Subclassification of patients with acute myelogenous leukemia based on chemokine responsiveness and constitutive chemokine release by their leukemic cells

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ABSTRACT

Background and Objectives

Chemokines are soluble mediators involved in angiogenesis, cellular growth control and immunomodulation. In the present study we investigated the effects of various chemokines on proliferation of acute myelogenous leukemia (AML) cells and constitutive chemokine release by primary AML cells.

Design and Methods

Native human AML cells derived from 68 consecutive patients were cultured *in vitro*. We investigated AML cell proliferation (^{3}H-thymidine incorporation, colony formation), chemokine receptor expression, constitutive chemokine release and chemotaxis of normal peripheral blood mononuclear cells.

Results

Exogenous chemokines usually did not have any effect on AML blast proliferation in the absence of hematopoietic growth factors, but when investigating growth factor-dependent (interleukin 3 + granulocyte-macrophage colony-stimulating factor + stem cell factor) proliferation in suspension cultures the following patient subsets were identified: (i) patients whose cells showed chemokine-induced growth enhancement (8 patients); (ii) divergent effects on proliferation (15 patients); and (iii) no effect (most patients). These patient subsets did not differ in chemokine receptor expression, but, compared to $CD34^-$ AML cells, $CD34^+$ cells showed higher expression of several receptors. Chemokines also increased the proliferation of clonogenic AML cells from the first subset of patients. Furthermore, a broad constitutive chemokine release profile was detected for most patients, and the following chemokine clusters could be identified: CCL2-4/CXCL1/8, CCL5/CXCL9-11 (possibly also CCL23) and CCL13/17/22/24/CXCL5 (possibly also CXCL6). Only the CCL2-4/CXCL1/8 cluster showed significant correlations between corresponding mRNA levels and NFκB levels/activation. The chemotaxis of normal immunocompetent cells for patients without constitutive chemokine release was observed to be decreased.

Interpretation and Conclusions

Differences in chemokine responsiveness as well as chemokine release contribute to patient heterogeneity in AML. Patients with AML can be classified into distinct subsets according to their chemokine responsiveness and chemokine release profile.

key words: acute myelogenous, leukemia, chemokine, NFKB, CXCR2

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Chemokines are a family of soluble proteins that are involved in a wide range of biological processes with relevance for hematologic malignancies, including cell trafficking, regulation of cell proliferation and apoptosis, immunoregulation, normal hematopoiesis and angiogenesis.[1-5] The chemokines are grouped into the two major subclasses, CCL and CXCL chemokines, which interact with CCR and CXCR membrane receptors, respectively. Another classification of chemokines is: (i) homeostatic (also called constitutive) chemokines that bind to single receptors; and (ii) inflammatory (also called inducible) chemokines that bind to several receptors, and each of these receptors can usually bind several chemokines.[1-5]

Acute myelogenous leukemia (AML) is an aggressive disorder characterized by accumulation of immature malignant cells in the bone marrow.[6] The overall disease-free survival for younger patients (<60 years of age) receiving the most intensive conventional chemotherapy is less than 50%.[6] However, AML is a very heterogeneous disorder,[6-10] and the recently published WHO classification is, therefore, based on a combination of clinical history (predisposition due to preleukemic disorders or previous chemotherapy), morphology and cytogenetic abnormalities.[9] Patients can thereby be subclassified according to prognosis, i.e. risk of primary chemoresistance or later AML relapse. We have previously described that cytokine-induced, receptor-mediated phosphorylation patterns of intracellular mediators can also identify subsets of patient with different prognoses.[10] Furthermore CXCR4 expression was recently described to have a prognostic impact in AML.[7] However, chemokines and their receptors form a complex network and it is therefore relevant to focus also on chemokine profiles and not only single chemokines. In the present study we investigated the release of a wide range of chemokines from primary human AML blasts and these cells' proliferative responsiveness to the cytokines.

Design and Methods

AML cells

The study was approved by the local Ethics Committee (Regional Ethics Committee III, University of Bergen, Norway) and samples were 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 (>7×10^9/L).[11,12] AML cells were isolated by density gradient separation and included at least 95% leukemic blasts.[11-14] A more detailed characterization of the patients is given in the online supplementary section.

Flow cytometric analysis of chemokine receptors and NFκB subunits

The expression of chemokine receptors and NFκB subunits by the AML cells was analyzed using flow cytometry, as described in the supplementary section. Anti-CXCR2-FITC, anti-CXCR3A-PE; anti-CXCR4-APC, anti-CCR3-FITC; anti-CCR1-PE; anti-CCR5-APC, anti-CCR4-FITC, anti-CCR2-PE and their corresponding isotypic controls were all purchased from R&D Systems (Abingdon, UK). Anti-CXCR1-APC, an alternative anti-CXCR2-FITC, anti-CD34-PerCP and corresponding isotype controls were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA). PE-conjugated antibodies and isotype controls against the NFκB subunits p50, p52 and p65 were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

In vitro culture of human AML cells

Reagents. The Stem Span SFEM™ culture medium (referred to as Stem Span™; Stem Cell Technologies; Vancouver, BC, Canada) supplemented with 100 μg/mL of gentamicin was used in all experiments.[11,12,15] Recombinant human cytokines were purchased from Peprotech (London, UK) except for CXCL14 that was purchased from R&D Systems. Chemokines were used at a final concentration of 20 ng/mL and hematopoietic growth factors (interleukin-3, IL3; stem cell factor, SCF; granulocyte-macrophage colony-stimulating factor, GM-CSF) at 50 ng/mL. Bortezomib was purchased from Jansen-Cilag (Beerse, Belgium) and BMS-345541 from Sigma Aldrich (St. Louis, MO, USA). Both drugs were dissolved in DMSO and later in culture medium. Pilot experiments demonstrated that bortezomib 25 nM caused strong inhibition of cytokine-dependent AML cell proliferation and the drug was used at this concentration. BMS-34551 was used at a final concentration of 10 μM.[16]

Proliferation assay. AML blasts (5×10^4 cells/well) were cultured in flat-bottomed microtiter plates in Stem Span™ (150 μL per well; Costar 3596 culture plates; Costar, Cambridge, MA, USA) for 6 days before ^{3}H-thymidine was added and nuclear radioactivity assayed 18 hours later.[11,12]

Analysis of AML colony formation. AML cells (10^6 cells/mL) were cultured in suspension cultures in growth factor-containing medium alone or medium with additional exogenous chemokines for 7 days before 100 μL of the cell suspension were mixed with 900 μL of methylcellulose medium containing erythropoietin, GM-CSF, SCF and IL3 (MethoCult H4434; Stem Cell Technologies).[11,12] The AML blasts were thereafter cultured (Costar 3524 24-well culture plates, 0.5 ml medium per well) for 14 days before colony numbers were determined by light microscopy.

