Supplementary Appendix

SUBCLASSIFICATION OF ACUTE MYELOGENOUS LEUKEMIA PATIENTS BASED ON CHEMOKINE RESPONSIVENESS AND CONSTITUTIVE CHEMOKINE RELEASE BY THE LEUKEMIA CELLS

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MATERIAL AND METHODS

Detailed description of AML patients and AML cell preparation

Patients. The study was approved by the local Ethics Committee (Regional Ethics Committee III, University of Bergen, Norway) and samples collected after informed consent. The study included 68 consecutive adult patients (31 females and 37 males; median age 64 years and range 29-82 years) with high peripheral blood blast counts (>7 x 10^9/l) (11, 12). Fifty patients had de novo AML, the remaining minority had AML relapse (2 patients), chronic myeloid leukemia in blast phase (2 patients) and AML secondary to chemotherapy or primary myelodysplasia (3 and 11 patients respectively). The patients showed the following FAB classification: M0 4 patients, M1 17 patients, M2 18 patients, M3 1 patient, M4 16 patients, M5 11 patients and M6 1 patient. Thirty-three patients had >20% $CD34^+$ AML cells. Cytogenetic analysis was performed for 46 patients and the abnormalities classified as described by Wheatley et al. (13): 24 patients had normal chromosomes whereas 4 patients had low-risk, 11 patients high-risk and 7 patients intermediate-risk abnormalities. Sixty-five patients were tested for genetic Flt3 abnormalities (12, 14); 27 patients had Flt3 abnormalities including 22 patients with internal tandem duplications alone, 3 patients with D835 mutations alone and 2 patients had both abnormalities. A consecutive group of 17 patients were included in all experiments; the characteristics of these patients are presented in Supplementary appendix, Table 1.

Cell preparation. Leukemic peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (Ficoll-Hypaque; NyCoMed, Oslo, Norway; specific density 1.077) from the peripheral blood of patients with at least 80% leukemia blasts among blood leukocytes. The percentage of blasts among leukemic PBMC exceeded 95% judged by light microscopy or flow cytometric analysis (11). Cells were stored frozen in liquid nitrogen, and thawed AML cells showed a viability exceeding 70% (11).

This experimental approach was based on previous methodological studies (11, 12, 15). The preparation of enriched AML blasts by density gradient separation alone will minimize the risk of inducing functional alterations, but this strategy requires selection of patients with high peripheral

blood blast counts. The results may therefore be representative only for this particular patient subset as described and discussed in detail previously (11, 12, 23).

$CD34^+$ $CD38^-$ and $CD34^+$ cells were prepared by immunomagnetic separation from primary AML cells according to the manufacturer's instruction (MACS Cell Isolation; Miltenyi Biotec, Bergisch Gladbach, Germany) and fractionated on an AutoMACS (Miltenyi Biotec).

Detailed description of flow cytometric analysis

Reagents. The following monoclonal antibodies and corresponding isotypic controls were purchased from R&D Systems: mouse anti-CXCR2-FITC, mouse anti-CXCR3A-PE; mouse anti-CXCR4-APC, rat anti-CCR3-FITC; mouse anti-CCR1-PE; mouse anti-CCR5-APC, mouse anti-CCR4-FITC; mouse anti-CCR2-PE. Murine anti-CD34-PerCP, anti-CXCR1-APC, an additional anti-CXCR2-FITC and isotype controls were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). PE-conjugated antibodies and isotype controls against NFκB subunits p50, p52 and p65 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Labelling. For analysis of chemokine receptor expression the AML cells were washed once and then resuspended in phosphate-buffered saline (PBS) with 0,5% bovine serum albumine (BSA) (Sigma, St. Louis, MO) before 1 x 10^5 cells were incubated with antibody (all from R&D Systems) for 30 minutes at room temperature in the dark. Thereafter cells were washed once and resuspended in PBS with 1% paraformaldehyde. For analysis of CXCR1/CXCR2 expression using the Becton Dickinson antibodies AML cells (5 x 10^5) were washed with PBS containing 0.1% NaN_3 and 1% bovine serum albumine (BSA) at 4°C and then incubated with the antibodies for 30 minutes in the dark at 4°C. For NFκB analysis AML cells (1x10^6) were (i) washed with cold phosphate-buffered saline (PBS) and fixed with 1% paraformaldehyde for 10 minutes; (ii) washed, added ice-cold methanol while vortexing and incubated for 15 minutes at 4°C; (iii) thereafter washed twice with PBS containing 0.1% NaN_3 + 2% BSA before incubated with antibodies against either NFκB-p50, p52, p65 or isotype controls for 1 hour at 4°C. Samples were finally washed with PBS before analysis.

