (Navios, Beckman Coulter). The settings were harmonized between instruments. Flow cytometry standard listmode data were analyzed centrally in a blind fashion using Kaluza software (Beckman Coulter). The monocyte subsets were identified following an exclusion gating strategy (Supplementary Figures 1-2). Repeatability and reproducibility of the analytic strategy was validated on 20 randomly chosen samples, as described in Supplementary methods.

**Cell sorting, cytological analysis and RNA-sequencing**

PBMC were stained with CD45, CD24, CD14, CD16, CD2 and CD56 antibodies and monocytes subsets were sorted (Influx cytometer, BD Biosciences), centrifuged on microscope slides, dried for 1h at room temperature, and stained with May-Grünwald-Giemsa. RNA sequencing was performed on sorted CD14+/CD16- monocytes from healthy donors (n=4) and patients with a reactive monocytosis (n=4) or a CMML (n=6). After having checked RNA integrity on Agilent 2100 Bioanalyzer (Agilent; Score ≥ 7.0), poly-A mRNA was fragmented, converted into double stranded DNA and used for library preparation using SureSelect Automated Strand Specific RNA Library Preparation Kit. The libraries were bar-coded, purified, pooled in equal concentrations and subjected to paired-end sequencing on HiSeq-2000 sequencer (Illumina). Bioinformatic analysis is described in Supplementary methods.

**Statistical analysis**

Principal component analysis was performed using SPADE (Spanning-tree Progression Analysis of Density-normalized Events) algorithm (Cytobank software). The non-parametric Kruskal-Wallis test was used to compare distributions between groups. Receiver Operator Characteristic curves (ROC) representing the relationship between sensitivity and specificity were compared with a nonparametric approach.
A cutoff was estimated in the training cohort by maximizing the Youden index ($J = \text{Sensitivity} + \text{Specificity} - 1$). The classification performance of the estimated cut-off was assessed in the validation cohort and compared to the classical monocyte count cut-off of $1.10^9/L$. The performance of using both MO1 percentage and monocyte counts for the classification of patients with a CMML or not a CMML in the validation cohort was further assessed with a multivariate logistic regression model. Analyses were done with SAS® version 9.3 (SAS, Cary, NC) and $P$ values lower than 0.05 were considered significant.
Results

Flow cytometry identification of monocytes subsets

The repartition of monocyte subsets in PBMC from healthy donors was analyzed by flow cytometry. We used an exclusion gating strategy (Supplementary Figure 1) to identify monocytes and to separate CD14+/CD16- classical monocytes (herein called “MO1”) from CD14+/CD16+ intermediate (“MO2”) and CD14low/CD16+ non-classical (“MO3”) monocytes (Figure 1A). Cytological examination showed that each sorted population consisted almost exclusively of monocytes, which were absent from the double negative cell population (Figure 1B). Variations in the expression of monocyte subset specific markers were validated by RT-qPCR or flow cytometry (Supplementary Figures 3A-B). We used a computational approach to objectively organize our cytometry data into a hierarchy of related phenotypes in an unsupervised and unbiased manner. SPADE analysis automatically formed a tree in which monocytes were separated from other blood cells and clustered as three subsets with distinct expression of CD14, CD16, CCR2 and CX3CR1 expression (Figure 1C). All these results enforced the relevance of our gating strategy. Finally the flow cytometry assay was adapted to eliminate the mononuclear cell-sorting step and measure the monocyte subset fractions on whole blood samples (Supplementary Figure 2).

A CMML signature defined by monocyte subsets

The percentage of MO1 cells in the peripheral blood monocyte population was 86.1% (SD 4.3%; 95%IC: 78.0-92.0%) in young healthy blood donors, and 84.0% (SD 6.9%; 95%IC: 71.8-93.6%) in age-matched healthy subjects (Figure 2A-B). No significant difference was observed between these two groups. Compared to controls, CMML patients demonstrated an increased percentage of MO1 and a decreased fraction of
MO2 and MO3 monocyte subsets (Figure 2B). The percentage of MO1 cells, assessed in a learning cohort of 53 CMML patients, was significantly higher than in healthy controls, reaching 96.6% (SD 1.7%; 95%IC: 92.9-99.1%, Figure 2A). The use of the SPADE algorithm independently showed an amplification of the MO1 at the expense of the MO2 and MO3 subsets (Supplementary Figure 4A-B).

