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Serum Chemokine-release Profiles in AML-patients Might Contribute to Predict the Clinical Course of the Disease

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ABSTRACT

In cancer or hematologic disorders, chemokines act as growth- or survival factors, regulating hematopoiesis and angiogenesis, determining metastatic spread and controlling leukocyte infiltration into tumors to inhibit antitumor immune responses. The aim was to quantify the release of CXCL8, −9, −10, CCL2, −5, and IL-12 in AML/MDS-pts' serum by cytometric bead array and to correlate data with clinical subtypes and courses. Minimal differences in serum-levels subdivided into various groups (e.g. age groups, FAB-types, blast-proportions, cytogenetic-risk-groups) were seen, but higher release of CXCL8, −9, −10 and lower release of CCL2 and −5 tendentially correlated with more favorable subtypes (<50 years of age, <80% blasts in PB). Comparing different stages of the disease higher CCL5-release in persisting disease and a significantly higher CCL2-release at relapse were found compared to first diagnosis – pointing to a change of 'disease activity' on a chemokine level. Correlations with later on achieved response to immunotherapy and occurrence of GVHD were seen: Higher values of CXCL8, −9, −10 and CCL2 and lower CCL5-values correlated with achieved response to immunotherapy. Predictive cut-off-values were evaluated separating the groups in 'responders' and 'non-responders'. Higher levels of CCL2 and −5 but lower levels of CXCL8, −9, −10 correlated with occurrence of GVHD. We conclude, that in AML-pts' serum higher values of CXCL8, −9, −10 and lower values of CCL5 and in part of CCL2 correlate with more favorable subtypes and improved antitumor'-reactive function. This knowledge can contribute to develop immune-modifying strategies that promote antileukemic adaptive immune responses.

KEYWORDS

Dendritic cells; acute myeloid leukemia; T-cells; immunotherapy; chemokines

Introduction

Acute myeloid leukemia (AML) and Myelodysplastic Syndrome (MDS) are characterized by clonal proliferation of myeloid precursor-cells resulting in abnormal cell-differentiation and ineffective hematopoiesis. After successful treatment by chemotherapy or allogeneic stem-cell

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-transplantation (SCT) relapses occur due to persistence of minimal residual disease (MRD) (Buccisano et al. 2012). Donor lymphocyte infusions (DLI) in relapsed patients (pts) after SCT can reinstall remissions thereby demonstrating the importance of T-cells in antileukemic reactions, but graft-versus-host disease (GVHD) can impair the result of SCT or relapse therapy (Dickinson et al. 2017; Jedlickova et al. 2016; Schmid et al. 2018).

As malignant diseases are characterized by an ineffective immune-response, interactions and functions of immunocompetent cells with tumor cells might be influenced by soluble factors like chemokines in the microenvironment of cells (Franciszkiewicz et al. 2012; Galluzzi et al. 2012). Chemokines or chemotactic cytokines are small proteins secreted by various cells like monocytes, lymphocytes, fibroblasts, keratinocytes and also tumor-cells, that are involved in a variety of immune- and inflammatory responses and act primarily as chemoattractants and activators of specific types of leukocytes, mediating migration of individual cells or groups of cells (Hughes and Nibbs 2018; Palomino and Marti 2015). In cancer or hematologic disorders chemokines, act as growth or survival factors, regulating hematopoiesis and angiogenesis, determining metastatic spread and controlling leukocyte infiltration into tumors to inhibit antitumor immune responses (Ruffini et al. 2007): **CXCL8** is mainly chemotactic for neutrophils and T-cells and functions as a mediator in diverse inflammatory disorders, angiogenesis and as a growth-factor in several tumor-models (Rollins 2009; Zhu and Woll 2005). It is a product of tumor-cells, phagocytes, DC, Tregs, fibroblasts and epithelial cells (Levina et al. 2008; Piqueras et al. 2006) (Table 1). **CCL2** is a potent mononuclear cell chemoattractant which promotes recruitment and activation of leukocytes during acute inflammation and also tumor-initiation and -progression (Ferreira et al. 2005; Yoshimura et al. 2015). It is released by stromal cells, various leukocytes, DC, endothelial cells, as well as tumor (e.g. CLL)-cells (van Attekum et al. 2017; Salcedo et al. 2000; Yoshimura et al. 2015). **CCL5** is a monocyte-, eosinophil- and lymphocyte-attractant released by fibroblasts, epithelial cells, DC, monocytes/macrophages, platelets, certain tumor cells and T-cells which plays a critical role in chronic inflammatory diseases and progression of malignant disorders (Aldinucci and Colombatti 2014). As an attractant of monocytes, T-cells and NK-cells **CXCL10** is secreted by various leukocytes, epithelial and endothelial cells and stromal cells in response to IFN-γ mainly in inflammatory, autoimmune and malignant diseases (Liu et al. 2011). The secretion of **CXCL9** is induced by IFN-α2a/-γ in macrophages, DC and B-lymphocytes and is associated with T-cell-infiltration in inflammatory and malignant diseases and has been identified as a biomarker of chronic GVHD (Kitko et al. 2014; Padovan et al. 2002). Both, CXCL9 and CXCL10 were shown to act as inhibitors of angiogenesis and tumor-growth (Chow and Luster 2014; Naschberger et al. 2008). **IL-12** is mainly produced by macrophages, monocytes and activated DC and promotes the differentiation of naïve T-cells into Th1-cells (Schulz et al. 2009). As an adjuvant drug, it has been applied in several immunotherapeutic strategies (Klinke 2015). Known functions of the listed chemokines as well as sources and target cells are given in Table 1 (row 1–5).

