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Heterogeneous expression of cytokines accounts for clinical diversity and refines prognostication in CMML

Sandrine Niyongere[1], **Nolwenn Lucas**[2], **Jun-Min Zhou**[3], **Samer Sansil**[4], **Anthony D. Pomicter**[5], **Maria E. Balasis**[1], **John Robinson**[4], **Jodi Kroeger**[4], **Qing Zhang**[1], **Yu Long Zhao**[1], **Markus Ball**[1], **Rami Komrokji**[1], **Alan List**[1], **Michael W. Deininger**[5,6], **Brooke L. Fridley**[3], **Valeria Santini**[7], **Eric Solary**[2,8], **Eric Padron**[1]

[1]Department of Malignant Hematology, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

[2]INSERM U1170, Gustave Roussy Cancer Center, Villejuif, France

[3]Department of Biostatistics and Bioinformatics, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

[4]Flow Cytometry Core, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

[5]Huntsman Cancer Institute, The University of Utah, Salt Lake City, UT, USA

[6]Division of Hematology and Hematologic Malignancies, The University of Utah, Salt Lake City, UT, USA

[7]Hematology, AOU Careggi, University of Florence, Florence, Italy

[8]Hematology Departement, Gustave Roussy Cancer Center, Villejuif, France

Abstract

Chronic myelomonocytic leukemia (CMML) is a clinically heterogeneous neoplasm in which JAK2 inhibition has demonstrated reductions in inflammatory cytokines and promising clinical activity. We hypothesize that annotation of inflammatory cytokines may uncover mutation-independent cytokine subsets associated with novel CMML prognostic features. A Luminex cytokine profiling assay was utilized to profile cryopreserved peripheral blood plasma from 215 CMML cases from three academic centers, along with center-specific, age-matched plasma controls. Significant differences were observed between CMML patients and healthy controls in 23 out of 45 cytokines including increased cytokine levels in IL-8, IP-10, IL-1RA, TNF-α, IL-6, MCP-1/CCL2, hepatocyte growth factor (HGF), M-CSF, VEGF, IL-4, and IL-2RA. Cytokine associations were identified with clinical and genetic features, and Euclidian cluster analysis identified three distinct cluster groups associated with important clinical and genetic features in

Eric Padron, Eric.padron@moffitt.org.

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CMML. CMML patients with decreased IL-10 expression had a poor overall survival when compared to CMML patients with elevated expression of IL-10 ($P = 0.017$), even when adjusted for *ASXL1* mutation and other prognostic features. Incorporating IL-10 with the Mayo Molecular Model statistically improved the prognostic ability of the model. These established cytokines, such as IL-10, as prognostically relevant and represent the first comprehensive study exploring the clinical implications of the CMML inflammatory state.

Introduction

Chronic myelomonocytic leukemia (CMML) is a hematopoietic neoplasm with features of both myelodysplastic (MDS) and myeloproliferative (MPN) diseases hallmarked by a peripheral blood monocytosis ($\geq 1 \times 10^9$/L) that constitutes greater than 10% of white blood cells [1]. CMML is often accompanied by cytopenias and/or the presence of constitutional symptoms such as fatigue, weight loss, night sweats, symptoms from organomegaly (early satiety or abdominal pain) [1]. Previous French-American-British (FAB) classifications separate CMML based on white blood cell count into MPN-CMML and MDS-CMML [2]. World Health Organization (WHO) classifications separate CMML based on bone marrow blast count in three prognostically relevant groups [1]. However, these classification systems do not fully exemplify the clinical heterogeneity observed in CMML because many clinically relevant symptoms such as constitutional symptoms, splenomegaly, and peripheral cytopenias are not captured. Although recurrently mutated genes in CMML have been rigorously annotated, the spectrum of mutations is homogeneous relative to other adult cancers and also does not reflect the clinical diversity seen in patients with CMML [3–6].

To address the wide clinical heterogeneity, many investigators have devised prognostic scoring systems that predict overall and leukemia-free survival [7–11]. We have demonstrated that, although valid and reproducible, these prognostic scores have only modest predictive capacity and are vulnerable to up-staging via competing models [12]. Numerous studies have also explored the prognostic capacity of somatic gene mutations in CMML [3, 13, 14]. Despite several independent studies, only *ASXL1* frameshift or nonsense mutations have demonstrated to be independently and reproducibly associated with survival [3, 15, 16]. This has led to at least three new prognostic scoring systems that incorporate *ASXL1* and clinical features [3, 12, 15–18]. Given these efforts, we reason that identifying non-mutational molecular characteristics may further refine prognostication in CMML.

We have demonstrated that ruxolitinib, a JAK1/2 inhibitor FDA approved for treatment of primary myelofibrosis (PMF), is an effective therapeutic in CMML [19, 20]. Similar to that seen in MPNs, CMML patients treated with ruxolitinib were found to have marked reduction in circulating inflammatory cytokines and improvement in constitutional symptoms and spleen volume [19, 21, 22]. Previous literature investigating JAK inhibition in MPNs has demonstrated that inflammatory cytokines are reduced or normalized after treatment correlating with symptom improvement [19, 22, 23]. This suggests that inflammatory cytokines may associate with clinical features not captured by current models or genetic

mutations. However, the spectrum of cytokine secretion in CMML has not been previously comprehensively evaluated in the literature.

To understand the non-mutational molecular correlates driving CMML clinical diversity, we comprehensively profiled the inflammatory secretome in CMML patients. We hypothesize that annotation of inflammatory cytokines may uncover cytokine subsets associated with CMML-specific clinical and genetic features and refine existing prognostic models.

Methods

Patient samples and controls

The study was approved by the Institutional Review Board (IRB) for each respective institution including Moffitt Cancer Center (Tampa, Florida), Institute Gustave-Roussy (Paris, France), and the University of Florence (Florence, Italy). All patients provided informed written consent for the collection of peripheral blood and bone marrow samples and required a confirmed WHO diagnosis of CMML [1]. Age-matched, center-specific controls ($n = 35$) were also obtained under institute-specific IRB-approved protocols. Fifteen single donor healthy control plasma samples were purchased from Innovative Research (Innovative Research, Inc., Novi, MI) with an average age 66 (range 60–80) and a total of nine men and six women. These 15 samples from Innovative Research, Inc. were used as comparison for samples from Moffitt Cancer Center and University of Florence. All healthy control plasma samples were obtained at an FDA licensed commercial donor center or facility within the United States and each sample was tested and found negative for Hepatitis B, Hepatitis C, HIV, and syphilis as well as negative for active diagnosis of cancer. Twenty single donor healthy control plasma samples were obtained by the Institute Gustave-Roussy as comparison for their samples. Pretreatment plasma was isolated from whole blood and bone marrow samples obtained from patients at each individual institution (Fig. 1).

