The plasmacytoid dendritic cell CD123+ compartment in acute leukemia with or without *RUNX1* mutation: high inter-patient variability disclosed by immunophenotypic unsupervised analysis and clustering.

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Abstract

Plasmacytoid dendritic cells (pDC) constitute a small subset of normal bone marrow (BM) cells but have also been shown to be present, sometimes in large numbers, in several hematological malignancies such as acute myeloid leukemia with *RUNX1* mutation, chronic myelomonocytic leukemia or, obviously, blastic plasmacytoid dendritic cell neoplasms. These cells have been reported to display somewhat variable immunophenotypic features in different conditions. However, little is known of their plasticity within individual patients. Using an unsupervised clustering tool (FlowSOM) to re-visit flow cytometry results of seven previously analyzed cases of hematological malignancies (6 acute myeloid leukemia and one chronic myelomonocytic leukemia) with a pDC contingent, we report here on the unexpectedly high variability of pDC subsets. Although five of the studied patients harbored a *RUNX1* mutation, no consistent feature of pDCs could be disclosed as associated with this variant. Moreover, the one normal single-node small subset of pDC detected in the merged file of six normal BM could be retrieved in the remission BM samples of three successfully treated patients. This study highlights the capacity of unsupervised flow cytometry analysis to delineate cell subsets not detectable with classical supervised tools.

Introduction

Acquired mutations of the *runt-related transcription factor 1* (*RUNX1*) gene are found in 5-15% of patients with de-novo acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) or myelodysplastic/myeloproliferative neoplasms (MDS/MPN) such as chronic myelomonocytic leukemia (CMML) [1, 2]. AML with RUNX1 mutation (*RUNX1*^{mut}) is a provisional entity in the WHO 2016 classification of hematopoietic neoplasms [3], to be considered after excluding other AML categories such as AML with recurrent genetic abnormalities, AML with MDS-related changes (AML-MRC) and therapy-related AML (t-AML). *RUNX1*^{mut} AML is most often purely blastic by cytological assessment and myeloperoxidase-negative, corresponding to AML with minimal differentiation [2,3,4]. In some leukemias with *RUNX1*^{mut}, mixed-phenotype acute leukemia (MPAL) characteristics can however be present [5] and *RUNX1*^{mut} has also been associated in AML cases with a plasmacytoid dendritic cell (pDC) component [6,7]. However, *RUNX1*^{mut} has only seldom been reported in blastic pDC neoplasms (BPDCN) [6, 8], while clusters of mature pDCs have been described in bone marrow (BM) biopsies from patients with MPN or MDS/MPN (ref Naresh et al, Am. J. Hematol. 85:893, 2010). An association of BPDCN with CMML has also been reported (ref). Finally, *RUNX1*^{mut} has been found in blast crisis of chronic myeloid leukemia and *RUNX1*^{mut} BP-CML patients showed a notable population of pDCs (Awad et al, Leukemia (2021) 35:1087–1099)

Conventional multiparameter flow cytometry (MFC) initial immunophenotypic definition of pDC was of cells negative for lineage markers (lin) coexpressing CD4 and CD56 (Facchetti et al., 1988 Am J Pathol; Chaperot et al. Blood 2001). Subsequently (Galibert et al., 2001, Sem Immunol, Feuillard Blood 2002; Garnache Ottou, 2010 BJH), these cells were recognized to express CD38, CD123, CD303 and HLA-DR, with a variable expression of CD33 and absence of CD64. Small fractions of normal/reactive PDCs positive for CD2, CD7 and/or CD56 have also been noted. (ref). Dendritic cell progenitors in the human BM are extremely rare and their immunophenotype as well

as stages of differentiation remain a matter of debate (refs). Blasts in BPDCNs with leukemic presentation show an immunophenotype similar to normal pCDs i.e. CD45dim/low SSC, $CD4^+$(bright), $CD123^+$ (bright), $CD56^+$ (heterogenous), $CD33^+$(dim/heterogeneous), $CD36^+$, $CD38^+$, HLA-DR^+ and are negative for CD10, CD11b, CD13, CD14, CD15, CD16, CD19, CD34, CD64, CD65, CD235a, cyt.MPO, cyt.CD3, cyt.CD22, and nTdT (ref).