The aim of this study was to characterize chemokine-release-profiles in serum of AML- and MDS-pts in correlation with disease subtypes and the clinical course, since CXCL8, CCL2, CCL5, CXCL9, CXCL10, and IL-12 have been proven to be important mediators of immune-reactions.

Table 1. Chemokines and their main functions.

Chemokine	Source	Target Cells	Known Functions	Known clinical correlations	Reference	Pts' response to IT, GVHD
'antitumor response related chemokines':						
CXCL9	macrophages, DC, lymphocytes, tumor cells	Neutrophils, $CD8^+$ T-cells	Inhibition of tumor growth and inhibition of angiogenesis, T-cell-infiltration in inflammatory disorders	Increased levels associated with shorter survival in MDS-pts	Padovan et al. 2002; Kitko et al. 2014; Wong, 2013	Higher release in serum associated with improved response to IT and no GVHD
CXCL10	leukocytes, epithelial and endothelial cells, stromal cells, DC, monocytes, tumor cells	Neutrophils, $CD8^+$ T-cells,	Inhibition of tumor growth and inhibition of angiogenesis, Autoimmune reactions		Liu et al. 2011; Wong et al. 2013; Pardanani and Tefferi 2012; Carvalheiro et al. 2018	Higher release in serum associated with improved response to IT and no GVHD
IL-12	DC, B-lymphocytes, macrophages	NK-cells, CTL	Mediation of antitumor responses (inhibition of tumor-growth, antiangiogenesis, immunomodulation), differentiation of naïve T-cells into Th1		Schulz et al. 2009	release in serum not predictive for response to IT or GVHD
'tumor growth promoting/ inflammatory chemokines':						
CXCL8	tumor cells, phagocytes, DC, Tregs, fibroblasts, epithelial cells	Neutrophils, T-cells, endothelial cells, monocytes	Promotion of tumor cell proliferation, survival, angiogenesis and metastatic spread, proinflammatory	Associated with poor prognosis in a subset of pts, lower levels associated with longer survival	Rollins, 2009; Zhu and Woll 2005; Piqueras et al. 2006; Levina et al. 2008; Kuett et al. 2015; Kornblau et al. 2010; Alfaro et al. 2016	Higher release in serum associated with improved response to IT and noGVHD
CCL5	fibroblasts, epithelial cells, DC, monocytes, macrophages, platelets, tumor cells, T-cells	MNC, $CD8^+$ T-cells, T_{reg}	Promotion of tumor progression and metastasis, increasing thepresence of tumor-associated macrophages and Treg, chronic inflammatory disorders	Higher levels associated with longer survival and remission	Aldinucci and Colombatti 2014; Tan et al. 2009; Chang, 2016; Wong, 2013; Kornblau, 2010	Lower release in serum associated with improved response to IT and no GVHD
CCL2	stromal cells, leukocytes, DC, endothelial cells, tumor cells	MNC, macrophages, tumor cells	Promotion of tumor initiation and progression and angiogenesis, proinflammatory		Yoshimura et al. 2015, Yoshimura 2018; Ferreira et al. 2005; Salcedo et al. 2000	Higher release in serum associated with improved response to IT and GVHD

[x]Functional potential of T-cells (stimulated with DC) as evaluated in this work.