Chemokine release assay. AML blasts (2×10^6 cells in 2 mL) were cultured in 24 well culture plates (Costar 3524 culture plates) for 48 hours before chemokine levels were determined by Quantikine enzyme linked immunosorbent assays (ELISA) from R&D Systems, except the CXCL4 analysis (CoaChrome; Wien, Austria). The minimal detection limits are included in Table 1.

Table 1. Chemokine release by native human AML cells derived from 68 consecutive patients.

		Patients with detectable chemokine release		
Chemokine	*Detection limit (pg/mL)*	*Number of patients*	*Median concentration (pg/mL)*	*Range (pg/mL)*
CCL1	4	43	617	6.1->1000
CCL2	**5**	**59**	**1720**	**7.3-5722**
CCL3	**75**	**53**	**5209**	**102-13,836**
CCL4	**150**	**53**	**2902**	**151-26,420**
CCL5	1.2	67	236	2.0-2288
CCL7	27	40	751	42-935
CCL11*	5	0	–	–
CCL13	5	45	47.4	5.4-238
CCL17	11	31	114	11.7-3704
CCL20	4.5	49	128	4.7-1393
CCL21*	20	0	–	–
CCL22	**260**	**41**	**1088**	**263->40,00**
CCL23	9	28	19.8	12.9-23.3
CCL24	13	39	394	15.2-5080
CCL25*	26	0	–	–
CCL26*	4.5	15	13.1	5.1-82.8
CCL27*	3	0	–	–
CCL28*	7	14	7.8	7.1-41.7
CXCL1	**60**	**50**	**7196**	**67-13,610**
CXCL4	**0.05**	**59**	**0.34**	**0.05-8.7**
CXCL5	**40**	**42**	**1067**	**41->20,000**
CXCL6	3.2	30	53.9	4.2-2328
CXCL8	**30**	**64**	**22,720**	**42-33,720**
CXCL9	60	30	822	78-15,815
CXCL10	**60**	**48**	**1782**	**64.2-24,906**
CXCL11	40	28	168	40.7-3980
CXCL12*	18	10	37.5	28.5-623
CXCL13	3.5	38	189	3.5-1303

*Native human AML cells were cultured for 48 hours and chemokine levels determined in the supernatants by ELISA. The results are presented as the concentration in pg/mL, except for CXCL4 whose unit of measurement is IU/mL. Chemokines marked in bold showed detectable levels for at least 40 patients and a median level >1000 pg/mL, whereas chemokines with undetectable levels for at least 50 patients were left out from the clustering analysis and are marked with *.*

Chemotaxis assay. Normal peripheral blood mononuclear cells (2×10^6 cells in 0.5 mL) were added to the upper chamber of transwell plates with a 3 μm pore size (Costar 3504);[13] 0.5 mL fresh medium and 0.5 mL 48 hours AML culture supernatant (*see above*) were added to the lower chamber. Cultures were incubated for 21 hours before cells in the lower chamber were counted and the fractions of $CD4^+$, $CD8^+$ and $CD56^+$ lymphocytes determined by flow cytometry (all antibodies being conjugated monoclonal mouse IgG1 antibodies supplied by Becton-Dickinson, San Jose, CA, USA).

Analysis and presentation of the data

Proliferation was assayed by ^{3}H-thymidine incorporation, and the mean counts per minute (cpm) of triplicate determinations were 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 concentrations were transformed to logarithmic values before levels were compared; the Wilcoxon's test for paired samples was then used for statistical analysis. Analyses of a linear relationship between continuous variables, hierarchical clustering, principal component analysis (PCA), calculations of correlation coefficients and ANOVA were performed as described in the Supplementary section.[17-20]

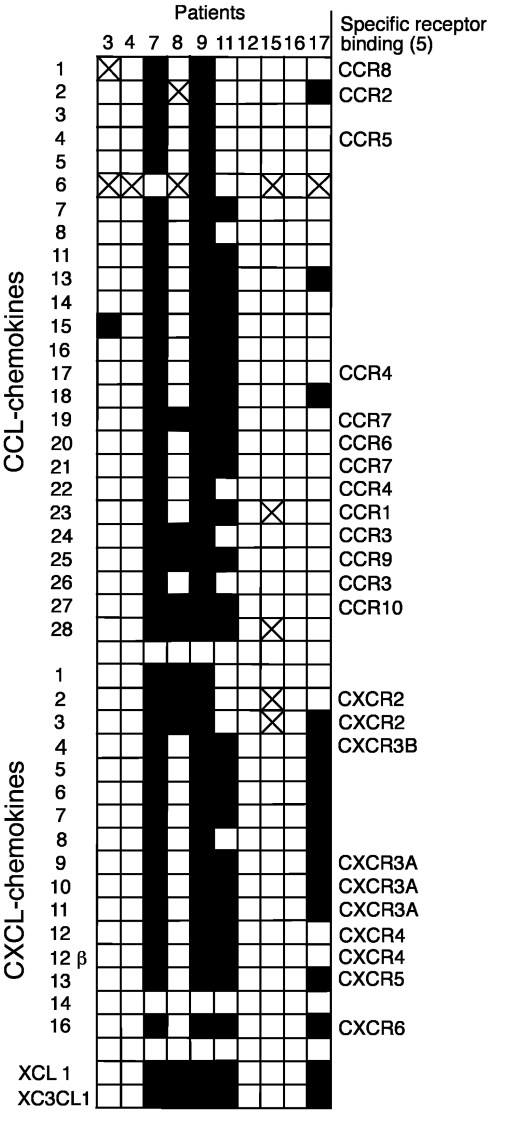

Figure 1. The effect of exogenous chemokines on growth factor-dependent (IL3+SCF+GM-CSF) proliferation of native human AML cells. AML blasts from 17 patients (Table 1) were cultured in growth factor-containing medium alone or in the presence of single exogenous chemokines (see left margin of the figure) and proliferation assayed as ^{3}H-thymidine incorporation. The figure presents the results only for those patients who showed detectable proliferation (corresponding to >1000 cpm). A significant alteration of proliferation was defined as (i) a difference corresponding to at least 20% of the control; and (ii) the absolute value of the difference being >2000 cpm. Proliferation could thereby be classified as increased (■), decreased (X) or unaltered (open squares) compared with that of the chemokine-free controls. Those chemokines that bind to only one specific CCR or CXCR receptor, according to the recent review by Allavera *et al.*,[5] are indicated to the right of the figure.

