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A specific bone marrow cytokine pattern in *de novo* acute myeloid leukemia

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ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous group of hematopoietic cancers. Cytokines play an important role in the regulation of normal and pathologic hematopoiesis. A pro-inflammatory state, described in hematopoietic malignancies, may participate in clonal selection. To identify recurrent cytokine patterns according to AML ontogenic subtypes, we quantified the concentration of 49 cytokines in the bone marrow (BM) plasma from 124 patients with AML or myelodysplastic syndrome (MDS), and from 94 healthy volunteers. We confirmed a pro-inflammatory profile in MDS and AML, with increased concentrations of CXCL8, CXCL10 and IL-6. Only a few cytokines varied when comparing AML to MDS. *De novo* AML subtypes carry a specific cytokine pattern dominated by the increase in CLEC11A concentrations and the decrease in FLT3 ligand concentrations. These cytokines could participate in clonal selection in this subtype of AML while being less critical in the other AMLs - *i.e.* secondary-like or *TP53*-mutated subtypes.

1. Introduction

The pathophysiology of acute myeloid leukemia (AML) has long been temptingly explained through the cell intrinsic functional consequences of most somatic lesions found in leukemic cells. However there is increasing evidence that bone marrow (BM) microenvironmental changes in AML could be directly involved in the *in vivo* natural selection of malignant cells [1,2]. The BM microenvironment is complex, composed of multiple extra-cellular matrix components and types of cells, including both hematopoietic and non-hematopoietic cells. These cells interact with each other through direct cell-to-cell contact or through the action of secreted soluble factors. These soluble factors include chemokines and cytokines, some of them being considered as hematopoietic growth factors. They form a tight network that regulates physiological hematopoiesis.

Several studies report pro-inflammatory BM microenvironment with increased interleukins (IL) levels, in particular those of CXCL8 and IL-6, which are common markers of inflammation, able to promote the

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proliferation, survival and invasion capacity of solid tumors [3] and various hematological diseases such as chronic lymphocytic leukemia [4], diffuse B large cell lymphoma [5], myelodysplastic syndromes (MDS) [6], myeloproliferative neoplasm [7] and AML [8–10]. CXCL8 is a pro-inflammatory chemokine, recruiting and activating granulocytes at sites of infection or inflammation [11]. Increased levels of CXCL8 produced by stromal cells may promote blast survival and proliferation during chemotherapy treatment [12,13]. Inhibiting the CXCL8-CXCR2 axis has also been shown a promising therapeutic approach in some myeloid malignancies [9]. IL-6 is a pleiotropic cytokine not only involved in inflammation but also in hematopoiesis and other fundamental biological mechanisms. In MDS, increased plasma level of IL-6 was associated with negative impact on overall survival and leukemia free survival that was independent from the International Prognostic Scoring System (IPSS) stratification [14]. IL-6 has been reported as secreted by the blasts, with autocrine and/or paracrine amplification through the constitutive activation of its downstream signaling that involves STAT3 [15,16].

Recently, a decreased concentration of FLT3-ligand (FLT3L) in blood plasma has been described in non-promyelocytic AML [17,18]. FLT3L is a key hematopoietic growth factor and its receptor FLT3 is mainly expressed by subsets of hematopoietic stem cells (HSCs), early myeloid and lymphoid progenitors [19]. *FLT3* mutations, especially internal tandem duplication (*FLT3-ITD*) in the juxta-membrane domain, represent the most common molecular abnormalities in AML. Beside FLT3L, by binding their specific receptors, several cytokines play a crucial role on hematopoietic cells and on extra-hematopoietic cells of the BM microenvironment. However, it is still unclear whether modifications of the cytokine composition in the BM plasma are causes or consequences of the mechanisms that underlie the initiation and clonal evolution of AML, and to what extent they participate in fitness changes of AML cells in their microenvironment.

In this work, we addressed the question of cytokine concentrations in the BM of AML and MDS patients, compared to those of healthy individuals. We particularly asked for association of changes in these concentrations with the ontogenic types of AMLs, *i.e.* AMLs with *de novo*-type mutations, secondary-type mutations, or *TP53* mutations.

2. Materials and methods

2.1. Patients and healthy controls

One hundred and two healthy individuals were enrolled in the HEALTHOX clinical trial (ClinicalTrials.gov Identifier: #NCT02789839) of Tours University Hospital (France) with written informed consent [20] (CPP Tours and AFSSAPS identifier ID-RCB: 2011-A00262–39; CPP Ile-de-France III: 2753).

One hundred and three non-promyelocytic AML were enrolled in the "MIF_AML" trial (ClinicalTrials.gov Identifier: #NCT03918655) in Saint Antoine Hospital and Tours University Hospital (France) with written informed consent. Samples from 50 MDS and 4 chronic myelomonocytic leukemias (CMML) were also collected with local written consent. Patients' diseases were characterized according to the 2016 WHO classification [21], in line with the standard of care for AML diagnosis including morphological examination of BM smear, immunophenotyping, karyotyping and sequencing analyses.

2.2. Sample collection

Peripheral blood (PB) and BM were concomitantly collected. Briefly, plasmas were separated from cells by double centrifugation (1200 *g*, 10 min, 20 °C). Plasmas were stored at −80 °C. BM and PB cells were used for DNA extraction and cryopreservation.