To determine whether this abnormal percentage of MO1 cells was specific of CMML, we measured the percentage of MO1 cells in the peripheral blood of patients with either reactive monocytosis (MO1: 79.1%; SD 10.5%; 95%IC: 67.4-93.7%) or another hematological malignancy, regardless of the monocyte count (MO1: 84.4%; SD 10.62%; 95%IC 70.2-96.9) (Figure 2A-B and Supplementary Figure 5). The proportion of MO1 cells in the monocyte compartment was significantly higher in CMML patients than in any other studied groups (P<0.0001, Kruskal-Wallis test). Differences between groups of the learning cohort but the CMML one were not significant.

The combination of CCR2 (CD192) and CX3CR1 expression also distinguishes three monocyte subsets in healthy donors, namely CCR2+/CX3CR1−, CCR2+/CX3CR1+, and CCR2low/CX3CR1+ subsets. A strong overlap between populations identified by the CD14/CD16 and the CCR2/CX3CR1 surface marker combinations was observed. CMML patients demonstrated an increased fraction of CCR2+/CX3CR1− cells and a decreased fraction of CCR2+/CX3CR1+ and CCR2low/CX3CR1+ cells, respectively (Supplementary Figure 4C), suggesting that the increase in MO1 fraction was not the consequence of a CMML-associated decrease in CD16 expression.

**MO1 percentage as a sensitive and specific tool for CMML diagnosis**

To further explore whether the increased MO1 monocyte subset could be helpful to diagnose CMML, we performed a ROC analysis using the learning cohort data. The
area under the ROC curve (AUC) was 0.977 (95% Wald confidence limits: 0.96-0.995, Figure 2C), indicating that the percentage of MO1 monocytes could be used to distinguish CMML from any other situation. The Youden index was defined for all points of the ROC curve in the learning population. The maximum value of the index was used as a criterion for selecting the optimum cut-off point of MO1 percentage to identify CMML. A cut-off value of 94.0% was calculated with a specificity of 95.1% and a sensitivity of 90.6%. We validated the cut-off value in the independent cohort (Figure 2D). In this validation cohort, the specificity and sensitivity of the “MO1 fraction >94.0%” criterion were 94.1% and 91.9%, respectively. MO1 percentage was 96.4% (SD 2.5%; 95%IC: 87.1-99.1%) in CMML, 85.1% (SD 5.6%, 95%IC: 69.5-93.8%) in age-matched healthy controls, 85.8% (SD 9.9%; 95%IC: 52.5-96.6%) in patients with a reactive monocyteosis and 81.9% (SD 11.5%, 95%IC: 49.3-95.7%) in non-CMML patients. Of note, we also tested the MO1/MO3 ratio in the learning and the validation cohort; this ratio was not statistically more efficient than MO1 percentage to identify CMML patients.

CMML diagnostic is currently based on the elevation of peripheral blood monocytes to more than 1.10^9/L. We performed a ROC analysis on the subset of patients with a monocyte count >1.10^9/L in the learning cohort. The AUC of the ROC curve drawn in a monocytosis context was 0.995 (95% Wald confidence limits: 0.988-1.00) (Supplementary Figure 6). The specificity and the sensitivity for a MO1 percentage cut-off point of 94.0%, were 100% and 90.4%, respectively. The validation of this cut-off in the patients of the validation cohort with a monocyte count >1.10^9/L was 94.5% and 91.7% for specificity and sensitivity, respectively. These data indicate that the MO1 percentage could be used to improve CMML diagnosis in this specific subset of patients with an elevated monocyte count.
**CMML patients accumulate abnormal classical monocytes.**