The identification of pDC in MFC relies on sequential supervised gating strategies and monoparametric or biparametric histograms tracking cells with these suspected immunophenotypic features (Breton et al, Frontiers). The recent development of tools for an unsupervised delineation of cell subsets within samples stained with targeted antibody panels allows for a more accurate identification of discrete cell subsets in normal BM and for detailed studies of the heterogeneity of malignant proliferations. FlowSOM is one of the new types of software, developed in the Bioconductor R system. This software has been applied to the definition of normal BM with myeloid, lymphoid, and erythroid antibody panels (refs) and related diseases. Here we explored how FlowSOM could dissect pDC-like populations in 7 patients with leukemic disorders involving these peculiar cells.

Materials and Methods

Bone marrow samples: Six normal bone marrow (NBM) samples were obtained from five healthy volunteers and one patient with idiopathic thrombocytopenia and reactive bone marrow. These six NBM samples were analyzed with the AML MFC panel (Suppl. Table 1, (ref)) at the Flow Cytometry Laboratory Lund's University Hospital Pathology Department, Sweden, as a part of a quality assurance study.

The same panel was applied to BM samples from 7 AML patients with potential pDC involvement, diagnosed between 2018 and 2021 according to routine procedures that included morphology, MFC, karyotype and molecular analyses. Patient characteristics are given in Table 1. MFC methodology is given in Suppl. Methods and Suppl. Table

1. Karyotype analysis was performed by routine methods at the Department of Clinical Genetics, Lund University Hospital. Next generation sequencing (NGS) was performed as a part of routine work-up, using Illumina Trusight Myeloid sequencing panel (Illumina, San Diego, CA) at the Diagnostic Molecular Center, Lund University Hospital. The diagnostic criteria of WHO 2016 classification were applied (ref). The samples were selected for detailed FlowSOM analysis based on the presence of *RUNX1*^{mut} and/or increased numbers of CD123^{+} cells in routine MFC analysis.

FlowSOM analysis: For detailed analysis of pDC maturation, the raw (.fcs) files of panel AML 2 that included CD123 together with immature progenitor markers CD34 and CD117, as well as CD7, HLA-DR, CD38, CD33, CD45, and markers of monocyte maturation CD36 and CD64 were used (Suppl. Table 1). Analysis was performed on the Kaluza software (Beckman Coulter, Miami, FL) equipped with FlowSOM plug-ins as previously described (Cytometry A ref). Briefly, all LMD files were checked for proper compensation and fluorescence was normalized by comparison to lymphocytes with a dedicated R script as reported (Cytometry A). The 6 normalized list-mode files of NBM samples were merged with the Kaluza merging tool. The resulting single merged NBM file was submitted to unsupervised analysis by FlowSOM with the set-seed option allowing the software to generate 24 minimal spanning trees (MST) of 100 nodes. Nodes with CD123 positivity were detected on the MST through back-gating and the major NBM subsets were determined by coloring a classical CD45/SSC dot plot as previously described (ref.Cytometry A). The graphical MST providing the best grouping, yet clear separation of the nodes of interest, was then chosen (Fig.1). Each node/group of nodes of interest was individually studied in Kaluza, recording the number of events, scatter properties and normalized mean fluorescence intensity (MFI) of each marker. The frequency of cells in each node/group of nodes was then calculated as a fraction of the whole sample. Based on the analysis of the NBM merged file, cut-offs for normalized marker expression were established (Suppl. Table.2). The cut-off for positive CD123 normalized expression was a mean

fluorescence intensity (MFI) of 2.0 while cells with a normalized expression >3.0 were considered as strongly CD123 positive.