2.3. Cytokine quantification

The quantification of cytokines was performed by using 48-plex human cytokine panel assay (Bio-Rad, Hercules, CA, USA): [FGF basic (named after FGF-2), Eotaxin (named after CCL11), G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-1α, IL-2Rα, IL-3, IL-12(p40) (named after IL-12B), IL-16, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (named after CXCL8), IL-9, GRO-α (named after CXCL1), HGF, IFN-α2, LIF, MCP-3 (named after CCL7), IL-10, IL-12(p70) (named after IL-12), IL-13, IL-15, IL-17 A, IP-10 (named after CXCL10), MCP-1 (MCAF) (named after CCL2), MIG (named after CXCL9), β-NGF, SCF (named after KITLG), SCGF-β (named after CLEC11A), SDF-1α (named after CXCL12), MIP-1α (named after CCL3), MIP-1β (named after CCL4), PDGF-BB (named after PDGF-B), RANTES (named after CCL5), TNF-α (named after TNF), VEGF (named after VEGF-A), CTACK (named after CCL27), TRAIL (named after TNFSF10), IL-18, M-CSF, TNF-β (named after LT-α)]. The assay was performed according to manufacturer's instructions. Fluorescence values were acquired on the Luminex 200™ system (Bio-Rad). Concentration data were obtained using a 5 parametric logistic curve regression on Bio-Plex manager software (version 6.1, Bio-Rad). MIF and FLT3L quantifications were performed using Human MIF Quantikine ELISA and Human FLT-3 Ligand Quantikine ELISA kits (R&D systems, Bio-Techne, Minneapolis, MN, USA). For MIF quantification, each sample was diluted (1:100 to 1:500) in RD5–20 diluent and manufacturer's instructions were followed. For FLT3L quantification, only volumes in the initial binding step were optimized: 10 μL of sample, standard or control was mixed to 40 μL of RD1W diluent directly into the ELISA plate. For each assay, controls (R&D systems) were used. Absorbance data were measured with the Infinite® 200 pro instrument (TECAN, Männedorf, Switzerland). For each cytokine, the highest lower limit of quantification and the lowest upper limit of quantification were applied between runs.

2.4. DNA extraction and FLT3-ITD detection

DNA extraction was performed using QIAsymphony® DNA kits (Qiagen, Hilden, Germany). *FLT3-ITD* detection was performed using fragment analysis method. Briefly, a 5'-TGGTGTTTGTCTCTTCTTCATTGT-3′ forward primer and 5'-GTTGCGTTCATCACTTTTCCAA-3′ reverse primer were used for initial PCR, and a fluorescent marker was added to the forward primer for subsequent capillary migration. An amount of 25 ng of extracted DNA was added to PCR mix containing 2.5 μL10X Gold Buffer, 1.5 μL $MgCl_2$ 25 mM, 2 μL DNTP 2.5 mM, 1 μL forward and reverse primers 10 μM, 0.2 μL Gold Taq polymerase and sterile purified water to an overall volume of 25 μL. Thermocycling program required initial activation 10 min at 95 °C, denaturation 45 s at 94 °C, hybridation 45 s at 60 °C and elongation 1 min at 72 °C during 40 cycles and final stage 7 min at 72 °C. PCR products were diluted into formamide and GeneScan™ 600 LIZ® Size Standard (ThermoFisher, Waltham, MA, USA) (1 μL PCR products, 0.5 μL size dye and 10 μL formamide) and analyzed using Applied Biosystems™ 3500 Genetic Analyzer instrument (ThermoFisher) and GeneMapper™ software (version 4.1, ThermoFisher).

2.5. Targeted next generation sequencing

Libraries construction and sequencing of a panel of 41 genes commonly mutated in myeloid malignancies were carried out as already described [20,22]. Read alignment and variant calling were performed using Sophia DDM® 5.0.12 software (Sophia Genetics, Lausanne, Switzerland) with a sensitivity of 1 %. Clonal hematopoiesis (CH) was defined as the presence of at least 10 reads carrying one somatic variant with a variant allele frequency (VAF) > 1 %.

2.6. Cytogenetic analysis

Cytogenetic analysis was performed using standard procedure for hematologic karyotyping. Briefly, BM samples were cultured 24 h to 48 h into fetal bovine serum and RPMI (ThermoFisher), 37 °C and 5 % CO_2. Subsequent steps were colchicine exposure, hypotonic choc and fixation until spreading and denaturation for R-banding. International System for Human Cytogenetic Nomenclature 2020 [23] was used to describe clonal chromosomal abnormalities. Revised International Prognostic Scoring System [24] and European Leukemia Net 2017 classifications [25] were used for prognostic categorization associated with the karyotype for MDS and AML patients respectively.

2.7. Ontogenic classification

For all AML, ontogeny type was defined according to Lindsley et al. classification [26]: *TP53* type for patients with *TP53* mutations; secondary type for patients carrying clonal mutations of *ASXL1, SRSF2, SF3B1, U2AF1, ZRSR2, BCOR, EZH2* or *STAG2*; *de novo* type for patients carrying clonal *NPM1* mutation or rearrangement of *KMT2A* or Core Binding Factor; and pan-AML type for patients not otherwise classified.

2.8. Statistical approach

Statistical analyses were performed using Python 3.8.5. Nonparametric Mann-Whitney or Kruskall-Wallis tests, Spearman correlation and Chi-squared test were used. *P* value <0.05 were considered significant. Recursive Feature Elimination (RFE) was applied for cytokine relevance analysis, using 1000 random forest trainings with different numbers of trees, depths and random seeds, and related features importance. Using a set of 10 different hyper-parameters and random seeds, cytokines that have been selected at least 100 times were isolated for PCA analysis. Logistic regressions were then applied on 3 main PCA axes. Cramer's V statistic was used to assess effect size of the association between patient cytokine profile, diagnosis and genetic mutations. This statistical method quantifies the magnitude of the variation of each quantitative variable of interest between different groups. Code is available under request.