Material and methods

Patients' characteristics, diagnostics and sample-collection (Table 2)

Thirty AML-/MDS-pts (14 at first diagnosis, 5 in persisting disease, 5 at relapse before and 6 at relapse after SCT). The median age was 52 years (range 35–80 years), the female:male ratio 1:1,1. FAB-types and blast-phenotypes are given in Table 2. Diagnostic data were obtained from accredited laboratories of the pts' treating institutions. After pts' or stem-cell donors' informed consent cells were obtained by aspiration of heparinized peripheral blood (PB) or bone marrow (BM).

'Response' to immunotherapy was defined as a complete remission achieved in 3 months following SCT or DLI, that persisted for at least 9 months. 'Non-responders' were pts who did not show a response to immunotherapy. Cases with early death after SCT were excluded from analyses.

Flow cytometry

To evaluate and quantify types of cells, flow-cytometric analyses were performed with a panel of mouse monoclonal antibodies (moAbs) purchased from Becton Dickinson (BD)/Pharmingen (Heidelberg, Germany)^{B}, Immunotech/Beckman-Coulter (Krefeld, Germany)^{I}, Miltenyi Biotec (Bergisch Gladbach, Germany)^{M}, Caltag (Hamburg, Germany)^{C} and Serotec (Duesseldorf, Germany)^{S} as already described (Vogt et al. 2014). Those moAbs were directly conjugated with fluorescein-isothiocyanate (FITC) (CD14^{I}, CD8^{I,} CD25^{I}, CD71^{I}), phycoerythrin (PE)(CD3^{I},CD25^{I}), tandem CY5-PE (PC7 CCR7^{B}) or allophycocyanin (APC CD33^{I}, CD45^{I}, CD56^{I}, CD117^{I}, CD34^{I,C}, CD13^{C}, CD4^{B,C}, CD71^{B,C}). For analysis and quantification of lymphocytes, monocytes, and leukemic-blasts before culture, the total MNC-fraction was gated. Proportions of positive events in defined gates compared with the isotype-controls were calculated using Cell-Quest-software (BD).

Cytometric Bead Array (CBA) for cytokine- and chemokine-analysis

Using the BD CBA Human Chemokine-Kit I and BD CBA Human IL-12-Kit chemokines (CCL2, CCL5, CXCL8, CXCL9, CXCL10, and IL12) were quantified in serum-samples (n = 29). Until analysis 500 µl serum was taken and stored at −80°C. The standard serial dilutions of positive and negative controls of the mentioned chemokines were included. For 3 h, 50µl mixed human Chemokine capture-beads and 50µl PE Detection-Reagent were incubated with 50µl of each antibody-labeled chemokine test-sample in assay-tubes (12x75mm, BD Falcon) at room temperature. After washing with 1 ml Wash-Buffer the assay-tubes were centrifuged at 200 g for 5 min and the bead-pellets resuspended in 300µl Wash-Buffer before flow-cytometric analysis. Chemokines in the samples were quantified according to the standard curve. The detection-limits were 0.2 pg/ml CXCL8, 1.0 pg/ml CCL5, 2.5 pg/ml CXCL9, 2.7 pg/ml CCL2, 2.8 pg/ml CXCL10 and 0.6 pg/ml IL-12.

Table 2. Pts' characteristics and source of T-cells for the mixed lymphocyte culture (MLC) are given.