Analysis. Events were collected using a standard FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with an Argon laser (488 nm) and a red diode laser (635 nm). A cytogram based on the forward light scatter versus the right angle scatter was used to eliminate aggregates and debris before fluorescence was detected: (i) green fluorescence (FL1) from FITC was detected through the 530/30 nm bandpass-filter, (ii) orange fluorescence (FL2) from PE through the 584/42 nm bandpass-filter, (iii) long red fluorescence (FL3) from PI through the 670 nm longpass-filter and (iv) far red fluorescence (FL4) from APC through a 661/16 nm bandpass-filter. All fluorescence measurements were collected in the logarithmic mode. Data from specific regions made in fluorescence were analyzed using Cell Quest Lysis II software (Becton Dickinson). The results are presented as the % positive cells when compared with the corresponding isotype controls.

Statistical analysis and presentation of the data

Analyses of experimental results. Proliferation was assayed by ^{3}H-thymidine incorporation, and the mean counts per minute (cpm) of triplicate determinations was used in all analyses (11, 12). A significant alteration of ^{3}H-thymidine incorporation was defined as a difference between incremental responses corresponding to (i) an absolute value of at least 2000 cpm; and (ii) this absolute value being >20% of the corresponding control (11, 12). Cytokine levels were transformed to logarithmic values before cytokine levels in cultures with and without bortezomib/BMS-345541 were compared; the Wilcoxon's test for paired samples was then used for statistical analysis.

Statistical analyses. The linear relationship between two continuous variables was determined by the correlation coefficient (r) and the corresponding significance level by a two-tailed t-test. The *p*-values were adjusted by multiplying them with the total number of tests, i.e. Bonferroni-correction. ANOVA was used for combined parametric and non-parametric variables (17).

Hierarchical clustering was used to visualize the similarity between several variables. The smaller node in the dendrogram the more similar are the variables. *Principal component analysis* (PCA) (18) calculates a few principal components (PC's) that describes most of the variation in the data set. Coordinates for each new variable are found by projecting the variables onto the PC's. Thereafter

these coordinates are plotted in a coordinate system spanned by e.g. the first and second PC. The obtained PCA plot displays the correlation between variables.

Calculations of correlation coefficients and ANOVA were performed in Matlab v. 6.5, the chi-squared test was run on http://faculty.vassar.edu/lowry/newcs.html, while the hierarchical clustering and principal component analyses were carried out in J-Express Pro v.2.7 (19, 20).

RESULTS AND DISCUSSION

In vitro culture of $CD34^+$ and $CD34^+CD38^-$ AML cells

AML stem cells are often regarded to be $CD34^+CD38^-$, but for exceptional patients these cells seem to be included among the $CD34^-$ cells (23). We investigated the number of $CD34^+CD38^-$ AML cells for 10 unselected patients, and for all patients this subset constituted <1% of the AML cells. A very low number of enriched $CD34^+CD38^-$ cells was achieved after immunomagnetic separation, and when these cells were seeded at a concentration corresponding to 500-1000 cells per well no detectable ^{3}H-thymidine incorporation (<1000 cpm) was observed (data not shown). These results suggest that ^{3}H-thymidine incorporation by this minor cell subset itself does not contribute to the proliferative responses seen in our suspension cultures of total AML cells. However, alternative experimental approaches have to be used to investigate whether exogenous chemokines also affect the proliferation of this small cell subset. Finally, additional experiments in three patients demonstrated that exogenous chemokines could alter the proliferation of $CD34^+$ enriched AML cells (data not shown).