Cytokine secretion profiling

Cytokine expression levels were measured, in duplicate, from plasma samples using a custom 45-plex Luminex-based inflammatory cytokine screening assay kit and Luminex 100™ according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Forty-five cytokines (Supplemental Table 1) were analyzed, which included 19 cytokines previously implicated in the pathogenesis of myeloid neoplasms [24]. Cytokine measurements for the CMML patients were normalized to the site-specific healthy control for each batch and a fold change was calculated for each cytokine measured for every CMML patient and healthy control enrolled in the study. Cytokine expression levels with a fold change above 1 in the CMML patients were deemed elevated compared to healthy normal controls, and cytokine expression levels with a fold change less than 1 in the CMML patients were deemed to be decreased compared to healthy normal controls.

Somatic mutation profiling

Gene mutation testing was achieved via specific, in-house, targeted next-generation sequencing platform. Both full gene and hotspot sequencing were generated on the following genes: *TET2, ASXL1, SRSF2, CSF3R, KRAS, NRAS CBL, DNMT3A, SF3B1,*

EZH2, JAK2, IDH1/2, FLT3, SETBP1, U2AF1, and *ZRSR2*. All variants reported were manually reviewed and validated to be variants of known significance as previously described [25].

Colony formation assay and gene expression analysis

Colony formation assays from cryopreserved primary CMML bone marrow mononuclear cells were conducted in duplicate using Methocult substrate (Stemcell, Vancouver, Canada) as previously described by our group in the presence or absence of human recombinant interleukin-10 (IL-10) or IL-8 (Stemcell) at a concentration of 10 μg/mL [26]. Cryopreserved primary CMML bone marrow mononuclear cells were thawed from seven CMML patients enrolled in our study for the assay. Colonies were counted using the StemVision automated colony-counting instrument and software (Stemcell). A paired *T*-test was used to compare colony output between patient samples. Multiplex gene expression analysis was performed using Nanostring nCounter PanCancer Pathway Panel and nSolver™ Analysis software (NanoString Technologies, Inc., Seattle, WA) on four CMML patients enrolled in our study.

Statistical methods

Cytokine expression levels were derived using Luminex software MasterPlex 2010 (MiraiBio Group of Hitachi Solutions America, Ltd). All analyses involving cytokines were completed after applying a log-transformation to reduce the impact of outliers. Principal component analysis (PCA) was completed with the first two principal components (PCs) plots to investigate potential batch effects. Cytokine expression levels were adjusted for batch effect (i.e., day the sample was run) using an ANOVA model for each cytokine (see Supplemental Methods). PCA demonstrated significant batch effect that was corrected by using an ANOVA model that considered the timing of experiments (Supplemental Fig. 1). Comparison of peripheral blood cytokines in CMML patients to each respective healthy age-matched and center-matched control was completed using Wilcoxon rank sum test. Cytokine levels in the peripheral blood of 20 CMML patients were compared to matched bone marrow samples using Pearson's correlation. Log-rank tests were used to determine if individual cytokines (high/low) or clusters of cytokines were independently associated with overall survival (OS). Cox proportional hazards models were used to incorporate clinical and gene variables into a multivariate survival model. Model development was completed by first including any clinical, genetic, or cytokine variable with $P < 0.05$ into an initial model, followed by backward elimination to remove variables with $P > 0.05$ from the final model. Proportional hazards assumptions were confirmed with Schoenfeld residual plots. Cluster analysis was completed on the log-transformed batch-adjusted cytokine data with Gaussian model-based clustering using the R package *mclust* [27, 28] and Recursively Partitioned Mixture Model with R package *RPMM* [29]. Determination of difference in OS between the clusters was completed using log-rank tests. Statistical analysis was completed in collaboration with the Department of Biostatistics and Bioinformatics at Moffitt Cancer Center.

Results

Patient characteristics

A total of 215 patients with a diagnosis of CMML and median age of 74 (range 29–91, 65% males) had diagnostic pretreatment peripheral blood plasma samples available for the study. Of the 215 CMML patients with available clinical and genetic information, 105 (61% of evaluable patients) patients were classified in the CMML-0 subgroup, 43 (25% of evaluable patients) patients in the CMML-1, 25 (14% of evaluable patients) patients in the CMML-2 subgroup by 2016 WHO classification[1], and 42 patients did not have clinical data available (Table 1). Overall, 122 (68% of evaluable patients) CMML patients had a hemoglobin level greater than 10 g/dL (median 11.2, range 5.9–16.7 g/dL), 58 (32% of evaluable patients) patients with hemoglobin less than 10 g/dL, and 35 patients did not have clinical data. *TET2* was the most common mutation seen in our cohort with 87 patients (58% of evaluated patients) with mutated *TET2*. Our patient demographics and clinical characteristics are summarized in Table 1 and Supplemental Table 2.

Concordance between bone marrow and peripheral blood samples

Because our study focused on annotating peripheral blood cytokine levels, we explored the concordance between peripheral blood and bone marrow plasma samples in 20 CMML patients with blood and bone marrow obtained concomitantly. When all data were aggregated across patients, we found a positive concordance between bone marrow plasma samples and peripheral blood samples of the majority of the cytokines evaluated ($r = 0.7891$, $P < 0.0001$, 95% CI 0.592–0.8971), suggesting that peripheral blood is a reasonable representation of the bone marrow cytokine milieu. However, certain cytokines appeared to be discordant suggesting enrichment or de-enrichment in the bone marrow compared to peripheral blood. These discordant cytokines included Granulocyte-macrophage colony-stimulating factor (GM-CSF) ($r = -0.1395$), vascular endothelial growth factor (VEGF) ($r = -0.1415$), and tumor necrosis factor-α (TNF-α) ($r = -0.1561$) (Supplemental Fig. 2).