3. Results

3.1. Cohort description

From the initial cohort of 259 individuals, we excluded 23 AML, 10 MDS and 8 healthy volunteers because of the absence of available material or karyotypic characterization. We finally analyzed 218 BM plasma (94 healthy volunteers, 44 MDS and 80 AML patients) (Fig. 1, Table 1). The sex ratio was 0.6, 2.4 and 1.5 in healthy volunteers, MDS and AML patients, respectively. The average age was 45.1 years for healthy volunteers, 72.5 years for MDS patients, and 56.9 years for AML patients. MDS with excess blasts represented 36.4 % ($n = 16$) of MDS patients, and 9.1 % ($n = 4$) CMML patients were included. Intermediate cytogenetic risk was observed in 70.5 % ($n = 31$) MDS and 65.0 % ($n = 52$) AML patients. Favorable cytogenetic risk was found in 13.6 % ($n = 6$) MDS and 5.0 % (n = 4) AML patients. Poor cytogenetic risk concerned 15.9 % ($n = 7$) MDS and 30.0 % ($n = 24$) AML patients. When classifying patients according to ontogenic subtypes (see 2.7. Ontogenic classification), 25.0 % AML ($n = 20$) belonged to *de novo* type, 15.0 % AML ($n = 12$) to *TP53* type, 35.0 % AML ($n = 28$) to secondary type, and the remaining 25.0 % AML (n = 20) to pan-AML type. When applying this ontogenic classification to MDS/CMML cases, no case belonged to *de novo* type, 11.4 % (n = 5) to the *TP53* type, 56.8 % ($n = 25$) to the secondary type, and the remaining 31.8 % cases ($n = 14$) to pan-AML type (Table 1, supplemental Table 1).

3.2. MDS and AML share similar BM pro-inflammatory cytokine profiles in comparison to healthy volunteers

In a previous study, the comparison of BM and blood plasmas from healthy individuals showed that most cytokines had uncorrelated concentrations [27]. We found a similar pattern in pathological samples by comparing cytokine concentrations from 45 paired BM and blood samples from MDS and AML patients, indicating that BM plasma concentrations really reflect this territory in the whole cohort (Supplemental Table 2). Assuming that the local BM microenvironment plays a major role in AML pathophysiology, we focused our analysis on the BM cytokine concentrations. We first interrogated the correlation between each cytokine and no strong correlation was observed (minimum Spearman r ranging from −0.30 to 0.79, supplemental Fig. 1). Violin plot graphs of cytokine concentrations in each group and descriptive statistics are shown in supplemental Fig. 2 and supplemental Table 3, respectively. Non-supervised clustering of cytokine concentrations allowed a clear distinction between MDS and AML patients *vs.* healthy volunteers (Fig. 2). The cytokine profiles reveal two distinct groups of patients, one of which approximates the profile of healthy volunteers. Of note, AML and MDS patients were still mixed in this first approach.

Among controls, as we previously described, individuals with CH had increases in MIF and IL-1β concentrations and decreases in IL-5 and IL-9 concentrations [27]. These cytokines were statistically significantly increased in MDS or AML patients when compared to all healthy volunteers with fold changes (FC) > 2 (IL-1β, IL-5 and IL-9) except for MIF, with smaller FC (FC = 1.54, $p = 7.73\text{E-}05$ for MDS and FC = 1.22, $p = 4.07\text{E-}01$ for AML) (supplemental Table 3). Moreover, when comparing MDS patients exclusively to CH individuals, IL-1β, IL-5 and IL-9 remained significantly increased. Altogether, as BM cytokines concentrations varied with aging in healthy volunteers [27], these results led us to keep CH individuals in further analysis.

Between MDS and healthy individuals, we highlighted a significant increase (FC > 2) in the concentrations of 34 cytokines (Fig. 3A, supplemental Table 3). No cytokine concentration was found decreased in MDS patients in comparison to healthy volunteers. The higher FC were found for CXCL8, IL-6 and CXCL10 (FC = 21.46, $p = 2.10\text{E-}18$, FC = 14.06, $p = 5.37\text{E-}12$ and FC = 11.02, $p = 3.59\text{E-}17$, respectively).

Comparison of AML patients to healthy volunteers revealed a significant increase in 31 cytokines (Fig. 3B, supplemental Table 3), again including CXCL8 (FC = 9.84, $p = 1.08\text{E-}25$), CXCL10 (FC = 9.60, $p = 4.84\text{E-}25$) and IL-6 (FC = 8.28, $p = 7.08\text{E-}23$). Two cytokines were decreased in AML patients: FLT3L (FC = 0.31, $p = 2.09\text{E-}25$) and PDGF-B (FC = 0.28, $p = 1.22\text{E-}16$).

These data indicate that in both MDS and AML, a large panel of cytokines is overexpressed within the BM plasma, in relationship with a global inflammatory context.

3.3. AML display specific changes in cytokine concentration compared to MDS

In the BM plasma, the concentrations of 44 cytokines did not change significantly between MDS and AML patients (Fig. 3C, supplemental Table 3). Interestingly, only one cytokine was higher in AML *vs.* MDS: CLEC11A (FC = 2.71, $p = 3.87\text{E-}04$). Four cytokines were significantly decreased in AML plasma: PDGF-B (FC = 0.25, $p = 1.40\text{E-}08$), VEGF-A (FC = 0.39, $p = 2.37\text{E-}03$), FLT3L (FC = 0.36, $p = 4.76\text{E-}11$) and IL-12 (FC = 0.40, $p = 1.14\text{E-}02$). CLEC11A, FLT3L and PDGF-B are highlighted in Fig. 3D. Considering these results, we chose to focus our analysis on the most informative cytokines according to the diagnosis by successively performing recursive feature elimination (RFE), PCA and logistic regression.