All patient samples were analyzed individually. The FlowSOM set-seed script was run for each sample requesting 100 nodes and 6 MST proposals. Each of the 6 MST for each sample was visually checked after back-gating and color-coding of the cell subsets on a standard CD45/SSC histogram. Nodes with marker expression levels and scatter properties similar as those identified in the NBM MST were recognized and color coded as such.

Results

Normal bone marrow pattern: FlowSOM analysis of the merged NBM file (Fig.1) showed 95 nodes that could be identified as cell clusters based on scatter characteristics and CD45 expression. Three nodes showed normalized CD123 expression >2. The node characterized as pDC (0.32% of all cells, strong CD123 with 4.66 MFI) was also strongly positive for HLA-DR, CD36 and CD38 with a borderline expression of CD33 (1.52) and intermediate CD45 (0.62). CD7, CD64, CD34 and CD117 were negative (Suppl. Table 3). However, separately performed manual analysis (Suppl.Fig1) showed in each normal BM a tiny CD7+ population of pDC 0.02-0.06% (mean 0.03%) of bone marrow cells and 3-10% of pDC. Obviously, this population was too small to allow for a separate node by the software. Another node with strong CD123 (MFI 4.47) was characterized as basophils due to negative HLA-DR, stronger CD33 and CD38 expression, all other markers in the panel being negative. The third CD123-positive node was characterized as myeloid DC (MDC) with an intermediate expression of CD123 (MFI 2.5), positivity for HLA-DR, weak CD33 and CD36 but none of the other markers. Marker expressions in other identified cell populations are given in Suppl. Table 3.

Abnormal bone marrow samples:

Cases 1-5 (Table 1) were *RUNX1*^{mut} and showed distinct CD123+ subsets while cases 6-7 had a clear pDC population with some aberrant features but were *RUNX1*^{wt}. Cases 1 and 2 (Table 1) were diagnosed as AML with *RUNX1*^{mut}. No follow-up samples were available in these cases. Case 3 was classified as AML-MRC due to del(7q) and a sample taken after induction therapy could also be studied. Case 4 was classified as t-AML due to previous chemotherapy for colon cancer. Case 5 was diagnosed as CMML-1, 6 months before the studied sample was taken. The patient developed a skin lesion with the morphology and immunophenotype of BPDCN. At the time of BPDCN diagnosis there were no signs of BM involvement, and an allogeneic hematopoietic stem cells transplant (Allo-HSCT) was planned. The sample taken before Allo-HSCT showed signs of involvement by BPDCN. Another sample taken after Allo-HSCT showed complete remission. Cases 6 and 7 were diagnosed as t-AML and T/myeloid MPAL, respectively. A follow-up sample after induction therapy was studied in case 7.

Case 1 is a *RUNX1*^{mut} AML that displayed a dominating CD34^{+}CD117^{+}CD45dim CD36^{-} CD33^{-}CD64^{-}CD7^{-} blast population with low SSC. Detailed analysis by FlowSOM (Fig.2, Suppl. Table 4) showed the near absence of granulocytes and absence of monocytes with 5 nodes of lymphocytes and four of remaining normal hematopoiesis. The bulk of the blastic population, quite homogeneous in SSC/CD45, was in fact split in 10 subsets based on differential expression of CD123, HLADR, CD34 and CD38. Three CD34+CD117+ subsets showed strong CD123 expression (>3) and differed by HLA-DR and CD38 (from strong through intermediate to dim). Five CD34+CD117+ subsets had intermediate CD123 and varied by the levels of HLA-DR expression. One CD34+CD117+ subset was CD123, CD38 and HLA-DR negative. One subset was CD123 intermediate and CD34 negative but CD117+. This subset was CD33 positive and had stronger CD45 but was negative for CD36 and CD64 suggesting differentiation towards promyelocytes.