Cytokine expression in CMML patients compared to matched controls

We evaluated the peripheral inflammatory secretome of CMML by annotating 45 chemokine/cytokine levels and compared these levels to age- and center-matched normal controls ($n = 35$) (Fig. 1a, b; Supplemental Table 3). After data normalization and quality control, significant differences in 23 of 46 cytokine levels between CMML and healthy controls were measured. We observed significantly downregulated FLT-3 ligand (fold change (FC) = 0.4), PDGF-AA (FC = 0.3), PDGF-BB (FC = 0.04), PDGF-CC (FC = 0.5), PDGF-DD (FC = 0.4), EGF (FC = 0.6), TRAIL/TNFS10 (FC = 0.5), and CCL5/RANTES (FC = 0.1) when comparing CMML patients to healthy age-matched controls ($P \leq 0.001$). IL-8 (FC = 8.3), IP-10 (FC = 1.2), and IL-1RA (FC = 1.5) were found to be significantly elevated in CMML patients compared to healthy age-matched controls ($P \leq 0.001$) (Fig. 1b, Supplemental Table 3). Proinflammatory cytokines including TNF-α (FC = 3, $P = 0.011$), IL-6 (FC = 2.6, $P = 0.0129$), MCP- 1/CCL2 (FC = 2.1, $P = 0.0114$), macrophage colony-stimulating factor (M-CSF) (FC = 2.6, $P = 0.0035$), HGF (FC = 2, $P = 0.0061$), VEGF (FC = 2.2, $P = 0.0223$), IL-4 (FC = 2.4, $P = 0.0499$), and IL-2RA (FC = 1.5, $P = 0.0269$) were found to be significantly elevated in CMML patients. We observed significant CMML

cytokine/chemokine heterogeneity within our cohort, suggesting that annotating the secretome may reveal relevant disease subsets. We therefore reasoned that cytokines/chemokines that were not observed to be statistically up- or downregulated should be considered in our downstream analysis (Fig. 1c).

Inflammatory cytokine signals are an established feature of stress-induced hematopoiesis and the secreted inflammatory response [30]. We hypothesized that CMML patients may exhibit distinct cytokine expression patterns due to underlying somatic mutations. To evaluate this, we used Pearson's correlation to determine pairwise correlations between cytokines measured in CMML and normal age-matched controls, respectively (Fig. 1d, e, Supplemental Tables 4–6). Cytokine associations were identified in CMML patients not seen in normal controls between IL-1β, IL-6, IL-8, and TNF-α previously identified in the literature in other proinflammatory diseases such as osteoarthritis [31–35]. Only in normal control patients, GM-CSF was positively correlated with IL-5 ($r = 0.577$, $P = 0.0002$) and CXCL7 ($r = 0.432$, $P = 0.009$), but negatively correlated with IL-2RA ($r = -0.449$, $P = 0.006$), HGF ($r = -0.341$, $P = 0.044$), and MIF ($r = -0.628$, $P < 0.0001$). A statistically significant, positive correlation was also observed between IL-10 and GM-CSF ($r = 0.151$, $P = 0.025$), M-CSF ($r = 0.138$, $P = 0.0417$), CCL3 ($r = 0.169$, $P = 0.0125$), FGF-basic ($r = 0.146$, $P = 0.032$), and TNF-α ($r = 0.197$, $P = 0.0035$) that was present only in CMML patients.

Clinical and genetic associations with the inflammatory secretome

When comparing the CMML secretome with clinical parameters, cytokine and chemokine secretion was associated with a variety of clinically relevant features. For example, patients who were classified as MPN-CMML by the FAB had significantly elevated inflammatory cytokine and chemokine secretion that included S100A8 (FC = 1.8, $P = 0.005$), HGF (FC = 1.3, $P = 0.004$), MIF (FC = 1.5, $P = 0.006$), and IL-1RA (FC = 1.3, $P = 0.001$). CCL3 was found to be downregulated in MPN-CMML patients compared to MDS-CMML patients (FC = 0.3, $P = 0.012$) (Fig. 2a; Supplemental Table 7). Other relevant clinical associations included IL-2RA (FC = 1.9, $P < 0.001$), which statistically associated with higher risk CMML (Fig. 2b; Supplemental Table 8) and IL-8 (FC = 2.1, $P = 0.041$) and IL-2RA (FC = 0.045, $P = 1.5$), which statistically associated with constitutional symptoms (Fig. 2c; Supplemental Table 9). IL-8 (FC = 6.1, $P = 0.04$) was found to be profoundly elevated in CMML patients classified in the CMML-2 subcategory by 2016 WHO classification system [1]. Interestingly, the association with constitutional symptoms and IL-8 (FC = 2.1, $P = 0.041$) is consistent with the clinical efficacy of ruxolitinib, an FDA approved JAK1/2 inhibitor, that potently down-regulates IL-8 and improves constitutional symptoms in patients with myelofibrosis and CMML [22]. Patients with enlarged spleens by physical exam were observed to have elevated CXCL9 levels compared to those without enlarged spleen (FC = 1.6, $P = 0.042$) (Fig. 2d; Supplemental Table 10). Finally, CMML patients with anemia had elevated levels of IL-5 (FC = 1.2, $P = 0.014$) while those with thrombocytopenias had elevated levels in IP-10/CXCL10 (FC = 1.9, $P = 0.005$) and IL2-RA (FC = 1.5, $P = 0.008$) (Fig. 2e, f; Supplemental Tables 11 and 12).

When comparing inflammatory cytokine secretion with the presence or absence of commonly mutated genes, we observed *TET2* mutant patients had statistically decreased levels of IL-6 (FC = 0.2, $P = 0.005$), PDGF-AA (FC = 0.4, $P < 0.001$), and PDGF-DD (FC = 0.5, $P = 0.007$) (Fig. 3a, Supplemental Table 13). Combining all mutated epigenetic regulator genes tested, significant increases in IL-1β (FC = 1.2, $P = 0.029$) secretion were observed (Fig. 3b, Supplemental Table 14). CMML patients with mutations in splicing regulators had statistically elevated levels of IL-2 (FC = 1.7, $P = 0.043$) and CD44 (FC = 1.6, $P = 0.041$) (Fig. 3c, Supplemental Table 15). However, other than PDGF-AA, no cytokines were found to be significantly different among patients with *ASXL1* mutation compared to those with wild-type *ASXL1* (Fig. 3d, Supplemental Table 16). CMML patients with signaling mutations, alternatively, were found to have the greatest increases in inflammatory cytokines to include IL-10 (FC = 3.8, $P = 0.006$), CCL2/MCP-1 (FC = 1.5, $P = 0.033$), CD44 (FC = 2.2, $P = 0.002$), IL-1RA (FC = 1.3, $P = 0.027$), IL-12 23p40 (FC = 6.6, $P = 0.019$), and CXCL7 (FC = 1.5, $P = 0.012$) (Fig. 3e, Supplemental Table 17). CXCL7 has been previously reported to be differentially methylated and overexpressed in CMML patients resistant to decitabine supporting its clinical relevance [36]. GM-CSF was observed to be elevated in patients with signaling mutations, with a fold change of 3.8 with $P = 0.073$, but it did not reach statistical significance. Overall, distinct cytokine signatures were associated with somatic mutations, but the greatest number and increase were in those with signaling mutations (Fig. 3e).