Results

In vitro proliferation of human AML cells in the presence of exogenous chemokines

AML blasts derived from 17 consecutive patients (*supplementary section, Table 1*) were cultured in medium alone and medium with the chemokines CCL1-8, CCL11, CCL13-28, CXCL1-14, CXCL16, XCL1 and CX3CL1 (Figure 1). The chemokines included ligands for the receptors CCR1-10, CXCR1-6, XCR1, CX3CR1, D6, DARC and PPR1 (Figure 1, right margin).[1-5] One patient showed autocrine AML cell proliferation, and for this particular patient some of the chemokines had growth enhancing (CCL11, CCL17) or inhibitory effects (CXCL1-3, CXCL11, CXCL14, CXCL16). For the other 16 patients a low proliferative response (corresponding to <2600 cpm) was observed only in the presence of CCL28 for patients 4 and 7 (*data not shown*).

The effects of the chemokines on AML cells from the same 17 patients were also tested when the medium was supplemented with GM-CSF+IL3+SCF and proliferation compared for cultures without and with exogenous chemokines. AML cells derived from patients 1, 2, 5, 6, 10, 13 and 14 showed undetectable proliferation (corresponding to <1000 cpm) in all cultures; the results for the other patients are summarized in Figure 1. These results suggest that the effect of exogenous chemokines on growth factor-dependent proliferation differs between patients. Several of the growth-modulating chemokines only bind to a single chemokine receptor (Figure 1, right part).[5] The overall results thereby suggest that for a subset of patients the AML cells express a wide range of chemokine receptors (including CCR1-10 and CXCR2-6) that mediate growth enhancement. Finally, similar chemokine effects were observed when we investigated the effects of exogenous chemokines (CCL2, CCL3, CCL6, CCL27, CXCL9-11) on AML cell proliferation in the presence of single growth factors (SCF, GM-CSF, G-CSF or IL3) for five randomly selected patients (*data not shown*).

Taken together our results suggest that chemokines affect AML cell proliferation mainly in the presence of exogenous growth factors, and that the effect of a limited number of chemokines can be used to predict differences in responsiveness between patients (Figure 1). For these reasons we only investigated the effects of selected chemokines on growth factor-dependent proliferation in the following experiments (*see top of Figure 2*); these chemokines are known to be ligands for a majority of the chemokine receptors (CCR1-3, CCR5, CCR6, CCR10, CXCR1-4, and DARC).[4,5]

The effects of chemokines on AML cell proliferation differ between patients

Based on the results summarized in Figure 1 we investigated the effects of a limited number of exogenous CCL (CCL5/13/15/18/23/28) and CXCL (1/2/7/9-12/14) chemokines on cytokine-dependent (IL3+SCF+GM-CSF) AML blast proliferation (n=64). The effects of the chemokines showed no correlations with clinical characteristics or biological AML cell characteristics (i.e. morphology, membrane molecule expression, genetic abnormalities). AML cells derived from 22 of these patients showed undetectable proliferation for all growth factor/chemokine combinations, the hierarchical clustering for the other patients is shown in Figure 2. From this analysis five additional patient clusters were identified: (i) several patients for whom exogenous chemokines did not alter AML blast proliferation (cluster B); (ii) patients for whom most CCL as well as CXCL chemokines increased proliferation (cluster D); and (iii) three clusters (A, C and E) of patients for whom some exogenous chemokines had divergent effects on AML cell proliferation.

CCR3 can bind several inflammatory chemokines, including CCL5 (also binding to CCR1 and CCR5), CCL13 (also binding CCR2), CCL15 (also binding CCR1) and CCL28 (also binding CCR10).[5] CCL13, CCL15 and CCL28 clustered together (Figure 2, top), an observation suggesting that common effects mediated through CCR3

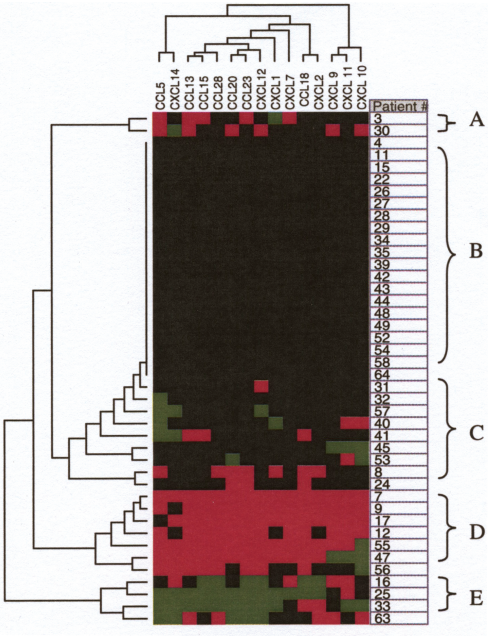

Figure 2. Hierarchical clustering analysis of the effects of chemokines on growth factor (IL3+SCF+GM-CSF)-dependent AML blast proliferation measured as ^{3}H-thymidine incorporation. The study included 64 patients, but 22 patients showed undetectable proliferation for all growth factor/chemokine combinations and the figure presents the results for the other 42 patients with detectable ^{3}H-thymidine incorporation (corresponding to >1000 cpm). A significant alteration of ^{3}H-thymidine incorporation was defined as (i) a difference corresponding to at least 20% of the control; and (ii) the absolute value of the difference being >2000 cpm. A significant increase according to these criteria is marked in red and significant inhibition in green. Five different patient clusters were identified (*A-E*), as indicated at the right margin of the figure.