Patient No.	Age at dgn	gender	FAB-type	Appearance	stage	blast phenotype [CD]	IC Bla%	Response to SCT/DLI	GvHD
P 201	48	female	AML M5	primary	Diagnosis	33, 34, 13, 117	93		
P 326	58	male	AML M2	secondary	Relapse after SCT	34, 13, 33	57	no response to SCT	GvHD II
P 328	59	male	AML M2	secondary	Relapse before SCT	34, 33, 13, 117	49	-	GvHD III-IV
P 338	65	male	AML M1	secondary	Diagnosis	-	-		
P 343	28	female	AML M5	primary	Diagnosis	-	-		
P 344	65	female	AML M5	primary	Diagnosis	13, 15, 65	85		
P 346	39	male	AML M5	primary	Diagnosis	-	-		
P 347	37	male	AML M5	secondary	Diagnosis	33, 64, 65	86		
P 348	35	female	AML M1	primary	Diagnosis	33, 34, 117	75		
P 350	63	male	AML M2	primary	Diagnosis	-	-		
P 351	73	male	AML M5	primary	Diagnosis	33, 65, 15, 64, 7, 117	13		
P 352	35	male	AML M2	secondary	Relapse after auto SCT	65	12	no response to 2nd SCT	no GvHD
P 362 *	53	female	MDS RAEB	n.a.	Diagnosis	13, 33, 34, 117	40	early death after SCT	GvHD III-IV
P 428	59	female	AML M4	primary	Relapse after SCT	13, 33,117	47	no CR after SCT	no GvHD
P 454	52	male	MDS CMML	primary	Relapse after SCT	14, 16, 33, 65	37	-	-
P 460	36	male	AML M0	primary	Relapse after SCT	7, 15, 13, 33, 56, 65	75	early death after SCT	no GvHD
P 466 *	62	female	AML M6a	n.a.	Persistence after SCT	13, 33, 34, 65, 117	20	CR after SCT	GvHD III
P 481 *	66	female	AML M4/M5	primary	Relapse before SCT	13, 15, 33, 34, 56, 117	93	} -	no GvHD
P 481	66	female	AML M4/M5	primary	Relapse before SCT	13, 15, 33, 34, 56, 117	93		
P 487	50	female	AML M1	primary	Persisting Disease	13, 15, 33, 34, 56, 93, 117	14	no response to SCT CR after SCTSCT CR after SCT	
P 90/491	51	female	AML M2	primary	Relapse before SCT	13, 33, 34, 117	50	CR after SCT	no GVHD
P 502	41	male	AML M4 eo	primary	Persisting Disease	13, 15, 33, 34, 117	92	} CR after SCT	GvHD I
P 502	41	male	AML M4 eo	primary	Persisting Disease	13, 15, 33, 34, 117	92		
P 518	55	male	AML M2	primary	Diagnosis	13, 33, 117	81	-	-
P 520 *	46	female	AML M1/M2	primary	Diagnosis	65, 14, 15, 19, 33, 34	70	-	-
P 545	44	female	AML M1	primary	Relapse before SCT	33, 34, 117	83	early death after SCT	GvHD III
P 546	39	male	AML M2	primary	Diagnosis	15, 33, 34, 65, 117	76	-	-
P 561	43	male	AML M2	primary	Diagnosis	13, 15, 33, 65, 117	65	CR after SCT	GvHD III
P 565	68	female	AML M5	primary	Relapse after SCT	13, 15, 33, 34, 65	88	no CR	GvHD III
P 584	52	female	AML M4	primary	Relapse before SCT	13, 33, 117	95	CR after SCT	GvHD I
P 601	80	male	n.a.	secondary	Persisting Disease	-	-		
P 618	66	male	MDS RAEBt	n.a.	Persisting Disease	33, 117	42	-	GvHD I

dgn: first diagnosis; IC-Bla: immunocytologically determined blasts; autologous: pts' T-cells; allogeneic: allogeneic stem cell donors' T-cells; after SCT: pts' T-cells at relapse after SCT; CR: complete remission; GvHD: Graft versus Host Disease.

Statistical methods

Applying statistical methods means, medians, standard deviations, interquartile ranges were calculated as well as groups compared using a one-tailed t-test. Tests were performed with a personal computer using Microsoft®-Excel 2007. Cut-off-values for all chemokines were defined that allowed the best distinction between particular groups compared. Significances were defined in cases with p-values <0.005 as highly significant, between 0.005 and 0.05 as significant and between 0.05 and 0.1 as tendentially significant.

Results

Characterization of MNC-fractions obtained from AML-/MDS-pts before culture

We analyzed uncultured MNC-fractions from 22 AML-/MDS-samples with high blast-counts by Flow-cytometry to quantify monocytes, lymphocytes, and blasts and to evaluate or confirm the blast-phenotype. On average AML-/MDS-samples presented with 6% B-cells (range 0.1–22), 9% T-cells (range 1–32), 4% NK-cells (range 0.5–10), 3% monocytes (range 0.2–9) and 61% leukemic cells (range 12–95). The myeloid blast-phenotype could be confirmed by detection of a patient-specific expression of myeloid leukemic markers (CD15, CD34, CD65, CD117) in all cases. In addition, an aberrant lymphoid antigen-expression (CD7, 56) was seen in five cases (pt 351, pt 460, pt 481, pt 487, pt 520). In cases with aberrant CD56-expression, no estimation of ($CD56^+$) NK-cells in the total MNC-fraction could be performed. In cases of $CD14^+$ myelo-monocytoid leukemia, no evaluation of ($CD14^+$) monocytes in the MNC-fraction was performed.