Unsupervised clustering analysis

Given these clinical and genetic associations with subsets of cytokine/chemokine secretion, we next tested whether unsupervised clustering of cytokines could identify clinical subtypes of CMML using two different methods, *RPMM* and *Mclust*. Using *RPMM* clustering, we found three distinct cluster groups when analyzed in 213 evaluable CMML patients (Fig. 4a, Supplemental Tables 18 and 19). A total of 69 patients (32%) were classified in cluster 1 driven by statistically significant increases in M-CSF (Supplemental Table 15). A total of 42 patients (20%) were classified in cluster 2 driven by statistically significant increases in 17 cytokines including IL-6, IL-8, and IL-12p70 cytokines (Supplemental Table 20). A total of 102 patients (48%) were classified in cluster 3 driven by statistically significant increases in IL-2RA (Fig. 4, Supplemental Table 18). We observed that a larger proportion of CMML patients with anemia with hemoglobin <10 (32 of 58 patients, 55.2%, $P = 0.058$) and thrombocytopenia with platelet count <100,000 (55 of 88 patients, 62.5%, $P < 0.001$) were categorized into cluster 3. Additionally, we observed that patients were significantly more likely to be lower risk by the Mayo score ($P = 0.003$) [17] and the Global MD Anderson score ($P = 0.08$) [10, 37] when assigned to cluster 2. Last, the presence of immature myeloid cells, an adverse clinical factor in CMML, was associated with Clusters 1 and 3 ($P = 0.012$). When comparing OS between our three distinct cluster groups, no statistical difference was observed (Supplemental Fig. 3). All tested variables and their statistical significance are presented in Supplemental Tables 20 and 21.

Impact of cytokines on survival

Using log-rank tests with all cytokines (measured as binary variable high/low), we identified M-CSF ($P = 0.012$, HR 0.74, 95% CI 0.58–0.94), IL-13 ($P = 0.035$, HR 5.59, 95% CI 1.34–

23.3), and IL-10 ($P = 0.037$, HR 0.8, 95% CI 0.65–0.98) to be associated with OS in CMML. Confirming this, we observed that CMML patients with decreased IL-10 expression (median IL-10 = 0.895939) had an inferior OS when compared to CMML patients with elevated expression of IL-10 by Kaplan–Meier analysis (median OS of 46.98 vs 79.84 months, $P = 0.0166$) (Supplemental Figs. 4 and 5A). Despite small numbers of patients in subgroup analysis, investigating IL-10 expression across Mayo prognostic risk groups in CMML patients consistently demonstrated that those with elevated IL-10 secretion had a favorable OS [17] (Supplemental Fig. 5B–I).

To evaluate whether cytokines can improve our current prognostic systems in CMML, we conducted log-rank tests on clinical and genetic variables, and found significant risk factors for survival that included anemia, absolute monocyte count, elevated WBC count, the presence of *ASXL1* mutation, and FAB-MPN status (Supplemental Table 22). To create a final model that could predict OS and leukemia-free survival, we used a backward selection to eliminate non-significant variables when considering the above clinical and genetic variables, including age, hemoglobin, platelet count, monocyte count, bone marrow blast percentage, presence of circulating immature cells, ASXL1, FAB-MPN, and CMML-specific cytogenetic risk score. (Tables 2 and 3, Supplemental Tables 22 and 23). When considering this, IL-10, hemoglobin <10 g/dL, ASXL1 mutation status, and FAB-MPN status remained statistically significant (Table 2). Because the vast majority of these clinical and genetic variables were previously identified by the Mayo Molecular Model [17], we tested the additive prognostic power of IL-10 expression (including ASXL1 mutation status) and found that IL-10 statistically improved the Mayo CMML prognostic score model [17] (C-Index of 0.6736317 (model without IL-10) vs 0.7367075 (model with IL-10), $P = 0.01871$) in our patient cohort (Supplemental Table 22).

Impact of IL-10 and IL-8 on colony formation in primary CMML patient samples

CFAs were performed in duplicate from cryopreserved bone marrow mononuclear cells of seven CMML patients. Four of the patients were found to have elevated IL-10 expression (above median IL-10 = 0.895939) using our custom luminex assay and three of the patients were found to have low IL-10 expression. We compared colony output between IL-10 treated patient sample, IL-8 treated patient sample, and untreated patient sample. We observed lower colony formation output in our CMML patients with decreased IL-10 expression ($P = 0.054$) (Supplementary Figure 6A). In CMML patients with elevated IL-10 expression, again we observed decreased colony formation potential compared to our untreated sample which was statistically significant ($P = 0.025$) (Supplementary Figure 6B). When all seven samples in duplicated treated with IL-10 was compared to the untreated samples, we persistently found decreased colony formation in IL-10-treated samples ($P = 0.0007$) (Supplementary Figure 5C). We, however, observed increased colony formation in IL-8-treated samples compared to our untreated samples which was statistically significant ($P = 0.017$) (Supplementary Figure 6D–F). RNA gene expression analysis comparing untreated cultured primary CMML bone marrow mononuclear cells to those treated with IL-10 found upregulation in cell cycle apoptotic pathways using Nanostring PanCancer Pathway (Supplementary Figure 6G, H, I).

Discussion

CMML is characterized by clinical heterogeneity that cannot be molecularly explained by known recurrent genetic mutations alone. Previous studies have demonstrated that elevated inflammatory cytokines in MPNs, such as PMF, correlate with clinical phenotypes and are associated with OS [24]. However, cytokine expression has never been evaluated in patients with CMML. Given the clinical responses to ruxolitinib, we reasoned that the inflammatory secretome may play an important role in the pathogenesis of CMML. To explore this, we profiled 45 inflammatory cytokines and chemokines in CMML patients, demonstrating that proinflammatory cytokines were heterogeneously expressed in CMML patients with 23 of 45 cytokines that were significantly discordant to matched healthy controls. Of interest, approximately 50% of these cytokine/chemokines were increased in CMML and 50% were decreased. Within clinical CMML subgroups, this heterogeneity appeared to coincide with clinically relevant phenotypes. As an illustration, proliferative CMML, which diversely combine leukocytosis, splenomegaly, and constitutional symptoms, was typically associated with a strong inflammatory cytokine profile. Cytokine heterogeneity also aligned with mutational signatures. For example, the largest number of inflammatory cytokines were, not surprisingly, associated with those patients that harbored a cytokine signaling mutation. However other genetic associations identified were unexpected. For example, it has been previously reported that TET2 function is associated with suppression of IL-6 and increased production of IL-6 in TET2-deficient mice; however, our results found the opposite effect [38, 39]. In our cohort, we observed decreased IL-6 expression in CMML patients with mutated TET2, suggesting that there are complex molecular interactions in human, polygenetic disease not predicted by single gene models (Fig. 3a).