are most important for these three chemokines. CCL5 did not cluster together with the other three CCR3 ligands; this may be due to additional effects mediated through CCR5, which does not bind the other three ligands.[5]

The three homeostatic chemokines CXCL9-11 clustered together; this was expected because all three bind only to the CXCR3A receptor.[5] The situation for CXCR2 and possibly CXCR1 (which binds CXCL1/6/8) seems more complex because the three CXCR2 ligands CXCL1/2/7 did not form a separate cluster like the CCR3 and CXCR3 ligands. We, therefore, analyzed CXCR1 and CXCR2 protein expression in the total AML cell population from 32 consecutive patients; 24 patients belonged to the five clusters identified in Figure 2 (cluster D, 3 patients; cluster B, 11 patients; clusters A, C and E, 10 patients) and eight additional patients belonged to the group of 22 patients with undetectable proliferation (see above). CXCR1 expression was low for all patients (<5.5% positive cells), whereas CXCR2 expression showed a wide variation (median 33.9% positive cells, range 5.8-73.1%) without any association with chemokine responsiveness. Taken together the results suggest that CXCR1 ligation is unlikely to affect AML cell proliferation, and for most patients CXCR2 ligation does not affect growth factor-dependent AML blast proliferation to such an extent that a separate CXCR2 cluster is formed.

Results from additional experiments suggest that the minor subset of $CD34^+CD38^-$ cells (<1% of AML cells) does not contribute to the proliferation detected in our suspension cultures (*see the Supplementary Appendix*).

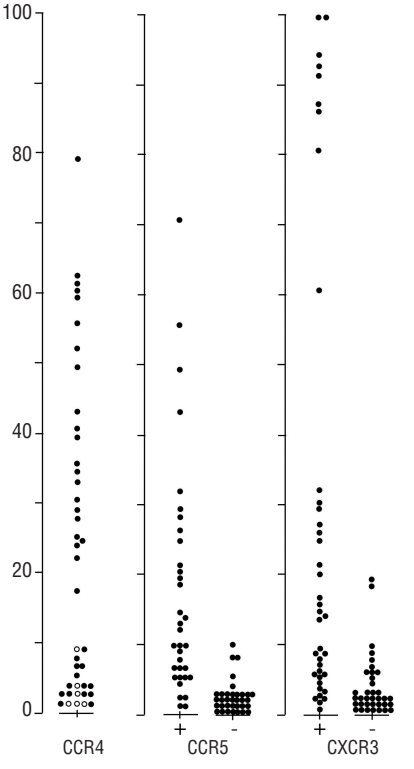

Figure 3. Chemokine receptor expression by primary AML cells. Chemokine receptor expression was investigated for 45 unselected patients. (left) Cells showing chemokine-induced growth enhancement (Figure 2, patient cluster D; open symbols) expressed significantly less CCR4 than did the cells from the other patients (dark symbols). (Middle and right part) When investigating 45 unselected patients it was found that the expression of CCR5 and CXCR3 was significantly greater by $CD34^+$ (+) AML cells than by $CD34^-$ (-) AML cells.

The chemokine receptor expression of $CD34^+$ and $CD34^-$ AML cell subsets differs

We investigated the expression of CCR1-5 and CXCR1/2/3A/4 by $CD34^+$ and $CD34^-$ AML cells for 45 randomly selected patients. The five CCR can bind 18 CCL chemokines (CCL2-5, 7, 8, 11-17, 22-24, 26, 28) and the four CXCR can bind 11 CXCL chemokines (CXCL1-3,5-12). Most of the receptors have relatively broad binding profiles, and can bind at least one of the chemokines used to identify the patient subsets defined according to AML cell proliferation (Figures 1 and 2). When considering the whole AML cell population, CCR/CXCR expression varied: (i) CXCR1 (see above) and CCR3 showed low expression with <20% positive cells for all patients; (ii) CXCR2, CXCR3A and CCR5 showed intermediate expression; and (iii) CXCR2, CXCR4, CCR1, CCR2 and CCR4 showed relatively high expression with >60% positive cells for several patients. Spontaneous AML blast proliferation was only observed for a minority of patients and showed no correlation with receptor expression or constitutive chemokine release (*see below*) (*data not shown*)

For each of the patients we also examined receptor expression by $CD34^+$ and $CD34^-$ AML cell subsets. The $CD34^+$ cell subset showed increased expression of several receptors; this was most clearly seen for CCR5 and CXCR3A (Figure 3, $p<0.0005$), but was also observed for CCR1, CCR2 and CCR4 ($p<0.03$). The levels of CXCR2 and CXCR4 did not differ, and for CXCR1 and CCR3 the levels were relatively low for both cell subsets (*data not shown*). Receptor expression in the subgroup of patients with increased proliferation in the presence of exogenous chemokines did not differ from that in the other patients except for significantly decreased expression of CCL4 (Figure 3). For 20 randomly selected patients we investigated the receptor expression profile for *in vitro* cultured AML cells (48 hours' incubation in medium alone or in the presence of GM-CSF+IL3+SCF): (i) receptor expression was usually slightly decreased during culture both in medium alone and in the presence of exogenous growth factors when compared with fresh cells; and (ii) the overall chemokine receptor expression profile and the difference between $CD34^+$ and $CD34^-$ cell subsets were maintained both in the presence and absence of exogenous growth factors (*data not shown*).

Exogenous chemokines alter the proliferation of clonogenic AML cells

We investigated the effect of six exogenous chemokines on clonogenic AML cell proliferation for the seven patients in cluster D (Figure 2) and three of the patients in

Table 2. The effect of exogenous chemokines on the proliferation of clonogenic AML cells; studies of the patient subset showing enhanced proliferation in the presence of exogenous chemokines (Figure 2, patient cluster D).