Chemokine levels in serum samples (Figures 1 and 2)

Pooling results of chemokine-levels obtained in all **stages and subgroups** of the disease showed median levels of 0 pg/ml IL-12 (range 0–2 pg/ml), 97 pg/ml CXCL8 (range 0–5908 pg/ml), 121 pg/ml CCL2 (range 0–2412 pg/ml), 132 pg/ml CXCL10 (range 0–431 pg/ml), 140 pg/ml CXCL9 (range 0–841 pg/ml) and 356 pg/ml CCL5 (range 0–3571).

We subdivided cases into different **age groups** (Figure 1): we didn't detect significant differences in chemokine levels in the group younger than 50 years (n = 12) compared to the group older than 50 years (n = 17, p = .09–0.4).

Comparing cases with different **blast-proportions** in the PB, only a tendentially higher release of CCL5 and CCL2 was observed in the group with less than 50% vs. more than 50% blasts in the PB (p = .06).

Chemokine release-profiles in serum were correlated with the **stages of the disease** (Figure 2). Levels of CXCL8 and CCL5 were high at diagnosis ('dgn', n = 10) and in persisting disease (n = 3) and lower at relapse (before (n = 4) and after SCT (n = 4)). In contrast, levels of CXCL9, CCL2, and CXCL10 were low at dgn compared to other stages of the disease. The release of CCL2 (median 578 pg/ml, range 27–2412 pg/ml) in serum of cases at relapse (n = 4) was significantly higher than at first dgn (85 pg/ml, range 0–455 pg/ml) (p = .03). There were no significant differences in blast counts between the four stages of the disease (dgn.: median 76%, range 13–86%, relapse before SCT: median 90%, range 49–95%, relapse after SCT: median 61%, range 12–91%, persisting disease: median 83%, range 42–92%, p = .2–0.4).

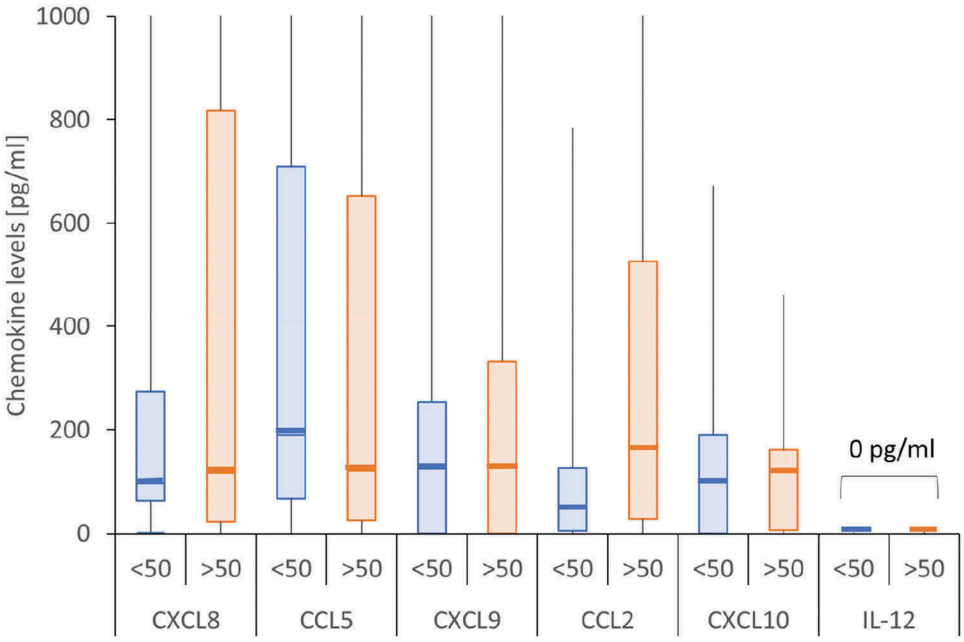


Figure 1. Chemokine-release in different age groups.