Using unbiased cluster analysis, we were able to further validate the capacity of cytokine secretion to account for CMML clinical diversity. Surprisingly, while the greatest number of elevated individual cytokines was found in those CMML patients with proliferative symptomatology, cluster analysis was able to identify associations with higher risk disease and cytopenias. These are important clinical variables, albeit not associated with proliferative disease, suggesting that complex networks of cytokines may also govern ‘dysplastic’ symptomatology. In support of this, cytokine association studies demonstrated that CMML patients have, in addition to discordance in individual cytokine secretion, loss of normal cytokine associations.

Although the present study represents the first interrogation of inflammatory cytokines/chemokines in CMML, circulating cytokine levels have been previously evaluated in PMF and other myeloid diseases [1, 24]. When comparing abnormal cytokine secretion between CMML and PMF, for example, several cytokines in CMML patients that were elevated compared to healthy controls were also increased in PMF patients, including IL-1RA, Il-2RA, IL-6, IL-8, TNF-α, HGF, IP-10, MCP-1, and VEGF [24]. Further, cytokine–phenotype associations discovered in our cohort were also seen in PMF, validating our findings. Specifically, statistically increased IL-8 expression in patients with constitutional symptoms was observed in our cohort and in PMF (Fig. 2c, Supplemental Table 6) [24]. However, not every cytokine association in CMML was similar to that in PMF. IFN-γ was found to be decreased in PMF, but in our cohort, IFN-γ was elevated in CMML patients

compared to healthy controls, though it did not reach statistical significance [24]. Further, 50% of cytokines differentially secreted were decreased in CMML, a number far greater than in PMF. Of these cytokines, RANTES/CCL5 was among the most decreased cytokines in CMML, with similar findings previously reported in MDS and aplastic anemia, providing cytokine-level support for the phenotypic overlap between bone marrow failure and myeloproliferation in CMML [40].

Prognostic risk models are valuable tools for assessing individual disease risk and clinical decision-making. In CMML, these risks models have modest predictive capacity even after adding prognostically informative genetic mutations. We identified IL-10 as an independent prognostic factor for OS in CMML, consistent with findings in other malignancies [41, 42]. This cytokine has been observed to suppress cytokine secretion and inhibit the induction of proinflammatory cytokines such as IL-1β, TNF-α, GM-CSF, and IFN-γ [43]. Further, CMML patients with downregulated IL-10 expression were identified to have poor OS when compared to CMML patients with elevated expression of IL-10 ($P = 0.0166$). Importantly, the addition of IL-10 statistically improved the predictive power of the Mayo Molecular Model [17] within our cohort. Although the magnitude of improvement was small, these data provide the foundational evidence supporting the notion that inflammatory cytokines represent a tractable, clinically relevant molecular characteristic of CMML. In fact, a sequential analysis of eight CMML patients treated with 13.75 mean cycles of 5-azacitidine demonstrated that 50% (16 of 32 tested) of cytokines tested were statistically augmented by therapy suggestive that inflammatory cytokines may have therapeutic relevance (Supplemental Figure 7).

IL-10 is a multifunctional cytokine produced by several cell types including monocytes and has been described to have both immunosuppressive and proinflammatory properties [43–45]. IL-10 has been implicated in the pathogenesis of multiple tumor types including CMML, and its potential to suppress cytokine expression has made it of particular interest as a possible therapeutic target [46–49]. Geissler et al. [50] observed that IL-10 could inhibit the growth of CMML primary cells in a dose-dependent manner in vitro potentially via the suppression of endogenous GM-CSF. The group observed that treatment of exogenous GM-CSF was able to reverse IL-10 growth inhibition of CMML primary cells, and observed that GM-CSF mRNA levels and protein levels were decreased after treatment with IL-10 [50]. In our cohort, we observed that CMML patients with high IL-10 expression had a favorable OS perhaps mediated by IL-10 growth suppression of CMML leukemia cells. In support of this notion, a small study of five CMML patients treated with recombinant human IL-10 observed some clinical efficacy with improvement in one of three patients with confirmed skin infiltration by CMML suggesting that IL-10 is a potential therapeutic target [51]. Our own experiment confirmed decreased colony formation in primary CMML patient samples treated with IL-10 compared to untreated samples and IL-8-treated samples (Supplementary Figure 6).

Our study comprehensively evaluated cytokine expression in CMML patients, which has not been evaluated in the literature to understand the pathophysiology as well as evaluate the potential for new therapeutic targets. The annotation of inflammatory cytokine/chemokines in this study also raises several important questions. For example, what is the individual

contribution of the stroma and the leukemic cells to the overall cytokine milieu? Additionally, can inhibition of cytokines critical to the symptomatology of CMML such as IL-8 be selectively targeted in CMML? Based on the findings from this study, further investigation is warranted into the inflammatory state of CMML, its therapeutic relevance, and its use to improve our current prognostic models. Last, our data demonstrate that IL-10 has the potential to improve existing clinical and genetic prognostic model and serve as a therapeutic target in CMML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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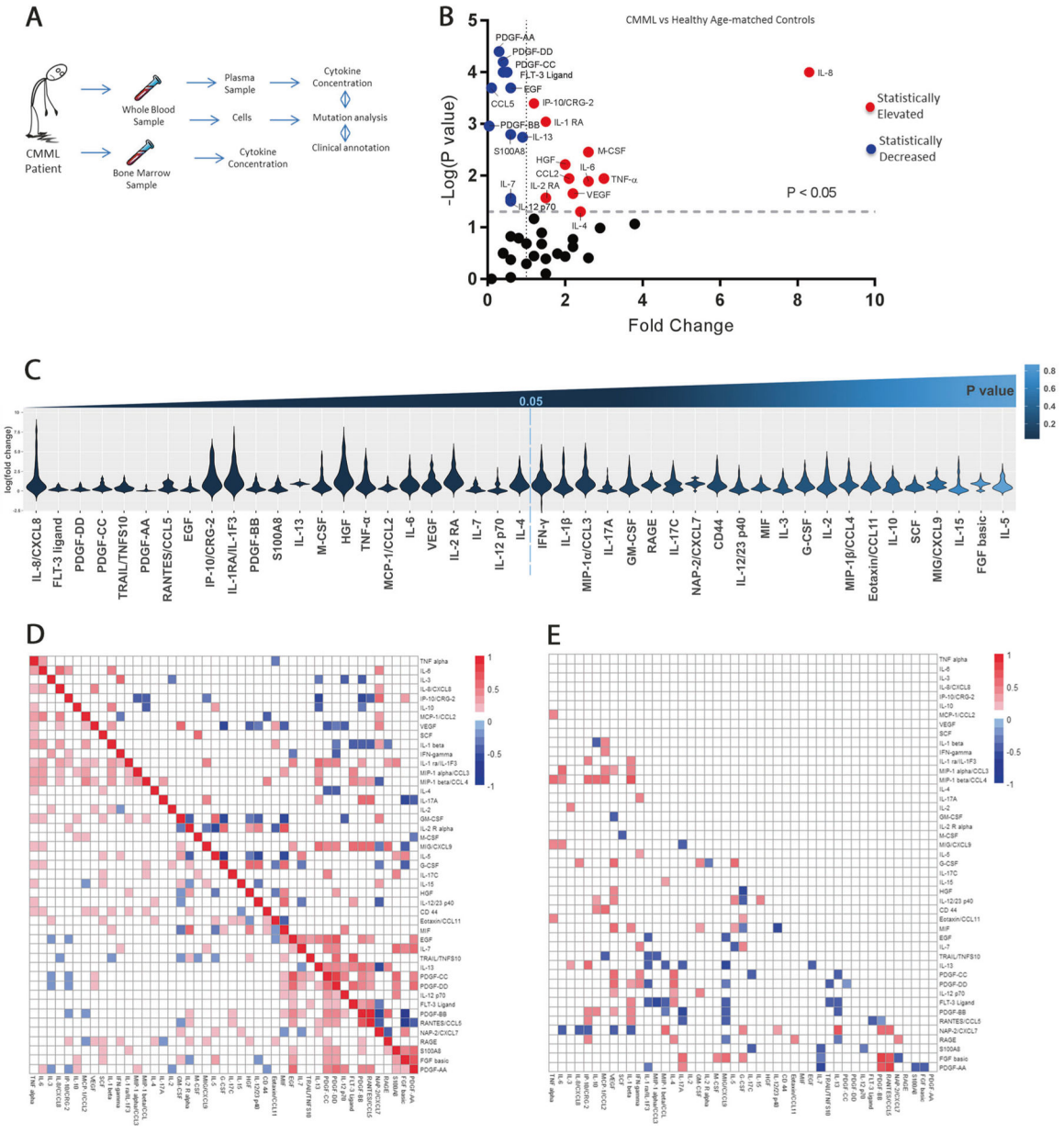