RFE allowed the extraction of cytokines by performing 1000 RFE to reach lists of only 10 cytokines in each iteration, using a random forest algorithm trained to match diagnosis (*i.e.*, 'Healthy', 'AML' or 'MDS') (Fig. 4A). We obtained a final selection of 18 cytokines. Interestingly,

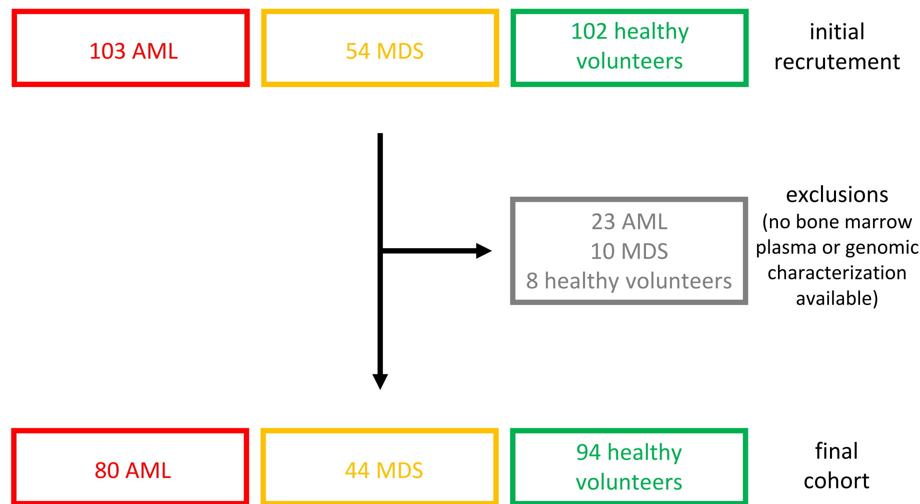

Fig. 1. Flow chart of the study.

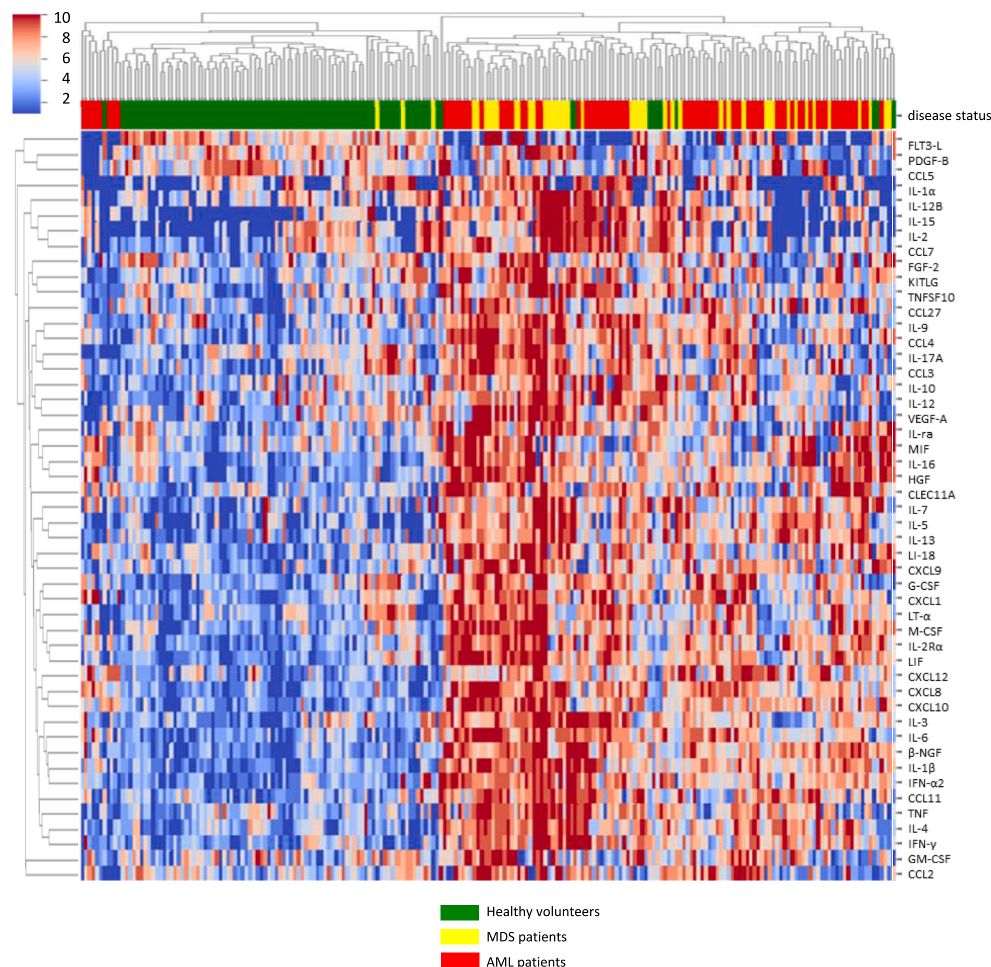

Fig. 2. Heatmap of BM cytokine concentrations in healthy volunteers, MDS, and AML patients. Concentrations for each patient are expressed in decile by cytokine. Disease status is depicted as follows: green, healthy volunteers; yellow, MDS patients, and red, AML patients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the most conservative cytokines were CXCL8, FLT3L, CXCL10 and PDGF-B, which reached the end of RFE in more than 90 % of occurrences. The other cytokines selected (ranked by decreasing order of RFE count) were IL-6, CXCL12, CLEC11A, β-NGF, IL-1ra, CXCL1, CCL11, IFN-α2, IL-2Rα, IL-1β, IL-16, LIF, VEGF-A, and CCL4. Among the 18 cytokines, 17 cytokines allowed a clear distinction between patients and controls (Supplemental Fig. 3) with IL-16, IL-1ra, CLEC11A, CCL11, CCL4, CXCL1, LIF, IL-2Rα, IL-6, β-NGF, IL-1β, IFN-α2, CXCL12, CXCL10,