Case 2 is another *RUNX1*^{mut} AML that had both an immature CD34^{+}CD117^{+} myeloid blast population and a clear population with differentiation towards pDC (Fig.3, Suppl. Table 5). Most blasts were negative for CD123 except for one subset (15%, Suppl. Table 5). This population had lower CD117 and CD45 expression but higher CD38 and no CD36. The pDCs represented 13.47% of the cells and were partitioned in three different subsets. One had the similar immunophenotype as the pDC node in NBM (Suppl. Table 3) yet with a somewhat lower CD36 expression. The second subset was CD36 negative and the third was CD36 and CD7 positive. Other BM subsets were well represented, with a high diversity among monocytes

Case 3 was classified as AML-MRC with *RUNX1*^{mut}. At diagnosis, 22% of the cells were identified as blasts with four subsets that differed in CD123, CD33, CD34 and CD117 expression (Suppl. Table 6, Fig.4a). There was a small population of classical pDC and a larger population of CD7^{+} pDC. Moreover, abnormal CD123^{+} populations of promonocytes (CD117^{+}) and monocytes were detected (Suppl. Table 6). After induction therapy (Fig.4b, Suppl. Table 6) the BM pattern was almost normal except for a small subset of CD123+ monocytes.

Case 4 was classified as t-AML with, among other anomalies, *RUNX1*^{mut} (Table 1). FlowSOM analysis (Fig.5, Suppl. Table 7) showed a major (~74% of the cells) population of CD34^{+}CD117^{+} blasts with low CD38 expression and absence of CD123. Amazingly, FlowSOM partitioned this bulk of cells in more than 60 individual nodes. These blasts were CD33 negative. However, two subsets of CD34^{+}/CD117^{+}/CD123^{+} blasts were found: one CD33/CD38 negative and one with CD38 and CD33 expression. Of note a minor population of blasts positive for CD34, CD117 and CD36 was also detected.

Case 5 is the sample from the CMML patient who was also diagnosed with BPDCN on a skin lesion biopsied 4 months before. At this time only one normal PDC node was found in the BM sample (data not shown). In the current BM sample collected before Allo-HSCT, routine analysis disclosed 3% of abnormal CD56^{+}CD4^{+}CD123^{+}HLA-DR^{+}

cells corresponding to early involvement by BPDCN (Fig.6). FlowSOM analysis (Fig.6, Suppl. Table 8) revealed two subsets of CD34+CD117+ blasts (8.2% of total) that were CD123 negative and differed by a low CD38 and HLA-DR in one of the subsets. Also, a minor population of $CD34^{+}CD117^{+}$ blasts with CD123 expression was noted (0.28%). Two populations of pDC were observed, one of them coexpressing CD7 (2.8%) corresponding to the CD56+ population found at routine analysis. Moreover, a diversified population of granulocytes, normal proportions of lymphocytes and four nodes of monocytes were found. One month after Allo-HSCT, only 0.78% of normal pDC were found in the reconstituted BM (data not shown).

Case 6 was a t-AML and *RUNX1*^{wt} (Table 1). Five CD117+ blast populations could be separated by FlowSOM including two CD123+ subsets (Fig.7, Supp. Table 9). Four were CD34+ and two were CD7+. One of the CD123+ subsets had strong CD123 and HLA-DR expression and was CD7neg while the other had intermediate CD123 and HLA-DR but was CD7 positive. Moreover, a population of cells identified as pDC precursors strongly expressing CD123 with weak CD34 expression and positive for CD36 and CD7, was noted together with a mature pDC population, also expressing CD7.

Case 7: was diagnosed as a T/myeloid MPAL due to the presence of populations of blasts coexpressing cyt.CD3, CD7, CD13dim, CD34, CD117, TdT dim, and MPO with low HLA-DR in a subset of blasts. All other tested T-cell markers (CD1a, CD2, mCD3, CD4, CD8) and CD56 were negative. FlowSOM analysis (Fig.8 and Suppl. Table) showed a major population of CD7+CD34+CD117+CD123- blasts. Another population in the blast region was negative for all markers in the panel except CD38 and a population showing granulocytic differentiation with CD33dim and CD64dim was found. Two populations of CD123dim blasts were noted and three subsets of pDC (7.47% totally) could be delineated (one CD36+CD7+, one CD36+CD7- and one negative for both CD36 and CD7.