		Exogenous cytokine added					
Patient	Chemokine free control	CCL5	CCL13	CCL23	CXCL12	CXCL2	CXCL10
7	975±105	1060 ±71	900 ±96	**1190 ±61**	**1270 ±31**	**1185 ±39**	**1215 ±90**
9	10±4	**19±7**	8±3	**19±1.5**	**28±7**	5±3	**22±6**
17	46±9	**62±5**	**68±16**	**96±2**	**114±31**	**108±26**	**86±24**
12	14±6	**36±11**	15±6	**26±14**	**33±9**	**25±1**	**32±9**
55	5±1	**88±18**	**28±14**	**36±16**	**30±2**	**64±2**	**84±9**
47	1200± 13	**1680± 43**	**1650± 80**	**1660± 81**	**1605± 26**	**1890± 92**	1420± 102

All seven patients in Figure 2 (patient cluster D) were investigated using the colony formation assay. Colony formation was detected for six of the patients and these results are presented in the Table. No colonies were detected in any culture for the last patient (patient 56). The results are presented as the number of colonies (mean ± standard deviation of duplicates) per 50,000 seeded cells. Colony numbers marked in bold represent a difference of at least 20% from the chemokine-free control.

cluster E (patients 25, 33 and 63). Our chemokine selection was based on the clustering in Figure 2 and the known receptor specificity of CCL5 (mainly mediated via CCR3/5, see above), CCL13 (representing CCR3 mediated effects), CCL23 (CCR1), CXCL2 (CXCR2), CXCL10 (CXCR3A) and CXCL12 (CXCR4).[5] The AML cells from patients in cluster D were characterized by increased proliferation (^{3}H-thymidine incorporation) in the presence of all or most exogenous chemokines, and either increased or unaltered clonogenic cell proliferation was then detected in the presence of chemokines. For a majority of patient/chemokine combinations an increase of more than 20% was detected (Table 2). In contrast, exogenous chemokines had divergent effects on clonogenic cell proliferation when testing AML cells from patients in cluster E (*data not shown*). Erythroid colonies could be detected for four patients, for all of whom an unaltered fraction of erythroid colonies, corresponding to <0.30, was observed with exogenous chemokines.

Constitutive chemokine release by native human AML cells

We investigated constitutive release of 28 CCL and CXCL chemokines from the AML cells of 68 consecutive patients; the results are summarized in Table 1. Chemokine levels varied widely even for those patients with detectable release. The highest levels were detected for CXCL8 with 64 patients showing detectable release; the median level was 22.720 pg/mL. In addition, detectable release for at least 40 patients and median levels exceeding 1000 pg/mL were observed for CCL2-4, CCL22, CXCL1, CXCL5 and CXCL10 (Table 1, marked in bold). This group includes both homeostatic and inflammatory chemokines.[1-5] CXCL4 release from the cells of 53 patients was also detected, but this chemokine was measured in IU/mL. In contrast, CCL11, CCL21, CCL23 CCL25-28 and CXCL12 release was not detected in any, or only in a few, patients. Thus, relatively few chemokines are released at high levels in most patients.

Classification of patients based on constitutive chemokine release from AML cells

We investigated correlations between constitutive release of various chemokines by hierarchical clustering (Figure 4) and principal component analyses (Figure 5) of chemokine levels for the 68 consecutive patients. In these last parts of the study we included only those chemokines with either detectable levels for at least 18 patients or maximal levels exceeding 30 pg/mL (see Table 1, chemokines left out from the analyses are marked with*). Both the cluster and principal component analyses identified three chemokine release groups: (i) CCL2-4/CXCL1/8; (ii) CCL5/CXCL9-11 and possibly also CCL23; (iii) CCL13/17/22/24/CXCL5 and possibly CXCL6. Notably, CXCL4, which is an anti-angiogenic CXCR3B ligand, showed no association with any other chemokine.

Based on the hierarchical clustering (Figure 4) our patients could be classified into three major subgroups depending on their chemokine release profile (Figure 4, left margin). Firstly, several separate clusters constituting a relatively large subset showed undetectable or low levels of most/all chemokines (lower patient clusters, 18 patients). Secondly, the majority of patients showed relatively high levels for the CCL2-4/CXCL1/8 chemokine cluster, in some cases in combination with other single chemokines (the 49 upper patients). Thirdly, a minority of patients showed high levels for the CCL2-4/CXCL1/8 cluster and also high levels of CCL13/17/22/24/CXCL5 and/or CCL5/CXCL9-11 (nine patients constituting the three intermediate patient clusters). No single chemokine or chemokine release cluster showed any correlations with clinical characteristics or biological AML cell characteristics (i.e. morphology, membrane molecule expression, genetic abnormalities); this is illustrated by the distribution of the genetic abnormalities presented in Figure 3 right part.

Finally we compared chemokine release and chemokine responsiveness. As described above, AML cells from 26 patients showed no detectable *in vitro* proliferation in response to any chemokine/growth factor combination. There was a significant correlation between undetectable proliferation and chemokine release (χ^2 test, $p<0.01$) as ten out of the 26 patients whose cells showed no detectable proliferation were included in the cluster of 13 patients with no or only minimal chemokine release.

The chemokine release profile affects T-cell chemotaxis

We investigated AML cell supernatants derived from 46 unselected patients in the chemotaxis assay. The number of cells in the lower chamber after 18 hours of incubation is expressed as the percentage of the total cell number seeded in the upper well. Control experiments demonstrated that normal peripheral blood mononuclear cells did not proliferate in response to AML supernatants (*data not shown*). We compared the results for three major patient subsets (Figure 4): (i) a group of patients formed of several subclusters and characterized by high expression of CCL2-4/CXCL1/8 (patient cluster 1-52, 27 patients examined); (ii) the two clusters of patients in which most patients showed high levels of CCL2-4/CXCL1/8 together with high levels of CCL13/17/22/24/CXCL5 and/or CCL5/CXCL9-11; and (iii) patients with very low or undetectable levels of all chemokines (Figure 4, patient cluster 38-17, nine patients examined). Patients without detectable chemokine release showed decreased chemotaxis (i.e. decreased total cell numbers in the lower chamber) compared with patients with high release (Figure 6; Wilcoxon's test, p=0.0047); the third group showed intermediate levels. Results were similar when comparing two different healthy peripheral blood mononuclear cell donors, and the results were reproduced for 14 unselected patients. Detailed flow cytometric characterization of the cells in the lower chamber showed that: (i) only one distinct lymphocyte population was detected and >80% of the cells expressed T-cell markers; (ii) the CD4/CD8 ratio