Fig. 1.

Inflammatory cytokines are heterogeneously expressed in CMML patients. **a** Study design. **b** Significant differences observed in measured inflammatory cytokines in patients with CMML compared to healthy aged-matched controls. *Y*-axis is negative log(*P*-value). *X*-axis is fold change of CMML compared to healthy aged-matched controls. **c** Violin plot displaying the variable distribution of cytokine expression for each cytokine in CMML patients. On the *y*-axis is the log of the fold change for CMML patients. On the *x*-axis is each of the 45 cytokines evaluated. Cytokines to left of vertical dotted line were statistically significant when comparing expression in CMML patients to normal controls ($P < 0.05$). **d** Pearson correlation displaying cytokine–cytokine interaction in 45 cytokines measured in CMML patients. Top figure displays positive and negative cytokine interactions in normal

patients and bottom section of figure displays cytokine interactions in CMML patients. Blue notes negative R coefficient correlation while red notes positive R coefficient correlation. **e** The difference of correlation between CMML patients and normal patients. The colored squares represent all statistically significant interactions. The more blue present in the square denotes more negative R coefficient correlation between two cytokines while the more red in the square denotes more positive R coefficient correlation. Interactions between cytokines that were not statistically significant are shaded white

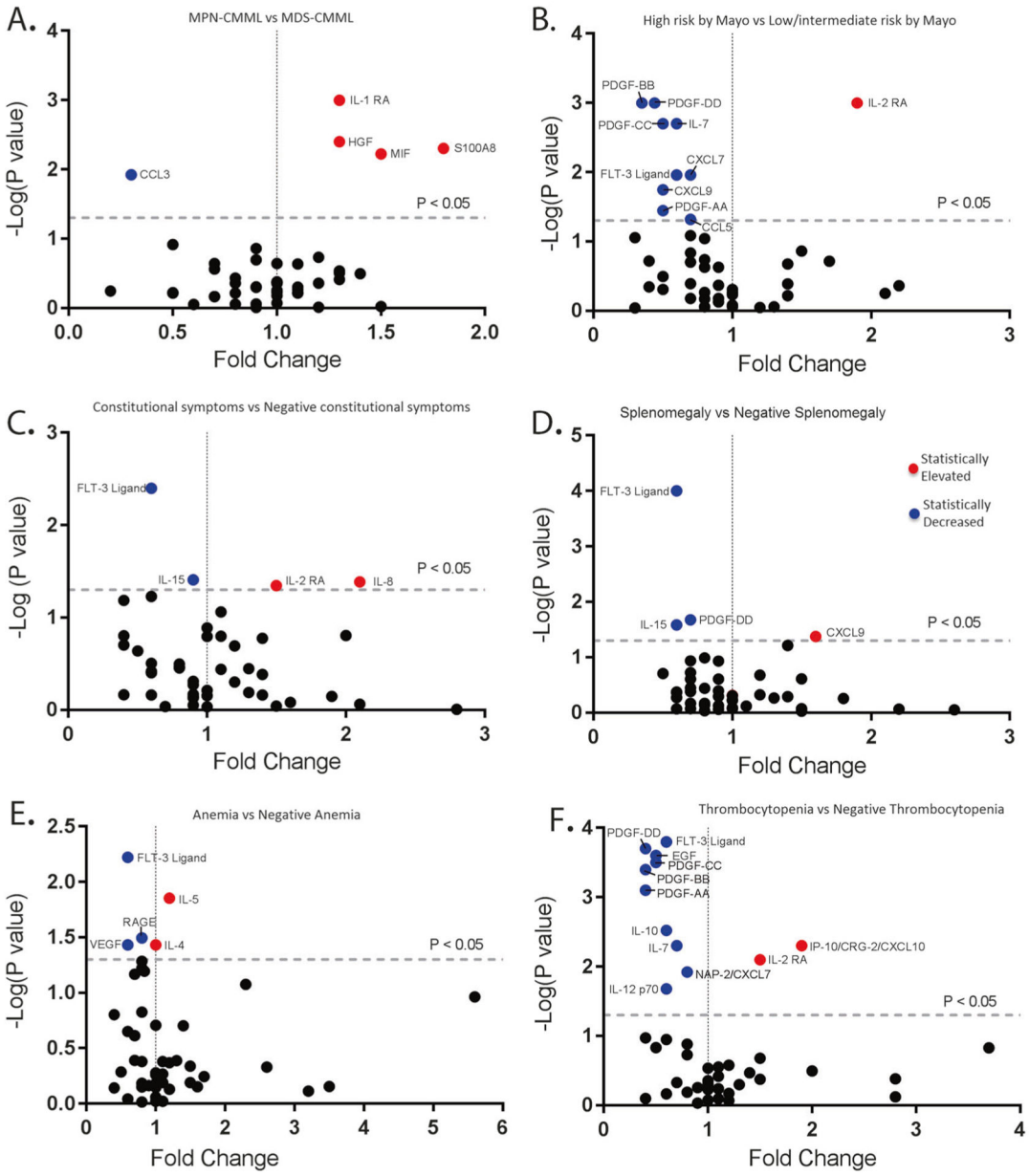

Fig. 2.