Discussion

The original approach of FlowSOM-aided unsupervised analysis of FCM data, applied here with a focus to CD123 expression, provides interesting and somehow unexpected new findings.

A first point of interest is the highly homogenous immunophenotype of normal pDCs, identified as a single node with a typical immunophenotype in a series of merged NBM. Of interest, this approach also straightforwardly singled out, among $CD123^{+}$ cells, a unique node of basophils and a unique node of myeloid DC with lower CD123 expression. Since the approach chosen by our group is to work with several samples of merged NBM to obtain the most accurate picture of normal patterns (Hemasphere papers), these findings indicate that these three subsets are robustly defined in NBM.

This gives even more weight to the astonishing diversity disclosed in the seven cases chosen here. Indeed, it could have been expected that the $RUNX1^{mut}$ signature would be associated with pDC differentiation (Xiao et al, Blood 2021, Zalmai et al) but several intriguing facts may be noted.

The first one is the observation in 4/7 cases of mature pDC with the expression of CD7. The significance of this population and its possible normal counterpart are unknown. A subset of CD7+ pDCs in non-neoplastic BM samples was described by Wang et al (Haematologica 2021) with a median frequency of 13% of pDCs (range, 0.3% to 21%). This population was not singled out by the present FlowSOM analysis of NBM although it could be retrieved by supervised manual analysis. The presence of CD7- pDCs certainly fits the earliest definition of pDC as lymphoid- (or more precisely T-lymphoid) derived (Lennert and Remmele, 1958 Acta Haematologica, Basel). This concept has again gained interest and a pathway for the pDC lineage to develop from a lymphoid progenitor that runs parallel to B cell development rather than being linked to the myeloid branch of common dendritic cell precursors was proposed (Rodrigues and Tussiwand, Molecular Immunology 2020).

Populations of pDCs detected by FlowSOM in neoplastic cases show variability and, in some cases, a small subset suggesting the presence of pDC precursors was found.

This was not fully described in previous studies (Hamadeh et al. Cytometry Part B (Clinical Cytometry) 98B:43–51 (2020).

Another interesting feature is that of the immunophenotypic variability of blast subsets associating with CD123 expression, suggesting perhaps their dependency on IL-3. If this is further demonstrated, the use of CD123 targeted therapies such as Tagraxofusp (Sapienza, Cancers 2019), CD123-directed CAR T-cells or bi-specifics could be better documented.

Xiao et al (Blood 2021) demonstrated in xenograft models of pDC-AML that targeting with Tagraxofusp led to the elimination of malignant pDCs and a two- to three-fold decrease in leukemia tumor burden, but not full elimination of leukemia.

A third important point, also regarding the potential therapeutic issues just mentioned, is that FlowSOM sequential analysis of patients' BM can provide a more precise appreciation of treatment efficacy. It is of major interest that in the three follow-up samples that were obtained in this series, normal pDCs and CD123 patterns were completely recovered after treatment.

In summary, although an extensive dissection of the subsets identified here is beyond the scope of this preliminary report, the analyses presented certainly point out the extraordinary flexibility of malignant cells in their immunophenotypic marker expression. Unsupervised analysis, although somewhat "simplified" here by grouping node clusters considered highly similar, discloses both the impressive homeostasis of NBM and the abundance of variegating patterns displayed by malignant cells. The relationship with just as variable molecular anomalies remains to be deciphered.