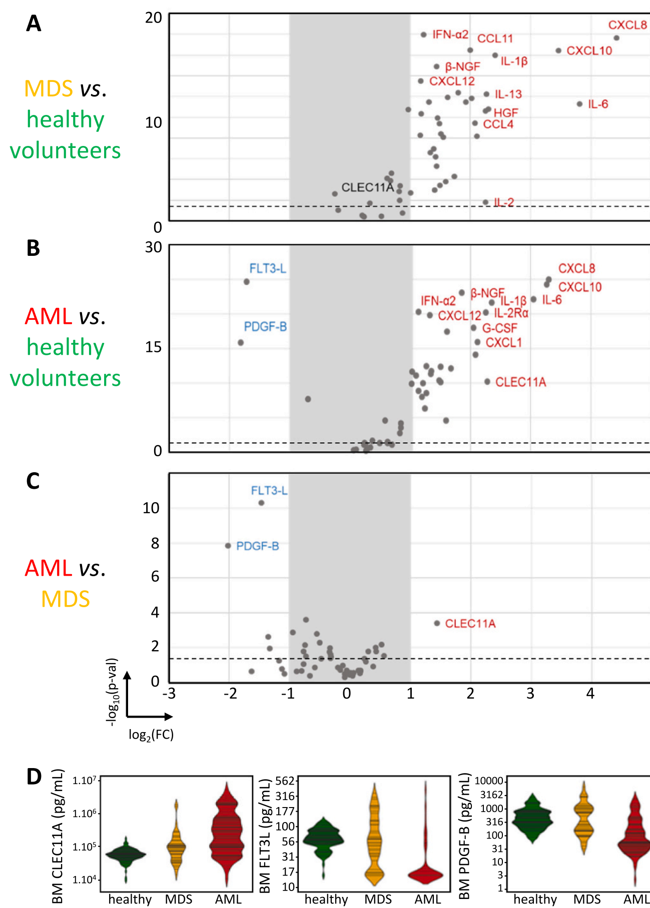

Fig. 3. Modifications of the 49 BM cytokine concentrations according to disease status. Volcano plots show the modifications found when comparing (A) MDS to controls, (B) AML to controls, and (C) AML to MDS. The dashed lines indicate the significance limit ($p < 0.05$). (D) Violin plots illustrate the concentration of 3 cytokines (CLEC11A, FLT3L and PDGF-B) in healthy volunteers ($n = 94$), MDS ($n = 44$) and AML ($n = 80$) patients.

Table 1
Description of the cohort.

		healthy volunteers	MDS/CMML	AML
	n	94	44	80
	male (%)	36 (38.3)	31 (70.5)	48 (60.0)
	age mean (standard deviation)	45.1 (15.2)	72.5 (9.4)	56.9 (17.2)
MDS/CMML	patients with excess blasts, n (%)		16 (36.4)	
	CMML patients, n (%)		4 (9.1)	
cytogenetics	favorable, n (%)		6 (13.6)	4 (5.0)
	intermediate, n (%)		31 (70.5)	52 (65.0)
	poor, n (%)		7 (15.9)	24 (30.0)
ontogeny type	*de novo*, n (%)		0 (0.0)	20 (25.0)
	TP53, n (%)		5 (11.4)	12 (15.0)
	secondary, n (%)		25 (56.8)	28 (35.0)
	pan-AML, n (%)		14 (31.8)	20 (25.0)

CXCL8 being more concentrated, and FLT3L and PDGF-B being lower in patients than in healthy volunteers, respectively (Fig. 3D). PCA allowed a better understanding of the links between these 18 cytokines (Fig. 4B, supplemental Fig. 4). The first 2 (PC1, PC2) and 3 axes (PC1, PC2 and PC3) explained a total of 56.4 % and 64.3 % of variance, respectively. The discrimination between patients and healthy volunteers was mainly due to the 15 cytokines previously cited as having high concentration in patients. The concentrations of PDGF-B, VEGF-A, FLT3L and CLEC11A emerged as relevant discriminators between AML and MDS based on both univariate analysis and supervised classification approaches (Fig. 4C, supplemental Fig. 5). A ROC analysis showed a strong discriminative capacity between AML and MDS (AUC = 0.82), confirming the relevance of these 4 cytokines as potential biomarkers (supplemental Fig. 5). Of note, PCA analysis revealed that healthy volunteers with CH ($n = 16$) exhibited a cytokine profile that was closer to that of healthy volunteers without CH than to those of MDS or AML patients (supplemental Fig. 6). Finally, we used logistic regression to assign a healthy, MDS or AML cytokine profile to all samples using the values of PC1, PC2 and PC3. A healthy cytokine profile was assigned to 92/94 healthy samples and 9/124 MDS or AML samples. A MDS cytokine profile was assigned to 1/94 healthy sample, 19/44 MDS samples, and 7/80 AML samples. An AML profile was assigned to 1/94 healthy sample, 19/44 MDS samples, and 70/80 AML samples (Fig. 4C, supplemental Fig. 7).

As show in Table 2, this strategy made it possible to classify the patients (92.7 %, $n = 115/124$) in 4 groups according to the disease diagnosis and the assigned cytokine profile (AML with AML profile, AML with MDS profile, MDS with MDS profile and MDS with AML profile).

3.4. *De novo* AML patients exhibit a specific cytokine profile

Using the Lindsley classification [26] (see 2.7. Ontogenic classification), 20 AML patients were classified as *de novo* type, 28 as secondary type, 12 as *TP53* type and 20 as pan-AML type (Table 1, supplemental Table 1).