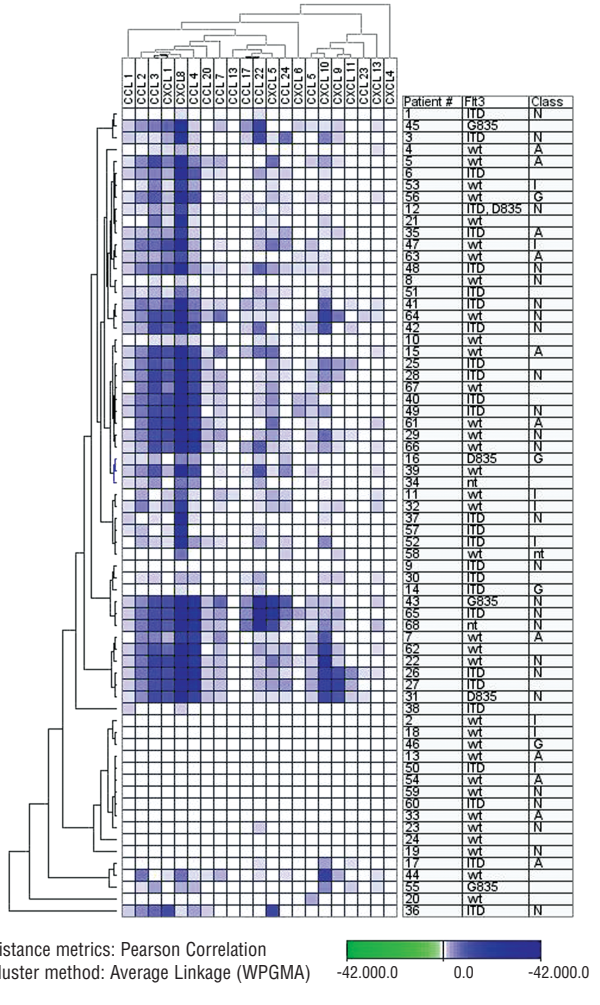

Figure 4. Classification of AML patients based on the chemokine release profile of their leukemic cells. We investigated 68 consecutive patients and the analysis was based on the results for 22 chemokines. The hierarchical clustering of chemokine release is shown at the top and the patient clustering at the left. Three major chemokine clusters were identified: (i) CCL2-4/CXCL1/8; (ii) CCL5/CXCL9-11; and (iii) CCL13/17/22/24/CXCL5/6. Three subsets of patients could be identified based on these analyses. One subset of patients showed undetectable or low levels of most chemokines (lower group of 18 patients). A second, large group of patients showed relatively high levels of the CCL2-4/CXCL1/8 chemokine cluster sometimes in combination with some other chemokines (the upper 49 patients). A third, small group of patients showed high levels for the CCL2/3/4/CXCL1/8 cluster in combination with high levels of the CCL13/17/22/24CXCL5/6 cluster and/or CCL5/CXCL9-11 chemokine cluster (the intermediate nine patients/three clusters). There were no correlations between cytogenetic abnormalities or Flt3 mutations and any patient cluster.

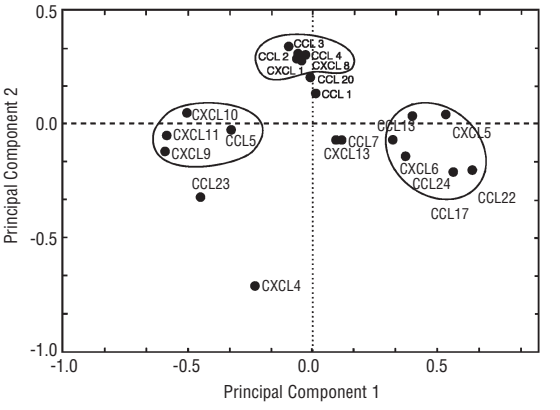

Figure 5. Classification of AML patients based on the chemokine release profile of their leukemic cells: a PCA plot of chemokine release by native human AML cells. We investigated 68 consecutive patients and the analysis was based on the results for 22 chemokines. PC 1 explains 27% of the variation in the data, while PC 2 explains 13% of the variation. Three major chemokine clusters were identified in the plot: (i) CCL 2-4/CXCL1/8; (ii) CCL5 (and eventually CCL23)/CXCL 9-11; and (iii) CCL13/17/22/24/CXCL5/CXCL6. CXCL4 did not correlate with any of the other chemokines. The three clusters correspond to those detected when correlation was used as a criterion for hierarchical clustering.

Table 3. Correlation analyses of chemokine-specific mRNA levels; analysis of the CCL2-4/CXCL1/8 cluster (CCL2 mRNA values were not available) for 25 randomly selected patients.

Bonferroni-corrected p values in correlation analysis

Chemokine	*CCL4*	*CXCL1*	*CXCL8*
CCL3	0.0044 (0.63)	0.0044 (0.63)	0.0011 (0.68)
CCL4		0.0463 (0.52)	n.s.
CXCL1			0.0015 (0.67)

The results are presented as the Bonferroni-corrected p-values (six comparisons), the corresponding correlation coefficients (r-values) are given in parenthesis.

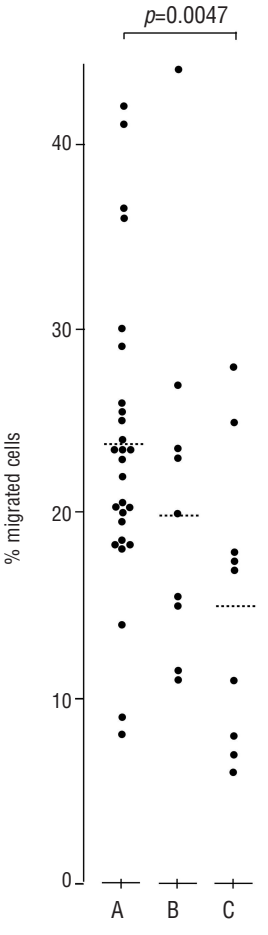

Figure 6. Chemotaxis of normal immunocompetent cells by primary AML cell culture supernatants. We investigated chemotaxis of normal peripheral blood mononuclear cells in a transwell assay in which AML cell culture supernatants were added to the lower chamber. The number of transmigrated cells were compared for (A) AML cells with high expression of CCL2-4/CXCL1/8 (27 patients); (B) AML cells from patients with high expression of the same chemokines together with high levels of CCL13/17/22/24/CXCL 5 or CCL5/CXCL9-11; and (C) AML cells from patients with undetectable chemokine release. The number of transmigrated cells differed significantly between group A and C (p=0.0047).