References

1. Sood, R.; Kamikubo, Y.; Liu, P. Role of RUNX1 in hematological malignancies. *Blood* **2017**,129, 2070-2082
2. Gaidzik, V.I.; Teleanu, V., Papaemmanuil, E.; Weber, D.; Paschka, P.; Hahn, J.; Wallrabenstein, T.; Kolbinger, B.; Köhne, C.H.; Horst, H.A.; et al. RUNX1 mutations in acute myeloid leukemia are associated with distinct clinico-pathologic and genetic features. *Leukemia* **2016,** 11**,** 2160-2168
3. Swerdlow S, Campo E, Harris N, Jaffz ES, Pileri SA, Stein H, Thiele J (eds). *WHO classification of tumours of haematopoietic and lymphoid tissues*. 4th ed. Lyon, France: IARC; 2017.
4. George, T.I.; Bajel, A. Diagnosis of rare subtypes of acute myeloid leukaemia and related neoplasms. *Pathology* **2021**, 3, 312-327
5. Takahashi, K.; Wang, F.; Morita, K.; Yan, Y.; Hu, P.; Zhao, P.; Zhar, A.A.; Wu, C.J.; Gumbs, C.; Little, L.; et al. Integrative genomic analysis of adult mixed phenotype acute leukemia delineates lineage associated molecular subtypes. *Nat Commun* **2018,** 1, 2670
6. Zalmaï, L.; Viailly, P.J.; Biichle, S.; Cheok, M.; Soret, L.; Angelot-Delettre, F.; Petrella, T.; Collonge-Rame, M.A.; Seilles, E.; Geffroy, S.; et al. Plasmacytoid dendritic cells proliferation associated with acute myeloid leukemia: phenotype profile and mutation landscape. *Haematologica* **2020** Oct 13; Online ahead of print. doi:10.3324/haematol.2020.253740
7. Xiao, W.; Chan, A.; Waarts, M.R.; Mishra, T.; Liu, Y.; Cai, S.F.; Yao, J.; Gao, Q.; Bowman, R.L.; Koche, R.P.; et al. Plasmacytoid dendritic cell expansion defines a distinct subset of RUNX1-mutated acute myeloid leukemia. *Blood*. **2021**, 137, 1377-1391
8. Renosi, F.; Roggy, A.; Giguelay, A.; Soret, L.; Viailly, P.J.; Cheok, M.; Biichle, S.; Angelot-Delettre, F.; Asnafi, V.; Macintyre, E.; et al. Transcriptomic and genomic heterogeneity in blastic plasmacytoid dendritic cell neoplasms: from ontogeny to oncogenesis. *Blood Adv* **2021**, 5, 1540-1551

PDC CD123 manuscript Figure legends.

Figure 1.

Merged normal bone marrow files showing the subsets delineated with the applied panel. Plasmacytoid dendritic cells (PDC) segregate as a single node between basophils and plasma cells on the right side of the minimal spanning tree.

Figure 2.

Case 1 minimal spanning tree. CD123+ cells constitute a highly clustered population on a classical CD45/SSC scattergram (left) but FlowSOM (right) separates this population in an unsuspectedly large number of nodes that supervised node-by-node analysis cluster in 10 different populations.

Figure 3.

Case 2 minimal spanning tree. Two different clusters of blast cells are separated on the basis of CD123 expression. Three different PDC clusters are segregated based on CD7 or CD36 expression.

Figure 4a.

Case 3 minimal spanning tree at diagnosis. The progenitor population is very heterogeneous and PDC segregate as two different clusters based on CD7 expression.

Figure 4b.

Case 3 minimal spanning tree after treatment. Normal partition of cell subsets, including recovery of a single classical PDC node segregated from basophils.

Figure 5.

Case 4 minimal spanning tree. FlowSOM segregates the apparently clustered population of progenitors (left CD45/SSC scattergram) in an impressively large number of nodes that may be re-clustered in 4 populations (see supplementary table). There is no clear PDC population in this case except perhaps cluster 1.

Figure 6

Case 5 minimal spanning tree. This is a CMML case with evolution towards BPCDN. FlowSOM segregates three nodes as normal PDC (n=2) or CD7+ PDC (n=1) that merge in a single cluster in the CD45/SSC scattergram (bottom left). The upper left scattergram, obtained from the same sample with another panel, confirms the CD56+/CD123+ immunophenotype of these cells, clearly separated from the small population of basophils (enhanced display).