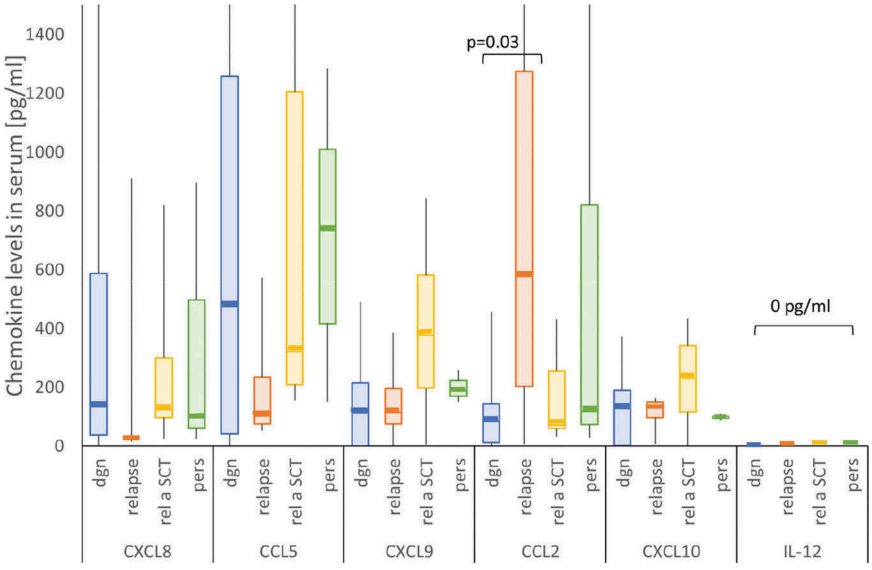


Figure 2. Chemokine-release in serum taken in different stages of the disease.

We observed variable, however not significantly different chemokine-release-patterns in different **FAB-subtypes**: In cases with an 'undifferentiated' AML (M0: n = 1, M1: n = 2, M2: n = 6) higher median levels of CXCL8, CCL2, CCL5 and CXCL9 were found compared to cases with a 'differentiated' AML (M4: n = 2, M4eo: n = 1, M5: n = 5) where median CXCL10-levels were higher. IL-12 was detected in low levels in both groups