Supplementary information, Table 1: Clinical and biological characteristics of patients included in the initial screening for chemokine effects on AML cell proliferation

Patient	Sex	Age (years)	Previous chemotherapy or preleukemic disease	FAB classification	Membrane molecule expression					Cytogenetic analysis	Flt3 ab-normalities
					CD13	CD14	CD15	CD33	CD34		
1.	M	79		M1	+	-	-	-	+	Normal	ITD
2.	F	40		M5	-	-	+	+	-	t(9,11) (p22;q23) +8	wt
3.	F	75		M5	+	-	+	+	+	Normal	ITD
4.	F	59		M2	+	-	-	+	+	-7	wt
5.	F	64		M1	+	-	+	+	+	(5)t(5;?8)(q14; q21),-7	wt
6.	F	81		M2	+	-	-	+	-	nt	wt
7.	M	74	MDS	M4	+	-	+	+	-	Multiple	wt
8.	F	45	Chemotherapy	M4	+	-	+	+	-	Normal	wt
9.	F	63		M4	+	-	-	+	+	Normal	ITD
10.	M	74		M0	-	-	+	+	+	90-94,XXYY,	wt
11.	F	45		M2	+	-	-	+	-	Normal	ITD, D835
12.	M	82		M5	+	+	+	+	-	45X	wt
13.	M	65	CML	M2	+	-	-	-	+	t(13;15)(q10;q10) -17,+21?,+22?	wt
14.	M	30		M3	+	-	-	+	-	t(15;17)	ITD
15.	M	43		M5	+	-	+	+	+	inv(16)(p13q22) (q21;?), inv(16), +20,+21	D835
16.	F	78		M4	+	+	+	+	-	-4,-5,+der(8)t(8;?)	wt
17.	F	64	MDS	M1	+	-	-	+	+	del(11)(q14), del(20)(q11),+21	wt

Abbreviations: female (F), male (M), acute myelogenous leukemia (AML), myelodysplastic syndrome (MDS), chronic myelogenous leukemia (CML). Flow cytometry was used for analysis of membrane molecule expression and patients were regarded as positive when more than 20% of the AML cells stained positive. The results from the analysis of the Flt3 genotype are referred to as wild type (wt), internal tandem duplications (ITD) and Asp(D) 835 mutations (D835) (for additional details see references 11-14).

Table 2. Bonferroni-corrected (21 parameters, 210 tests) p-values of correlations of constitutive AML blast release of various chemokines.

Chemo-kine	CCL2	CCL3	CCL4	CCL5	CCL7	CCL13	CCL17	CCL20	CCL22	CCL23	CCL24	CXCL1	CXCL4	CXCL5	CXCL6	CXCL8	CXCL9	CXCL10	CXCL11	CXCL13
CCL1	$<10^{-5}$	$<10^{-5}$	$<10^{-3}$								0.021	$<10^{-7}$				$<10^{-9}$				
CCL2		$<10^{-12}$	$<10^{-9}$	$<10^{-3}$	$<10^{-3}$	.002		$<10^{-5}$				$<10^{-13}$		.005		$<10^{-12}$		$<10^{-3}$		.006
CCL3			$<10^{-16}$	$<10^{-5}$	$<10^{-3}$			$<10^{-10}$				$<10^{-18}$		.008		$<10^{-16}$		.003		
CCL4				$<10^{-7}$	$<10^{-3}$	.001	.042	$<10^{-9}$	.005			$<10^{-11}$		$<10^{-3}$		$<10^{-9}$	.007	$<10^{-3}$	.034	
CCL5					.020			.037		.013		$<10^{-5}$				$<10^{-3}$	$<10^{-3}$	$<10^{-4}$	.049	
CCL7						$<10^{-3}$	.046	$<10^{-4}$	.002	$<10^{-3}$	.025	.0005		$<10^{-3}$		.003				
CCL13							.002		$<10^{-6}$		$<10^{-4}$			$<10^{-7}$						
CCL17									$<10^{-21}$		0.010			$<10^{-6}$						
CCL20		$<10^{-3}$							.038			$<10^{-8}$		$<10^{-4}$		$<10^{-5}$				
CCL22											$<10^{-9}$			$<10^{-12}$						
CCL23																	0.003	$<10^{-3}$		
CCL24														$<10^{-4}$						
CXCL1														$<10^{-4}$		$<10^{-10}$		.007		
CXCL5															.040					
CXCL9																		$<10^{-5}$	$<10^{-12}$	
CXCL10																			.002	

CXCL4, CXCL6, CXCL8, CXCL11, CXCL13: No additional significant correlations

Native human AML blasts derived from 68 consecutive patients were cultured in vitro for 48 hours before chemokine levels were determined in the supernatants. The patients were in addition examined for release of CCL11, CCL21, CCL23 CCL25, CCL26, CCL27, CCL28, CXCL9, CXCL11 and CXCL12; these chemokines showed undetectable levels for at least 50 patients.