Figure 7

Case 6 minimal spanning tree. The apparently homogeneous blastic population objectived by a CD45/SSC scattergram (bottom left) is dismembered in 5 progenitor clusters while PDC segregate in two nodes based on CD7 expression.

Figure 8

Case 7 minimal spanning tree showing how a heavily infiltrated BM, as exemplified on the CD45/SSC scattergram (bottom left) is segregated by FlowSOM in 7 progenitor subsets and, even more surprisingly, 8 PDC subsets (shades of dark blue), four of them being characterized by the absence of CD36 expression and 2 by the presence of CD7.

Table 1: Patient characteristics

Case #	Age (years)	Gender	Hb g/L	WBC x10^9/L	Plt x10^9/L	Diagnosis WHO 2016	Karyotype	Molecular data	%CD34	%CD123	% blasts by cytology
1	73	M	59	35	12	*RUNX1*^{mut}AML	46,XY	RUNX1 46%, IDH2 45%, TET2 47%, SRSF2 50%	90%	88%	88%
2	75	F	94	5.6	25	*RUNX1*^{mut}AML pDC differentiated	NA*	RUNX1 41%, ASXL1 35%, IDH248%, NRAS 45%, SRSF2 45%	21%	12%	20%
3	29	M	114	2.5	149	AML-MRC	46XY, del 7(q32), ?inv.dup.21 (q22;q11)[19]/46,XY[9]	RUNX1 53%, WT1 10%	30%	4%	31%
4	79	M	93	1.6	28	t-AML	47, XY,+8 [14]/ 48, XY, +8, +8 [11]	RUNX1 45%, IDH2 41%, SRSF2 44%, CUX1 5%	84%	10%	64%
5	71	F	134	4.4	92	CMML** and BPDCN	46,XX	RUNX1 41%, 2xTET2 (50 and 46%), PHF6 36%, SRSF2 50%, NRAS 10%,	9%	3%	19%
6	79	M	107	1.5	159	t-AML	46,XY	BCOR 43%, BCORL1 43%, U2AF1 20%	2%	1.7%	21%
7	62	M	96	8.3	84	MPAL (T/Myeloid)	46,XY,add(14)(q32)	2xFLT3 (21% and 5%), DNMT3A (48%), ASXL1 (47%), JARID2	55%	4%	81%

	(15%), 2xBCL11B (9% and 5%)

* no fusion transcripts by RNA analysis

** CMML-1 diagnosed 6 months earlier

Supplementary Methods

Flow cytometry: Bone marrow samples were bulk-lysed using Pharm-lyse (Becton Dickinson, San Jose, CA). Aliquotes of $250x10^3$ nucleated cells were incubated with antibody panels (Suppl. Table 1) in the dark at room temperature for 10 minutes. Acquisition was performed on a Navios Flow cytometer (Beckman Coulter, Miami, Florida, BC) within 2 hours of staining. Instrument settings on the three flow cytometers used at the laboratory were harmonized according to the Harmonemia protocol (ref) to ensure data reproducibility. Initial analyses were performed with the Kaluza Software (Beckman Coulter, Miami, FL, USA) for routine immunophenotyping of diagnostic samples.

Supplementary Table S1. Combinations of antibodies in the comprehensive myeloid panel (AML Tubes 1-3) applied at diagnosis

	FL1 FITC	FL2 PE	FL3 ECD	FL4 PC5.5	FL5 PC7	FL6 APC	FL7 A700*	FL9 A750	FL9 PB/BV421	FL10 KO
AML Tube 1	CD56	CD13	CD14	CD10	CD117	CD11b	CD34	CD33	CD16	CD45
AML Tube 2	CD36	CD7	CD64	HLA-DR	CD117	CD123	CD34	CD33	CD38	CD45
AML Tube 3	CD99	CD135	CD2	CD4	CD117	CD133	CD34	CD33	CD19	CD45

Antibody suppliers: CD133-APC from Miltenyi Biotec, Bergisch Gladbach, Germany; CD99-FITC from BIO-RAD, Hercules, CA; CD56-FITC, CD13-PE, CD7-PE, CD38-BV421, CD135-PE, CD19-BV421 from BD Biosciences, San Jose, CA; All other antibodies are from Beckman Coulter, Miami, FL.