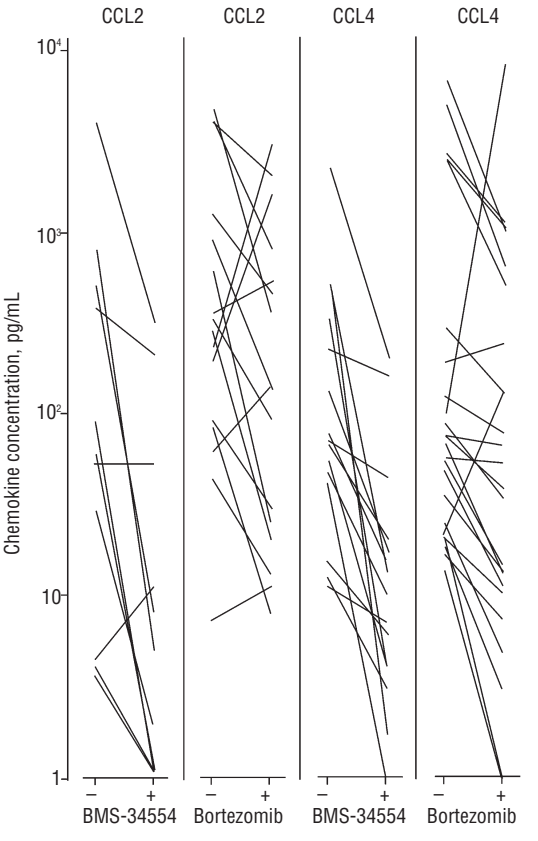

Figure 7. Effects of NFκB inhibition on the release of CCL2 and CCL4. Primary AML cells were cultured with the proteasomal inhibitor bortezomib (24 unselected patients) or BMS-345541 (15 unselected patients), and the chemokine levels were examined after 48 hours of culture for drug-free controls (–) and cultures containing bortezomib or BMS-345541 (+). The results are presented as the chemokine concentration (pg/mL) in culture supernatants. The figure shows the results only for those patients with detectable chemokine release).

was increased in cultures containing AML supernatants compared with control cultures prepared in medium alone, but this was observed for all patients and did not differ between the three groups of patients; (iii) the percentages of total $CD56^+$, $CD4^+CD56^+$ and $CD8^+CD56^+$ cells did not differ between the subsets of patients. Thus, the AML chemokine release affects total chemotaxis, but the effects on various T cell subsets do not differ between patients (*data not shown*).

Biological characterization of chemokine release clusters

The relative chemokine-specific mRNA levels were determined from microarray analyses for 24 randomly selected patients.[20] The mRNA levels showed significant correlations for the CCL2-4/CXCL1/8 cluster (Table 3), whereas the levels showed no statistically significant correlations for the CCL5/CXCL9-11 and CCL13/17/22/24/CXCL5 clusters (*data not shown*).

The transcription factor NFκB is important for transcriptional regulation of several chemokines[21,22] and we, therefore, investigated the protein levels of this factor for a consecutive subset of 30 patients from the whole study population. Most patients showed high levels of NFκB p65 (median number of positive cells 85.2%) and all except two patients had more than 70% $p65^+$ cells. The levels of the p50 and p52 forms showed wider variation and significant correlations (Table 4). Furthermore, the percentage of p50 and p52 positive cells was statistically significantly correlated with protein levels of the CCL2-4/CXCL1/8 cluster. We investigated the effect on CCL2 and CCL4 levels of (i) the proteasome inhibitor, bortezomib (24 unselected patients examined); and (ii) BMS-345541, targeting the inhibitor of κ B kinase (IKK) complex, which is a key regulator of NFκB signaling (15 unselected patients examined.[16, 21, 22] Both bortezomib and BMS-345541 decreased CCL2 (Figure 7, Wilcoxon's test for paired samples, p=0.04 and 0.004, respectively) and CCL4 levels (p<0.0001 and 0.0028, respectively).

Discussion

In this study we used highly standardized methodological approaches[23] and showed that AML cells usually release several CCL and CXCL chemokines involved in: (i) the regulation of angiogenesis; (ii) local T-cell recruitment and regulation of antileukemic T-cell reactivity; or (iii)

AML cell growth regulation.[1-5] Only the CCL12/CXCR4 system has been previously investigated in AML,[7,24] and in our present study we describe that AML cells release several other chemokines involved in angioregulation as well as in the chemotaxis of monocytes, T cells and NK cells.[4,5]

Our initial studies (Figure 1) showed that single chemokines usually did not affect AML cell proliferation in the absence of other exogenous growth factors, and we therefore investigated cytokine-dependent (GM-CSF+SCF+Flt3L) proliferation in the other experiments. When investigating the same 17 patients we concluded that many chemokines could alter AML cell proliferation through ligation of a wide range of CCR and CXCR receptors (Figure 1), and effects in individual patients could be predicted by testing a limited number of chemokines. This could have been expected because many inflammatory chemokines bind to more than one receptor and one receptor often binds several chemokins.[3-5] We selected a limited number of exogenous chemokines for the study of the whole group of 64 consecutive patients, and found that patients could be classified according to the chemokine responsiveness of their cells in suspension cultures (Figure 2): (i) in one relatively large cluster of patients there was no effect of exogenous chemokines on AML blast proliferation; (ii) in another major cluster there was increased proliferation in the presence of most chemokines; (iii) three minor clusters showed relatively weak and divergent effects of a few chemokines; and (iv) a large group of patients showed undetectable AML cell proliferation for all growth factor/chemokine combinations. Additional studies demonstrated that the chemokine-induced growth enhancement could be detected also for the clonogenic subset.