Cytokine–phenotype associations in CMML. **a** Cytokine expression in patients with MPN-CMML compared to patients categorized in MDS-CMML. *Y*-axis is negative log(*P*-value). *X*-axis is the calculated fold change from the mean of the batch-adjusted cytokine levels normalized to healthy controls. **b** Cytokine expression in patients with high-risk CMML by Mayo Prognostic Model compared to patients categorized to low-risk CMML by Mayo Prognostic Model. **c** Cytokine expression in CMML patients with constitutional symptoms compared to CMML patients without constitutional symptoms. **d** Cytokine expression in CMML patients with splenomegaly compared to CMML patients without the presence of splenomegaly. **e** Cytokine expression in CMML patients with hemoglobin <10 g/dL compared to CMML patients with hemoglobin >10 g/dL. **f** Cytokine expression in CMML

patients with platelet count <100,000/μL compared to CMML patients with platelet count >100,000/μL

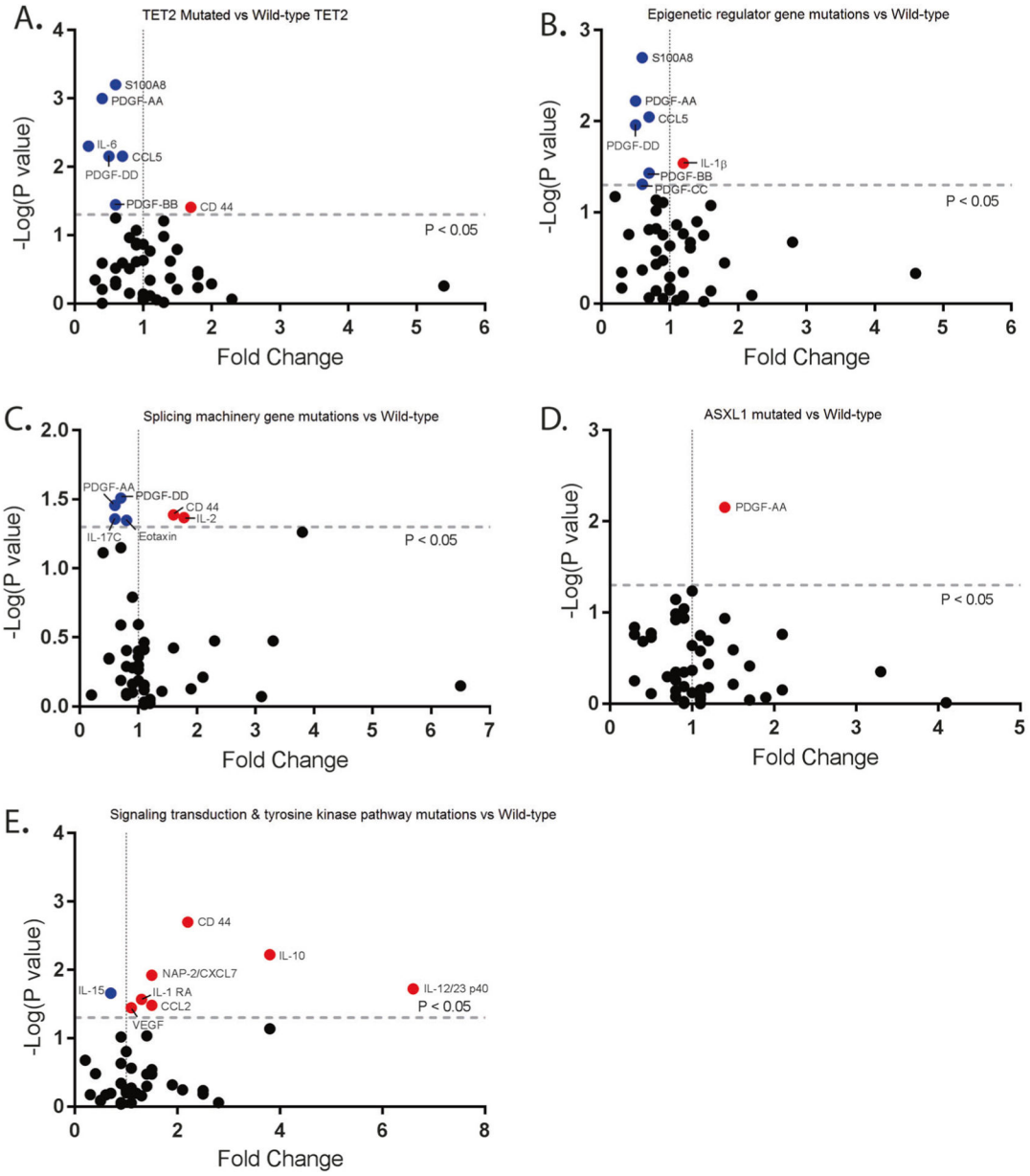

Fig. 3.

Cytokine genotype associations in CMML. **a** Cytokine expression in patients in CMML patients with the TET2 mutations compared to CMML patients without TET2 mutations. *Y*-axis is negative log (*P*-value). *X*-axis is the calculated fold change from the mean of the batch-adjusted cytokine levels normalized to healthy controls. **b** Cytokine expression in patients in CMML patients with the mutations in epigenetic regular genes compared to CMML patients without those mutations. **c** Cytokine expression in patients in CMML patients with mutations involving splicing machinery compared to CMML patients without those mutations. **d** Cytokine expression in patients in CMML patients with ASXL1 mutations compared to CMML patients without ASXL1 mutations. **e** Cytokine expression in

patients in CMML patients with mutations involving signal transduction and tyrosine kinase pathway compared to CMML patients without those mutations