Supplementary Table S2: Cut-offs for the classification of normalized marker expressions in FlowSOM analysis of AML tube 2

AML Tube 2	CD36	CD7	CD64	HLA-DR	CD117	CD123	CD34	CD33	CD38	CD45
Low/neg	<2	<2.5	<1.5	<2	<2	<2	<2	<1.5	<2.5	<0.5
Intermediate	2-3	2.5-3.5	1.5-2.5	2-3	2-3	2-3	2-3	1.5-2	2.5-3.5	0.5-0.6
High	>3	>3.5	>2.5	>3	>3	>3	>3	>2	>3.5	>0.6

Supplementary Table S3: Cell subsets identified by FlowSOM in the merged file of 6 normal BM analyzed with panel AML 2 and their respective normalized MFI

Subset	CD123	HLA-DR	CD33	CD36	CD64	CD34	CD117	CD45	CD7	CD38	No of nodes	% of total
PCD	4.66	4.66	1.52	4.16	1.14	1.47	1.07	0.64	1.6	3.82	1	0.32
MDC	2.5	4.3	1.58	2.45	1.4	1.07	1.03	0.71	1.77	1.86	1	0.17
BASOPHILS	4.47	1.11	2.14	1.85	1.06	1.16	1.06	0.6	1.27	4.65	1	0.29
EARLY MONOCYTES	1.17	4.57	1.56	3.71	1.64	1.2	1.26	0.47	2.5	3.72	1	0.35
MONOCYTES	1.6	3.95	3.65	4.82	3.79	1.1	1.08	0.7	2.15	3.9	6	4.61
GRANULOPOIESIS	1.14	1.08	1.81	1.58	1.69	1.02	1.01	0.63	1.71	1.4	61	67.35
B-PRECURSORS	1.14	4.6	1.01	0.91	1.01	1.06	1.01	0.56	1.08	5.37	1	1.37
MATURE B	1.06	4.74	1.01	1.01	1.03	1.02	1	0.67	1.1	2.06	2	2.2
PLASMA CELLS	1.29	1.61	1.08	2.01	1.14	1.05	1.03	0.58	2.02	7	1	0.18
T/NK cells	0.99	1.1	1	0.98	1	1	1	0.7	4.36	2.06	8	11.8
T/NK DR+	1.02	3.1	1	1.08	1.02	1	0.98	0.74	3.85	2.37	1	0.26
T/NK CD36+	1.07	1.33	1.04	3.93	1.22	1.05	1.04	0.69	4.3	2.07	1	0.25
ERY PRECURSORS CD117+	1.2	1.64	0.97	5.19	1.13	1.22	2.64	0.44	2.05	3.5	1	0.37
ERY PRECURSORS CD117-	1.34	1.45	1.04	5.46	1.22	1.19	1.18	0.39	1.77	2.19	1	0.43
CD34+/dim CD7+ DRneg PRECURSORS	1.16	1.89	1.19	1.5	1.07	2.43	1.02	0.66	2.92	1.73	1	0.2
CD34+CD117-	1.3	3.73	1.27	1.5	1.07	3.37	1.24	0.45	1.34	4.56	2	0.47
CD34+CD117+	1.65	3.76	2.09	1.83	1.17	4.22	3.21	0.52	1.69	3.69	2	0.52
CD117+CD34-CD33+ PROMYELOCYTES	1.34	2.68	2.18	1.5	2.76	1.4	3.26	0.49	2.28	4.2	2	0.54
CD7/DR NEG LYMPH0CYTES	0.98	1.25	1.02	1.03	1.04	1.01	1	0.71	1.27	1.01	1	1.25