Then, the distribution of the patients between the four ontogenic groups and their matching cytokine profile and diagnosis were compared. For *TP53*, pan-AML and secondary types AML, the distribution of all patients between the 4 groups was not different, meaning lack of correlation between specific cytokine profile and these ontogenic subtypes. Interestingly, *de novo* type was significantly correlated with AML cytokine profile ($p = 0.001$) (Table 2). The subgroup of MDS patients with AML profile tended to have a higher percentage of bone marrow blasts, higher IPSS-R scores, and a higher frequency of activated signaling mutations compared to other MDS patients. Conversely, AML patients with MDS profile tended to have a lower percentage of bone marrow blasts, and a lower frequency of activated signaling mutations compared to other AML patients (Table 2).

The concentration of each cytokine in AML subtypes was compared to healthy volunteers (Fig. 5A), and between them (Fig. 5B). This revealed significant differences in concentrations of CLEC11A (increased) and FLT3L (decreased) in *de novo* AML when compared to the 3 other AML subtypes. The *de novo* AML group included mostly *NPM1* mutated AMLs (66.6 %, $n = 14/20$), where the highest concentrations of CLEC11A were found (Fig. 5C). No major cytokine modification was found in the 3 other ontogenic subtypes of AML (Table 2). Altogether, these results suggest a potential key role of CLEC11A in the pathophysiology of *de novo* AML, especially those with *NPM1* mutations.

4. Discussion

Cytokine network is a key component regulating hematopoietic homeostasis in the BM. There are multiple studies in the literature showing major variations of these molecules in hematopoietic malignancies, with the increase of pro-inflammatory cytokines and decrease of anti-inflammatory cytokines. The resulting imbalance is thought to be

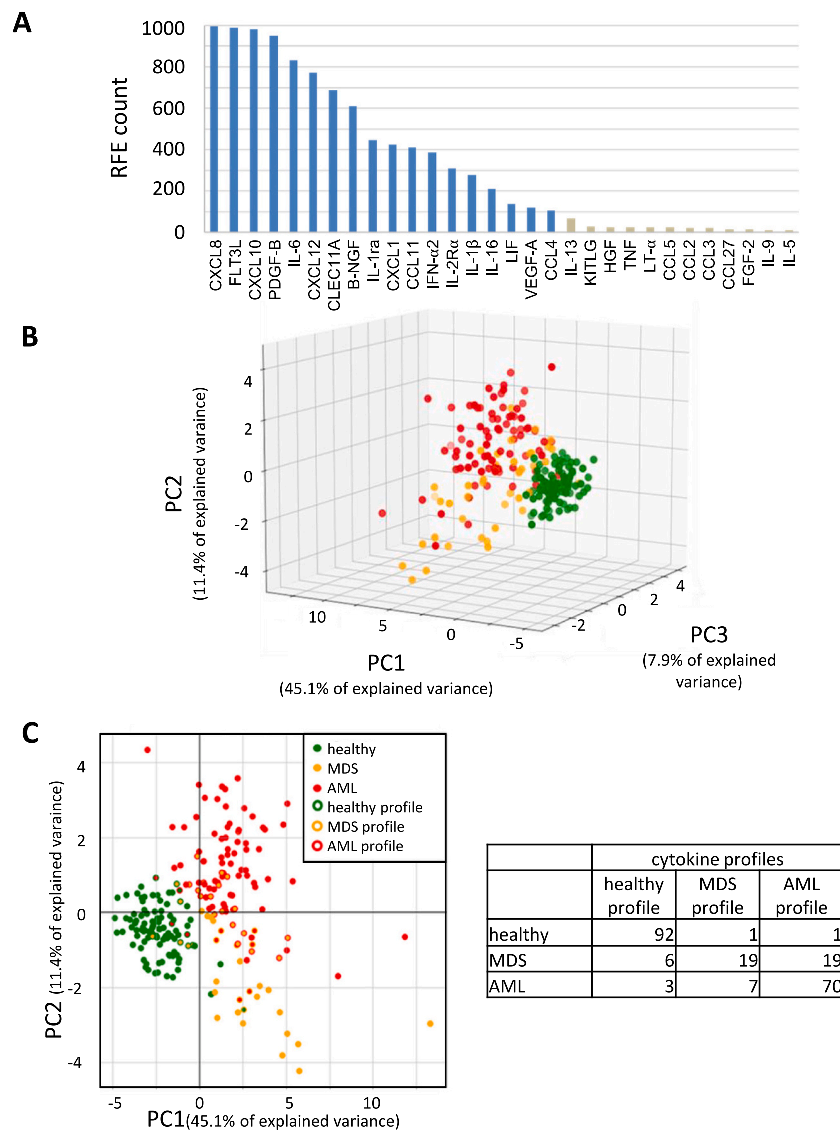

Fig. 4. Distinction of disease status according to cytokine profile. (A) Selection of the 18 most informative cytokines (blue bars) among the 49 cytokines after 1000 recursive feature eliminations (RFE) using the random forest algorithm. (B) Tridimensional principal component analysis representation using the 18 most informative cytokines selected in A. (C) Matching between disease status and cytokine profile using logistic regression model, as shown by PCA analysis or confusion matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Percentage of bone marrow blasts, IPSS-R score, frequency of activating mutations, and ontogeny distribution for each new group derived from PCA analysis, excluding healthy volunteers.