The MO1 percentage for CMML patients was observed to be independent of the absolute number of circulating monocytes (Figure 3A), the gene mutation pattern (Table 1 and not shown), the proliferative versus dysplastic status of the disease according to the FAB criteria (leukocyte count cut-off value $13 \times 10^9$/$L$, not shown), and the disease subtype (type 1 versus type 2) according to WHO criteria (not shown). The characteristic repartition of monocyte subsets in the peripheral blood of CMML patients reflects an increase in the absolute number of MO1 together with a decrease in the absolute number of MO3 monocytes as compared to monocyte subsets in healthy donor peripheral blood. Conversely, patients with a reactive monocytosis show a significant increase in the number of cells in the MO1 and MO3 monocyte subsets (Figure 3B). In 18 CMML patients, the increased percentage of MO1 cells identified in the peripheral blood was also observed in the bone marrow (not shown). Therefore, the decrease in MO3 cells observed in the blood of CMML patients may not related to their bone marrow retention.

CMML MO1 cells did not show major cytological alteration compared to healthy donor MO1, although the monocytes nuclei look more undifferentiated in CMML patients (Supplementary Figure 7A). RNA sequencing analysis of MO1 subset sorted from healthy donors, patients with a reactive monocytosis, and CMML patients showed a distinct pattern of gene expression in the three situations, respectively (Figure 3C, Supplementary Figure 7B). Moreover, the expression of CD56, CD115, and CD62L was significantly higher at the surface of CMML MO1 cells compared to healthy donors MO1 cells (Figure 3D).

**Monocyte subset profile is a biomarker of CMML response to demethylating agents**
The increase in MO1 monocyte subset remained constantly higher than 94.0% in 21 untreated CMML patients repeatedly analyzed for up to 26 months (Figure 4A). We observed also a normalization of monocyte subset repartition, together with a decrease in the monocyte count below $1.10^9$/L, in seven CMML patients who responded to azacytidine therapy (Figure 4B, exemplified in 4C, and Supplementary Figure 8A), whereas monocyte repartition remained unchanged in non-responding patients (Figure 4D) and a characteristic phenotype re-appeared in a patient who relapsed after initial response (Figure 4E). Lastly, in a CMML patient who relapsed on azacytidine therapy, we observed a normalization of monocyte count and MO1 fraction in response to decitabine (Supplementary Figure 8B). Altogether, these results indicate that the monocyte subset repartition could be used as a biomarker of demethylating agent activity in CMML patients.

Monocyte subset profile is more efficient than monocyte count to diagnose CMML

Finally, we compared the “MO1 percentage > 94.0%” and the “monocyte count >1.10^9/L” as criteria to identify CMML at initial work up, always using the WHO criteria to validate CMML diagnosis. For that purpose, we used the entire validation cohort (that includes 65 MDS). The AUC of the ROC curve was 0.968 with the percentage of MO1 (95% Wald confidence limits: 0.946-0.99) and 0.836 with the monocyte count (95% Wald confidence limits: 0.792-0.88) (Figure 5A). The difference between the ROC curves was highly significant (P<10^-4, Chi-square). The specificity and sensitivity of MO1 percentage were 94.1% and 91.9% respectively, whereas the specificity and sensitivity of the monocyte count were 95.3% and 62.0%, respectively. These results demonstrate that the MO1 percentage is a better parameter than the monocyte count to diagnose a CMML at initial workup. In a
multivariate logistic regression model, in which both MO1 percentage and monocyte count were entered, only the MO1 percentage was retained to classify the patients in the CMML group.

Looking in more details to 11 MDS patients with a MO1 fraction higher than 94.0%, all had a fluctuating monocyte count around 1.10^9/L, precluding the diagnosis of CMML according to the WHO (exemplified in Figure 5B). These observations suggest that the flow cytometry assay identifies CMML in situations in which the WHO criteria are (still) not fulfilled. Conversely, in a patient classified as CMML-1 according to the WHO that we serially explored over a two-year period (Figure 5C), we never detected the characteristic MO1 accumulation and bone-marrow examination revealed a sideroblastic anemia with mutations in *SF3B1* and *DNMT3A* genes. Further investigation may distinguish a CMML with an unusual phenotype, e.g. due to a specific background of genetic alterations, from a sideroblastic anemia with monocytosis.21,22