We investigated the expression of CCR1-5 and CXCR1-4 on primary AML cells. These receptors bind most CCL (CCL2-5, 7, 8, 11-17, 22-24, 26, 28) as well as CXCL chemokines (CXCL1-3, 5-12), including those chemokines used for the subclassification of patients based on effects on cytokine-dependent proliferation (Figure 2). Many receptors were more highly expressed by $CD34^+$ AML cells than by $CD34^-$ ones, but the receptor expression profile did not differ between the patient subsets identified in Figure 2. These observations suggest that the patient subclassification is most likely to be caused by differences in intracellular events downstream of receptor ligation rather then differences in receptor expression. Furthermore, *in vitro* culture in the presence of hematopoietic growth factors did not alter chemokine receptor expression, an observation indicating that the effect of chemokines only on cytokine-dependent but not spontaneous AML cell proliferation cannot be explained by cytokine-induced receptor expression.

Only a limited number of chemokines were released at high levels for most patients, but several of these chemokines are involved in angioregulation and chemotaxis of immunocompetent cells.[1-5] Pharmacological targeting of the corresponding chemokine receptors may, therefore, become a future therapeutic strategy in AML.[5] The quantitatively most important chemokine was CXCL8, suggesting that pro-angiogenic signaling mediated through CXCR2 by CXCL8 is usually stronger than the anti-angiogenic/angiostatic signaling mediated through CXCR3 by CXCL4/9/10/11.

Table 4. Flow cytometric analysis of the expression of NFκB p50, p52 and p65 by native human AML blasts from 30 consecutive patients; correlations with CCL2-4/CXCL1/8 protein levels.

	Percentage of positive cells		*Statistical correlation analysis*	
NFκB molecular form	*Median*	*Range*	*Protein levels*	*p value*
p50	39.5%	1.4-95.2%	p52	0.04
			CCL2	0.03
			CCL4	0.03
			CXCL8	0.03
p52	45.3%	5.0-79.8%	CCL4	0.04
p65	85.2%	18.1-98.7%	No significant correlations	

NFκB expression was determined by flow cytometry and correlations analyzed by Kendall's test. We investigated correlations between (i) the three NFκB forms and (ii) these three forms and CCL2-4, CXCL1/8 levels. Only statistically significant correlations are presented.

To evaluate the possible functional importance of constitutive chemokine release we investigated the effect of AML cell supernatants on normal peripheral blood mononuclear cell migration. Total lymphocyte chemotaxis was lower for patients with low/undetectable chemokine release compared with patients who showed higher levels of several chemokines. Chemokine release thus increased the chemotaxis of normal T cells, especially $CD4^+$ cells (increased CD4:CD8 ratio). However, no differences in chemotaxis of normal immunocompetent cells were observed between subsets of patients.

We examined CCL and CXCL chemokine release for 68 consecutive patients (Table 2), but chemokines whose levels were mainly undetectable or very low were excluded from the bioinformatic analyses (Figures 4 and 5). The correlation and principal component analyses demonstrated that three clusters could be identified based on chemokine release: (i) CCL2-4/CXCL1/8 (and possibly CCL23), a cluster including the chemokines usually released at high levels; (ii) CCL5/CXCL9-11; and (iii) CCL13/17/22/24/CXCL5. The significant correlations between chemokine mRNA and protein levels for the CCL2-4/CXCL1/8 cluster suggests that the common regulation of these chemokines occurs, at least partly, at the transcriptional level. Furthermore, NFκB is a transcription factor known to affect the expression of several chemokines in this cluster,[16,21,22] and the importance of this factor in the regulation of CCL2-4/CXCL1/8 release is further demonstrated by: (i) correlations between NFκB positivity (both p50 and p52 isoforms) and these chemokine levels; (ii) decreased

CCL2 and CCL4 levels in the presence of both bortezomib that inhibits proteasomal NFκB activation, and BMS-345541, which targets IKK and thereby reduces NFκB activation.[16,21,22]

Angioregulation and chemotaxis are functional characteristics of the three chemokine release clusters.[1-5] Firstly, the CCL2-4/CXCL1/8 cluster mediates pro-angiogenic effects through CXCR2 ligation, and several of these chemokines have additional chemotactic effects for a wide range of normal leukocytes including monocytes, dendritic cells, T cells and NK cells.[4,5] Secondly, the levels of CCL5, CXCL9-11 and possibly CCL23 were correlated but high levels were seen only in a subset of patients (Figure 4). CXCL9-11 and CCL23 (Figure 4) all show anti-angiogenic activity[5] and have chemotactic effects for various T-cell subsets and NK cells,[1-5,13] including chemotaxis towards AML cells.[13] However, it should be emphasized that anti-angiogenic effects are also mediated by CXCL4,[5] which showed no correlation with any other chemokine. Thirdly, the CCL13/17/22/24/CXCL5 cluster can mediate pro-angiogenic effects through CXCL5/CXCR2 ligation and has chemotactic effects mainly on NK cells.[4,5] In conclusion, these chemokine release profiles demonstrate that the angioregulatory and immunoregulatory characteristics of AML patients could be expected to differ. Even though the constitutive chemokine release profiles do not correlate to known prognostic parameters for patients receiving chemotherapy (e.g. genetic abnormalities), they should be investigated as possible prognostic parameters, especially in studies of immunotherapy or angiotargeting.

To conclude, our present results demonstrate that AML patients can be classified into distinct subsets according to their chemokine responsiveness and chemokine release profile. This chemokine organization may, therefore, contribute to clinically relevant heterogeneity with regard to angioregulation, chemosensitivity or antileukemic immunoreactivity.

Authors' Contributions

ØB: major responsibility for planning, designing and performing the study, responsible for analysis and presentation of the results and for writing the manuscript; AR: responsible for performing, analysis, interpretation and presentation of flow cytometric analysis. AMO: responsible for data analysis, presentation of results and writing the manuscript. LS: major responsibility for analysis of the results, presentation of results; AMaO, KHK: responsible for sample preparation, microarray analysis and interpretation of these results; BTG: major contribution to planning the study, analysis of the results, presentation of the data and writing the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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