	AML	AML	MDS	MDS	p value
	AML profile	MDS profile	AML profile	MDS profile	
n	70	7	19	19	
bone marrow blasts (%, mean [min - max])	55.9 [12.0–96.0]	28.1 [20.0–44.0]	8.2 [0.0–19.0]	3.7 [0.0–18.0]	<0.001
IPSS-R (mean [min - max])	NA	NA	3.9 [1.0–8.5]	2.6 [0.0–8.0]	0,029
activating mutations (%)[#]	42,9	14,3	26,3	5,3	0,010
de novo type[✤]	20 (28.6 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0.001
secondary type[†]	24 (34.3 %)	2 (28.6 %)	11 (57.9 %)	11 (57.9 %)	0.103
TP53 type[ˀ]	9 (12.9 %)	2 (28.6 %)	2 (10.5 %)	2 (10.5 %)	0.636
pan-AML[‡]	17 (24.3 %)	3 (42.9 %)	6 (31.6 %)	6 (31.6 %)	0.684

Ontogeny type was defined according to Lindsley et al. Classification:
[#] include mutations of *CBL, FLT3, HRAS, KRAS, NRAS, KIT, NF1, PTPN11*.
[✤] patients carrying clonal *NPM1* mutation, rearrangement of *KMT2A* or Core Binding Factor.
[†] patients carrying clonal mutations of *ASXL1, SRSF2, SF3B1, U2AF1, ZRSR2, BCOR, EZH2* or *STAG2*.
[ˀ] patients with *TP53* mutations.
[‡] patients not otherwise classified.

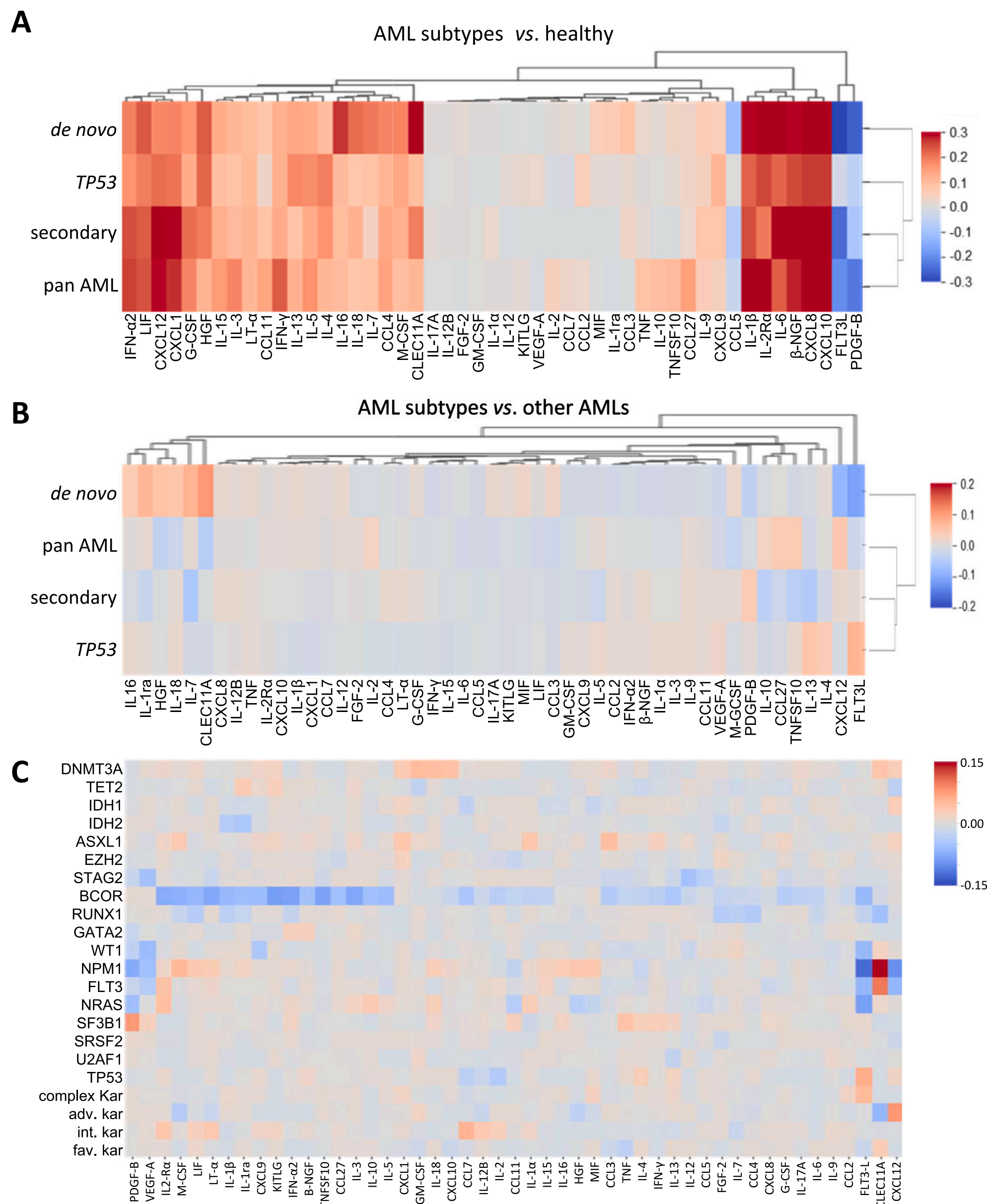

Fig. 5. BM cytokines levels effect size according to ontogenic subtypes in AML and for each cytogenetic abnormality or gene mutation. Unsupervised clustering on cytokine level effect size (A) in each ontogenic subtype AML compared to controls, (B) in each ontogenic subtype AML to other AMLs and (C) in each cytogenetic abnormalities or gene mutations. Kar = karyotype; adv = adverse; int = intermediate; fav = favorable.

responsible for a deregulation of the functions of HSCs and progenitors [28,29] and to promote the development of the leukemic clone [30]. However, most of these studies investigated these changes in the PB compartment and not directly in the BM because of the difficulty of obtaining the control material from strictly healthy individuals. Our study of cytokines in MDS and AML, although not exhaustive, highlights the interest of BM sampling to describe the cytokine compartment of the leukemic microenvironment. The sampling conditions were standardized to all patients and healthy volunteers as controls and were like those currently used for the diagnosis in onco-hematology in France.