**Discussion**

We show that CMML is characterized by an increase in the fraction of classical CD14^+/CD16^- cells (MO1) among circulating monocytes, whatever the genetic background of the disease. This increase can be rapidly identified using a robust multi-parameter flow cytometry assay performed on peripheral blood, and distinguishes CMML from reactive monocytosis and myeloid malignancies with a borderline monocyte count. Importantly, normalization of the monocyte subset repartition could be used as a biomarker of treatment efficacy in this disease.
The development and biological significance of monocyte subsets remains a matter of active investigation.\textsuperscript{9,23–28} Whatever their respective functions and their developmental relationships, CD16\textsuperscript{+} monocyte subsets expand in a variety of clinical situations,\textsuperscript{29} including auto-immune diseases,\textsuperscript{30} bacterial and viral infections,\textsuperscript{31–33} asthma,\textsuperscript{34} stroke, and coronary artery disease\textsuperscript{35} Conversely, a reduction in MO1 monocytes was observed after treatment with intravenous immunoglobulins\textsuperscript{36} or blockade of the M-CSFR pathway.\textsuperscript{37} Nevertheless, an increase in the MO1 subset had never been associated with a human disease. MO1 monocytes express high levels of CCR2, the receptor of the cytokine MCP-1, whereas CD16\textsuperscript{+} monocytes express high levels of the fractalkine receptor CX3CR1.\textsuperscript{9} The expression of these two surface markers distinguishes the three monocyte subsets in a manner similar to CD14 and CD16. A loss of CD16 expression was described in paroxysmal nocturnal hemoglobinuria\textsuperscript{38} and in patients with a genetic polymorphism.\textsuperscript{39} Since the increased fraction of MO1 in CMML patients was detected with the CCR2/CX3CR1 combination, it may not be related to an altered regulation of CD16 expression in the context of monocyte dysplasia.\textsuperscript{40} This accumulation of MO1 fraction could indicate the abnormal differentiation of specific monocyte subsets, as observed in Nr4a1\textsuperscript{−/−} mice.\textsuperscript{41}

CMML-associated monocyte dysplasia can be difficult to assess morphologically. We have shown previously that CMML cells identified morphologically as monocytes could include a variable fraction of immature granulocytes endowed with immunosuppressive properties.\textsuperscript{17} The aberrant expression of CD56 was shown to be a characteristic feature of monocyte dysplasia in a fraction of CMML patients, together with the decreased expression of HLA-DR and the aberrant expression of CD2.\textsuperscript{42–49} We observed an aberrant expression of CD56, CD62L, and CD115 on MO1 monocytes that accumulate in CMML patients. The dysplastic features of these
cells were further indicated by the principal component analysis of gene expression that separated CMML from healthy donor cells. Interestingly, MO1 monocytes sorted from reactive monocytosis blood samples demonstrated a pattern of gene expression that was distinct from both healthy and CMML MO1.

Mutations in epigenetic modifying enzymes such as \textit{TET2} and \textit{ASXL1} are highly prevalent in CMML\textsuperscript{20,50} and associated with DNA hypermethylation\textsuperscript{51} (Meldi K et al, submitted), whereas epigenetic extinction of specific genes such as \textit{TRIM33} might play a role in disease occurrence or progression.\textsuperscript{52} We have shown that re-expression of \textit{TRIM33} could be used as a biomarker of response to the nucleoside analog decitabine.\textsuperscript{52} Here, we show that the restoration of a normal monocyte subsets repartition in the peripheral blood is another biomarker of response to demethylating drugs. Flow cytometry analysis of monocyte subsets might be useful to monitor the efficacy of other therapeutic approaches tested in CMML.