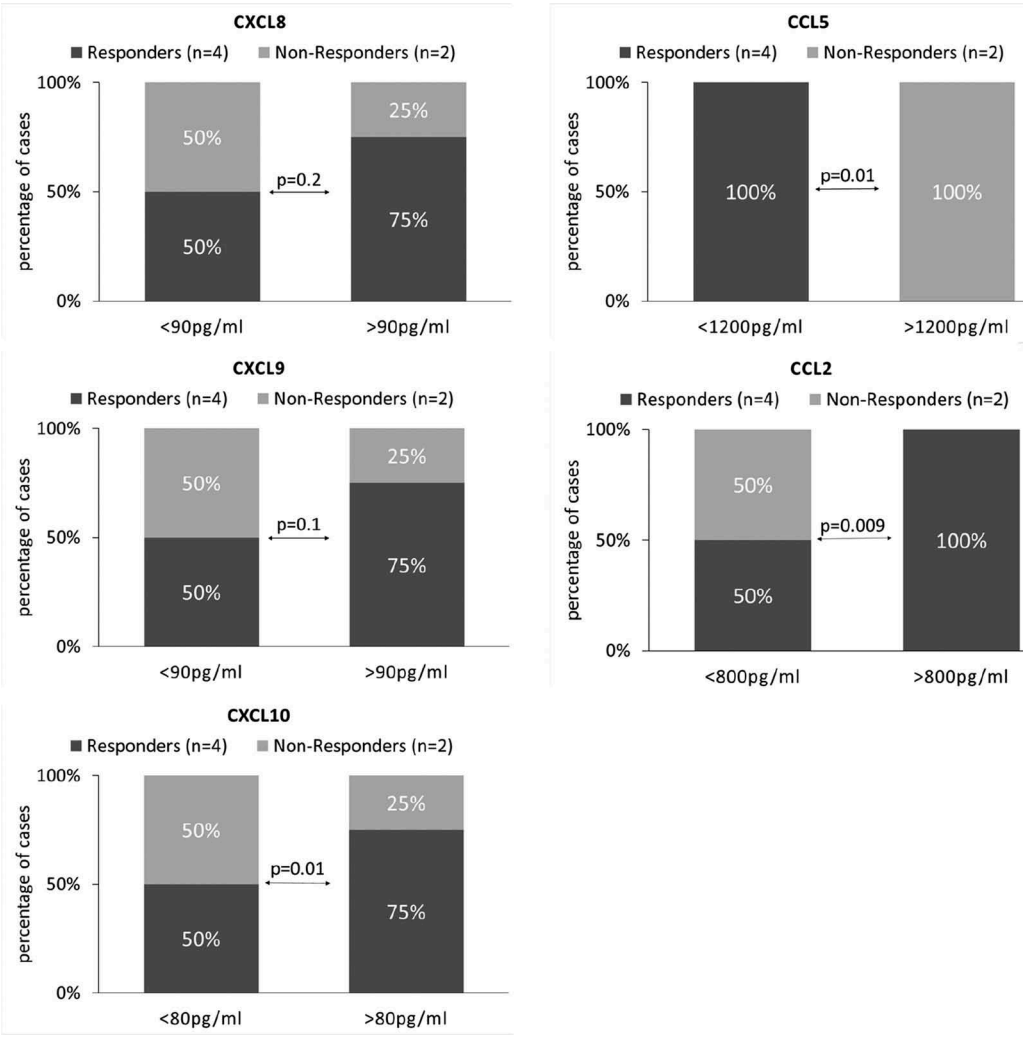


Figure 3. Predictivity of chemokine-release in serum on the response to immunotherapy.

and median release was comparable (0 p/ml each). Those differences were not statistically significant (p = .4–1.0, data not shown).

Comparing different **cytogenetic risk-groups** (studying cases at first dgn) no significant differences in chemokine levels were detectable (p = .2–0.5, data not shown). In the eight cases with intermediate risk, higher median levels of CCL5 (49 vs. 14 pg/ml), CXCL9 (2 vs. 0 pg/ml), and CCL2 (61 vs. 4 pg/ml) compared to the group with unfavourable risk (n = 5) and lower median levels of CXCL8 (61 vs. 96 pg/ml) and CXCL10 (4 vs. 5 pg/ml) were observed (data not shown). IL-12-levels were low and comparable in both groups (median release 0 pg/ml each). Due to only one single pt in the favorable-risk-group no further comparisons were performed.

In summary, our data in general show minimal differences in median serum chemokine-levels detectable in **disease-subtypes**. Comparing different **stages** of the disease, a high serum-release of 'inflammatory' chemokines (CXCL8, CCL2) and a low release of 'anti-tumor-response-related' chemokines (IL-12, CXCL9, CXCL10) was detected at first dgn, at

relapse and during persisting disease whereas the serum-samples taken at relapse after SCT showed an increased release of 'antitumor' response-related factors (CXCL9 and CXCL10).

Chemokine-release-profiles could contribute to predict the clinical course of the disease

Chemokine-release in serum to predict associations with clinical subgroups

We correlated chemokine concentrations in serum with the **clinical subtypes**: In the group with less than 30% blasts higher CXCL8-levels (p = .02) were observed and tendentially lower CXCL10-levels (p = .05) compared to the group with more than 30% blasts (data not shown).

Moreover, **cut-off values** were defined: In all cases pooled the release of more than 50 pg/ml CXCL8, 150 pg/ml CXCL9, 310 pg/ml CXCL10 and less than 1000 pg/ml CCL2 and 400 pg/ml CCL5 correlated with a lower **age** (<50 years) and **lower blast** proportions (<80%) in the PB (p = .001–0.02, data not shown).

Chemokine-release in serum to predict the response to immunotherapy

In order to correlate chemokine concentrations in serum with the clinical course of the disease, we analyzed chemokine-secretion in serum-samples of **clinical responders** and non-responders to immunotherapy. Immunotherapy in 17 cases consisted in allogeneic SCT for pts, who had relapsed before SCT or second SCT for pts who had relapsed after SCT. We grouped cases according to their response to the initiated therapy in 'responders' (n = 4) and 'non-responders' (n = 2) in the following 2–4 months after immunotherapy that persisted for at least 9 months. In the remaining 11 cases, no response to SCT could be evaluated due to early death, infections or clinical data were not available. For analysis of the response to immunotherapy, a different pts' collective was used than for analysis of the functional potential and occurrence of GVHD. Due to low sample counts no statistically significant results were found. The release of more than 90 pg/ml CXCL8, 90 pg/ml CXCL9, 80 pg/ml CXCL10 and 200 pg/ml CCL2 and less than 1200 pg/ml CCL5 in serum was associated with a higher probability of response to immunotherapy (p = .01–0.2, Figure 3). No cut-off values could be defined for IL-12.

In summary, this means that (significant) and predictive cut-off values could be evaluated for chemokines released in serum in correlation with response to immunotherapy: Higher serum values of CXCL8, −9, −10 and CCL2 and lower values of CCL5 correlated with a higher probability of response to immunotherapy.