As early as the MDS stage as well as at AML diagnostic, the BM microenvironment presents abnormal cytokine profiles with increased pro-inflammatory mediators such as CXCL8, IL-6 and CXCL10. This pro-inflammatory state in AML has already been described and seems to participate to leukemogenesis [31–33]. Recently, the knockout of *Il6* gene in a del(5q) murine model with monocytic AML showed a reduced risk of leukemic transformation [34]. Another recent study demonstrated an amplification loop between mesenchymal stem cells (MSCs) and leukemic blasts. MIF secretion by blasts increased CXCL8 expression in MSCs that triggered leukemic survival [13]. MIF overexpression in monocytes was shown to be related to TET2 depletion and its transcriptional activation through EGR1 [35]. We also observed an increase in BM levels of anti-inflammatory IL-4 and IL-10 in patients that was already described [36] and thought to be the activation of a negative feedback against inflammation. Although these cytokines seem to participate to blast proliferation and survival, they were not correlated to any specific clonal architecture in our study. We hypothesize that, alone, they do not exert sufficient selection pressure to select clones with specific ontogenic mutations or translocations.

In this work we explored cytokine changes in the BM plasma of MDS and AML patients, considering their somatic mutations and the inferred ontogenic type of their disease, according to the classification of Lindsley and colleagues [26]. We found a specific cytokine profile in *de novo* type AML, with particularly increased concentrations of CLEC11A

and decreased concentrations of FLT3L. Note that CLEC11A increased concentration is mainly observed in *NPM1* mutated AMLs.

CLEC11A, also called osteolectin, is a C-type lectin, first described as the Stem Cell Growth Factor because of its function as a growth factor for primitive hematopoietic progenitor and leukemic cells [37–39]. More recently, its role in osteogenic differentiation of mesenchymal stem cells has been established [40]. CLEC11A+ Leptin Receptor+ (LepR+) stromal cells are also critical for lymphopoiesis, through the stimulation of lymphoid progenitor proliferation and differentiation by the secretion of Kit ligand [41]. It has also been implicated in cancer [42], myeloma [43] and leukemia [44,45]. For instance, it appeared to be secreted by leukemic cell lines and participates to cell growth [38]. We quantified the β isoform of the protein, which is the shortest form lacking 78 AA of the C terminal lectin domain. BM plasma of MDS and AML patients were enriched in this isoform with specific correlation with *de novo* type AML patients. The putative receptor of CLEC11A is the integrin receptor α11β1 mostly expressed by LepR+ cells and osteogenic cells with activated β catenin/Wnt signaling pathway [46]. We still lack information on its role in leukemogenesis, as it may promote *de novo* type leukemogenesis directly or by specific BM microenvironment changes.

We found a dramatic decrease in BM FLT3L concentrations in patients with *de novo* type AML. FLT3L is a hematopoietic growth factor for subsets of HSCs, multipotent progenitors, myeloid progenitors, lymphoid progenitors and dendritic cell precursors that all express FLT3 receptor. It is unclear whether FLT3L promotes lympho-myeloid differentiation or is only responsible for the proliferation of these progenitors [19,47]. As FLT3 is its only known receptor, with an expression restricted to hematopoietic cells, modifications of FLT3L concentrations may play a direct role on clonal selection. Variations of FLT3L have been described in acquired aplastic anemia and in Fanconi anemia with increased concentrations in the PB [48]. On the contrary, MDS and AML patients display decreased concentrations of this growth factor. Moreover, FLT3L concentrations seem to correlate with prognosis, showing increasing values upon chemotherapy [17,18,49]. Altogether, these observations suggest that depletion in FLT3L may play an important role in the selection of AML cells, particularly those with gain of function mutations in *FLT3* or in the RAS pathway.

5. Conclusions

Leukemia-associated BM microenvironment is a pro-inflammatory milieu that favors the survival and proliferation of leukemic clones. In this work, we compared BM cytokine profile among different clonal architecture of MDS and AML. We observed a shared pro-inflammatory state between ontogenic subtypes that may favor leukemic proliferation and may be responsible for a defective hematopoiesis. Interestingly, *de novo* patients exhibited a specific pattern with an increase in CLEC11A and a decrease in FLT3L concentrations. Keeping in mind that a validation study using another cohort would reinforce our results, these data suggest that microenvironment changes and clonal selection are closely related. On one hand, clonal selection may depend mainly on the intrinsic characteristics of the blasts and their fitness in a pro-inflammatory microenvironment. On the other hand, specific modifications may be required for the emergence of clones. Thus, leukemogenesis may be in part driven by the BM microenvironment, for instance through the low concentrations of FLT3L, PDGF-B, and high concentration of CLEC11A, which may participate in the selection of *NPM1* and *FLT3* mutated cells. Further analyses are required to explore mechanisms implicated in the deregulation of these cytokines.

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CRediT authorship contribution statement

Noémie Ravalet: Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Hélène Guermouche:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Pierre Hirsch:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Formal analysis, Data curation, Conceptualization. **Frédéric Picou:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Vincent Flament:** Formal analysis, Data curation. **Caroline Deswarte:** Formal analysis. **Amélie Foucault:** Resources, Formal analysis. **Jenny Beaud:** Resources, Formal analysis. **Emmanuelle Rault:** Resources, Formal analysis. **Emeline Saindoy:** Formal analysis. **Sébastien Lachot:** Resources. **Mara Memoli:** Resources. **Nawa Hachem:** Writing – review & editing. **Jean-Alain Martignoles:** Writing – review & editing. **Valérie Gissot:** Resources. **Ludovic Suner:** Writing – review & editing, Resources, Formal analysis. **Emmanuel Gyan:** Resources. **Ollivier Legrand:** Resources. **Olivier Hérault:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **François Delhommeau:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

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Data availability

Interested collaborators may direct data requests to the corresponding author.

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