Altogether, while the diagnostic hallmark of CMML is a blood monocytosis for at least three months, the reliable identification of CMML can become challenging when dysplasia is not prominent. Identification of clonal genetic abnormalities can support CMML diagnosis in these confounding situations but none is specific. Given its simplicity and robustness, the flow cytometry demonstration of an increased fraction of classical monocytes may become an essential argument for CMML diagnosis and replace the three-month delay currently recommended by the WHO to diagnose this disease.
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Authorship Contributions

D.S-B. designed and performed experiments, analyzed results, and wrote the manuscript. O.W-B. designed and performed experiments, wrote the manuscript. V.S., V.B., L.B., C.D. and E.B. performed experiments. M.M. performed sample collection. E.M. collected patient informations. R.I., A-M. N., P.F., T.B., C.W., B.Q. L.A. and M.F. provided samples. P.R. performed cell sorting and analyzed results. N.D. designed and performed experiments, supervised genotyping and RNA sequencing analysis. S.K. performed statistical analysis, analyzed results and wrote the manuscript. E.S. provided samples, analyzed results, supervised the work, and wrote the manuscript. All authors approved the final manuscript.

Conflict of interest disclosures

The authors declare no competing financial interests.

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References


developmental relationship and distinct biological functions of CD16+ and CD16-


Legends

Figure 1: Monocyte subsets in peripheral blood mononucleated cells (PBMC). PBMC from a representative healthy blood donor were explored. A. PBMC were labeled with anti-CD45, -CD24, -CD14, -CD16, -CD56, -CD115, -CD62L, -CD64, -CCR2 and -CX3CR1 antibodies. Monocytes were identified using an exclusion gating strategy (described in Supplementary figure 1) and subsets were separated on CD14 and CD16 expression. MO1: classical CD14+/CD16- monocytes; MO2: intermediate CD14+/CD16+ monocytes; MO3 CD14low/CD16+ monocytes. The fraction of each subset is indicated. B. MGG staining of sorted MO1, MO2 and MO3 cells (gates defined in A), DN: remaining CD14-/CD16- cells. C. Multiparametric analysis of single cells monitored with 10 surface markers (Supplementary table 1) using SPADE algorithm. SPADE organizes cells in a hierarchy of related phenotypes. Flow cytometry data from 19 healthy donors PBMC were gated on morphology, then on CD45+/SSC intermediate and used to construct the SPADE tree that automatically separates, based on the hierarchy of related phenotypes, MO1 (CD14+/CD16-), MO2 (CD14+/CD16+), MO3 (CD14low/CD16+), Natural Killer cells (NK, CD56+), B-lymphocytes (CD24+), and residual granulocytes (Gran, CD24+/CD16+). The fraction of each subset in the monocyte population is indicated. Circles indicate the size of cell populations and colors are based on CD14 expression. D. Color representation of CD16, CCR2, or CX3CR1 expression in the monocyte subsets delineated in the SPADE tree.

Figure 2: Abnormal repartition of monocyte subsets in CMML. A. Percentage of classical MO1 monocytes in a learning cohort of CMML compared to healthy blood donors (Co), age-matched healthy donors (Aged-Co), patients with diverse hematological malignancies (Non-CMML), and those with a reactive monocytosis.
(Reactive). Mean ± SEM is shown; *** P<0.0001, Kruskal-Wallis test. B. Multicolor representation of monocyte subset repartition in PBMC collected from the distinct groups of the learning cohort. Percentage of each monocyte subset is indicated. C. Receiver operating characteristic (ROC) curve analysis of diagnostic sensitivity and specificity of MO1 percentage in peripheral blood monocytes, established on the learning cohort (young and aged-match controls, other hematological malignancies, reactive monocytosis and CMML defined in A.). D. Percentage of MO1 monocytes in a validation cohort of CMML compared to age-matched healthy donors (Aged-Co), patients with a myelodysplastic syndrome or a myeloproliferative neoplasm (Non-CMML), and those with a reactive monocytosis (Reactive). Mean ± SEM is shown.