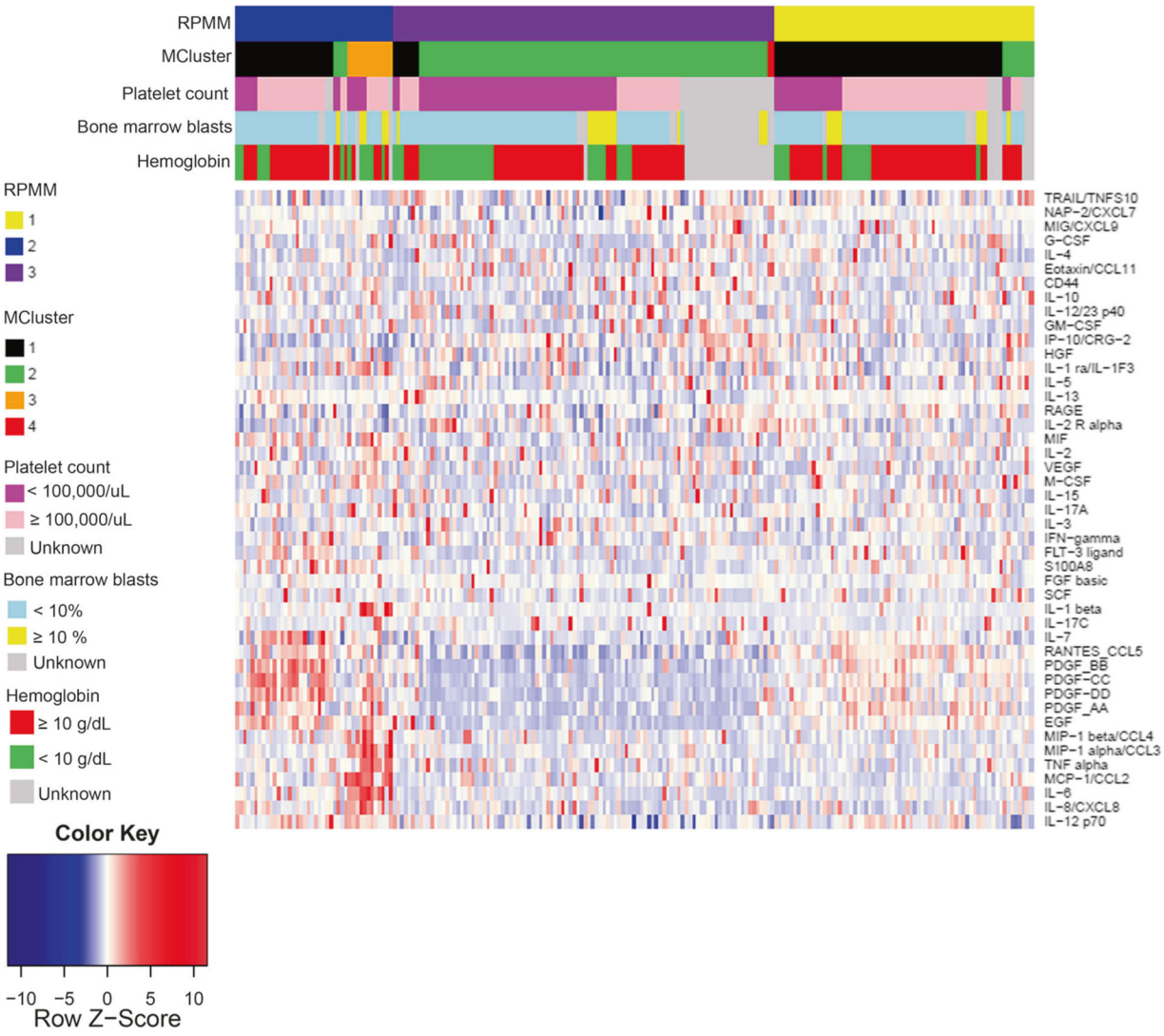

Fig. 4.

Unsupervised cluster analysis identifies three groups driven by cytokine signatures. Heatmap of batch-adjusted cytokine levels with cluster assignments denoted on the legend based on either RPMM (3 clusters) or Gaussian mixed-model clustering (4 clusters) when cytokine expression was analyzed in CMML patients ($N = 213$)

Table 1

Patient demographics and clinical characteristics

Characteristics	Median (range) CMML	CMML (%) (*N* = 215)	Control (*N* = 35)
Age, years	74 (29–91)		66 (60–80)
<65		32 (14.9%)	4 (11.4%)
≥65		176 (81.8%)	11 (31.4)
Data not available		7 (3.3%)	20 (57.1%)
Gender			
Male		139 (64.7%)	9 (25.7%)
Female		76 (35.3%)	6 (17.1)
Data not available			20 (57.1%)
WHO subtype			
CMML-0		105 (48.9%)	
CMML-1		43 (20%)	
CMML-2		25 (11.6%)	
Data not available		42 (19.5%)	
Hemoglobin level, g/dL	11.2 (5.9–16.7)		
<10g/dL		58 (27.0%)	
≥10 g/dL		122 (56.7%)	
Data not available		35 (16.3%)	
Leukocyte count, $\times 10^9$/L	19.2 (1.0–235)		
FAB-MDS		107 (49.8%)	
FAB-MPN		83 (38.6%)	
Data not available		25 (11.6%)	
Platelet count, $\times 10^9$/**uL**	101 (8.0–812.0)		
<100,000/uL		89 (41.4%)	
≥100,000/uL		90 (41.9%)	
Data not available		36 (16.7%)	
Blasts in BM, %			
<10%		148 (68.8%)	
≥10%		25 (11.6%)	
Data not available		42 (19.5%)	
Presence splenomeg;			
Yes		61 (28.4%)	
No		111 (51.6%)	
Data not available		43 (20.0%)	
Presence of circulating immature cells			
Yes		88 (40.9%)	
No		74 (34.4%)	
Data not available		53 (24.7%)	
ASXL1 status			
Mutated		49 (22.8%)	

Characteristics	Median (range) CMML	CMML (%) (*N* = 215)	Control (*N* = 35)
Wild type		121 (56.3%)	
Data not available		45 (20.9%)	
TET2 status			
Mutation		87 (40.5%)	
Wild type		62 (28.8%)	
Data not available		66 (30.7%)	

Table 2

Significant variables associated with overall survival (OS)

Covariate	Level	*N*	Hazard ratio (95% CI)	HR *P*-value	Overall *P*-value	Log-rank test *P*-value
IL-10	High	84	0.48 (0.26–0.89)	0.019	0.019	0.017
	Low	90	-	-		
Mayo CMML prognostic model	High	43	4.20 (1.71–10.31)	0.002	0.002	<0.001
	Low	66	-	-		
ASXL1	Positive	44	3.07 (1.52–6.21)	0.002	0.002	0.001
	Negative	97	-	-		

Table 3

Overall survival according to the defined risk categories in the training cohort determined using Mayo Molecular Model with addition of IL-10

	Number of patients (%)	Mean (months)	Proportion alive at 5 year (%)	Log- rank test (*P*-value)
				<0.001
Low	39 (22.4)	68.0	23.1	
Intermediate	122 (70.1)	56.0	8.2	
High	13 (7.5)	13.6	0	
Pairwise comparisons	Low vs Intermediate, $P = 0.031$ Low vs High, P<0.001 Intermediate vs High, $P = 0.003$			