**Figure 3: CMML patients accumulate abnormal classical at the expanse of non-classical monocytes. A.** Lack of significant correlation between the percentage of MO1 classical monocytes and peripheral blood monocyte count in reactive monocytosis (black circles) and CMML (red open circles) samples. Reactive monocytoses and CMML from learning and validation cohorts were pooled. B. Absolute number of MO1 and MO3 monocyte subsets in the peripheral blood of CMML patients compared to age-matched controls (Aged-Co), non-CMML patients, and patients with a reactive monocytosis. The learning and validation cohorts were pooled (*** P<0.0001; Student t-test). C. Component principal analysis of gene expression after RNA sequencing (DESeq2 analysis) in sorted classical monocytes (MO1) sorted from the blood of healthy controls (green) and patients with a reactive monocytosis (blue) or a CMML (red). D. Heatmap established by using Cytobank software to summarize the flow cytometry analysis of 8 markers at the surface of MO1 monocyte subset of six age-matched healthy donors and 17 CMML samples.
Figure 4: Monocyte subset profile as a biomarker of disease evolution. A. Repeated evaluation of MO1 subset fraction in 21 untreated CMML patients followed from 6 to 26 months. B. Evaluation of MO1 subset fraction in seven CMML patients before and after treatment with azacytidine (AZA). These seven patients responded to treatment.\textsuperscript{11} C, D, E. Evolution of monocyte subset repartition and monocyte count in CMML patients during treatment with a demethylating agent. C. Patient who responded to azacytidine. D. Patient who only transiently responded to azacytidine. E. Patient who relapsed after initial response.

Figure 5: Comparison between monocyte subset profile and monocyte count diagnosis. A. ROC curves of the “MO1 percentage > 94.0\%” and the “monocyte count >1.10^9/L” criteria to identify CMML at initial work up in the validation cohort. The difference between the two curves was highly significant (P<10^{-4}, Chi-square). B. Monocyte subset repartition analysed in two independent samples obtained from three patients classified as MDS and showing a fluctuating monocyte count around 1.10^9/L that precludes their classification as CMML according to the WHO criteria. C. Monocyte subset repartition analysed in two independent samples obtained from a patient who could be classified as a CMML according to the WHO but rather demonstrates a sideroblastic anemia with monocytosis, based on cytological and molecular analyses.

Table 1: Baseline characteristics of the studied patients and age-matched controls. The characteristics of younger healthy donors, who are voluntary blood donors, are unknown. In these series, CMML patients were studied before any treatment.
Selimoglu-Buet D et al, Table 1. Baseline characteristics of the patients in learning and validation cohorts

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<td>Mutations (48 CMML)</td>
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<tr>
<td>TET2 (mutated/ studied)</td>
<td>36/ 48</td>
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<td>SRSF2 (mutated/ studied)</td>
<td>21/ 48</td>
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<td>ASXL1 (mutated/ studied)</td>
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<td>RAS Path (CBL+NRAS+KRAS)(mut/ studied)</td>
<td>12/ 48</td>
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<tr>
<td>AML1 (mutated/ studied)</td>
<td>6/ 48</td>
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<td>JAK2 (mutated/ studied)</td>
<td>1/ 48</td>
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<tr>
<td>IDH1/IDH2 (mutated/ studied)</td>
<td>1/ 48</td>
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<td>ZRSR2 (mutated/ studied)</td>
<td>4/ 48</td>
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<td>DNMT3A (mutated/ studied)</td>
<td>3/ 48</td>
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<td>EZH2 (mutated/ studied)</td>
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<tr>
<td>SF3B1 (mutated/ studied)</td>
<td>1/ 48</td>
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<tr>
<td>FLT3 (mutated/ studied)</td>
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<tr>
<td>NPM1, c-KIT, U2AF35 (mutated/ studied)</td>
<td>0/ 48</td>
<td></td>
</tr>
</tbody>
</table>
Selimoglu-Buet D et al, Figure 1

(A) CD16 vs CD14 plot showing MO1, MO2, MO3, DN, and MO1 subpopulations.

(B) Microscopy images of MO1, MO2, and MO3.

(C) Heatmap and scatterplot showing NK, T cells, B cells, and residual granules.

(D) Heatmap showing CD16, CX3CR1, and CCR2 expression levels.
Selimoglu-Buet D et al, Figure 3

A

Monocyte count (10^9/L)

MO1 (% in total monocytes)

B

Number of MO1 (10^9/L)

NS

***

C

PC2: 18% variance

PC1: 25% variance

D

CMML

Co

CD14
CD16
CCR2
CX3CR1
CD64
CD62L
CD115
CD56
Characteristic repartition of monocyte subsets as a diagnostic signature of chronic myelomonocytic leukemia


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