Predictivity of chemokine-release in serum on the occurrence of GVHD

Chemokine-release in serum of cases with **GVHD** (n = 9) was compared to cases without GVHD (n = 3) after SCT (Figure 4). Three pts suffered from GVHD I, one from GVHD II, 4 from GVHD III and 2 from GVHD III – IV. No significant differences were found in all GVHD-subtypes with respect to the release of all chemokines (p = .05–0.5, data not shown), but higher CXCL-10-levels (median release 309 vs. 138 pg/ml) in cases without GVHD vs. GVHD (p = .02).

In summary, few differences in chemokine-release in serum of pts with response vs. no response and GVHD-subtypes were seen.

The release of less than 100 pg/ml CXCL8, 200 pg/ml CXCL10, 300 pg/ml CXCL9 and more than 100 pg/ml CCL2 and 300 pg/ml CCL5 in serum was associated with a higher

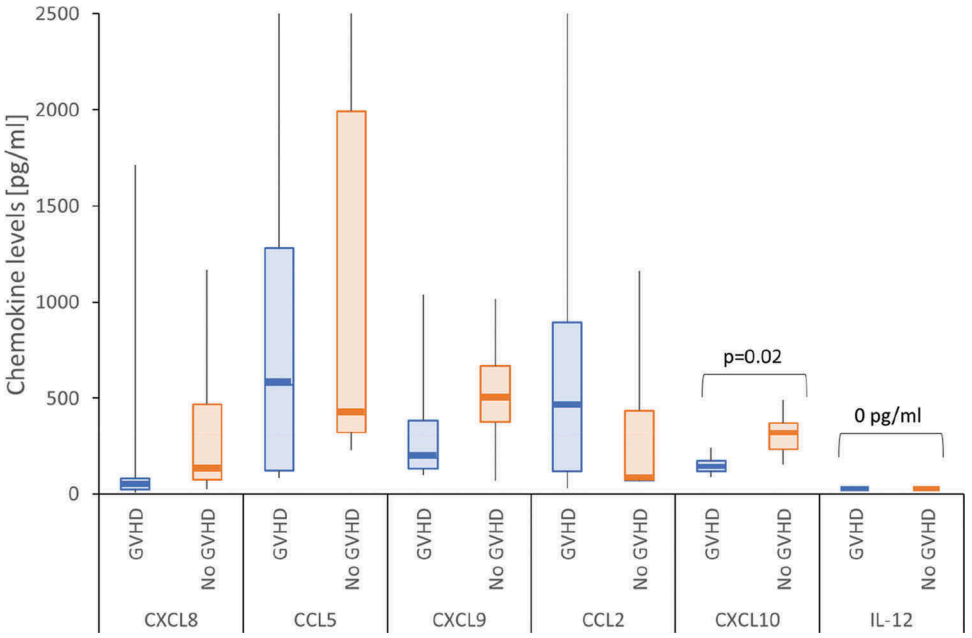


Figure 4. Chemokine-release in serum and occurrence of GVHD.

probability of an occurrence of GVHD. No cut-off values could be defined for IL-12 (Figure 5).

In summary, this means that significant and predictive cut-off values could be evaluated for chemokines released in serum in correlation with occurrence of GVHD: Lower serum values of CXCL8, −9, −10 and higher values of CCL2 and −5 correlated with a higher probability of occurrence of GVHD.

Discussion

AML and MDS: clonal diseases with high risk of relapse

Allogeneic SCT using the 'graft versus leukemia' (GvL) effects mediated by CTL and NK-cells. Therapeutic DLI can be used to restore remissions in case of relapses, although not in all cases (Dickinson et al. 2017; Schmid et al. 2018). There is a need for new (immunotherapeutic) treatment options for AML-patients.

Role of chemokines in tumor immunology and in immunotherapy

In this study, we tried to contribute to the characterization of the partners involved in the mediation of anti-leukemic or leukemia-stimulating reactions in vivo with a focus on the role of soluble factors.

Chemokines play a central role in the recruitment of cells involved in the induction of an immune-response (Brenner et al. 2017; Brenner and Reikvam 2016; Karin and Wildbaum 2015). In cancer, chemokines are secreted by tumor cells, immune cells and stromal cells in the tumor microenvironment (Nagarsheth et al. 2017). They were found to act as both, stimulators of tumor-development by targeting tumor cells and vascular endothelial cells and thus

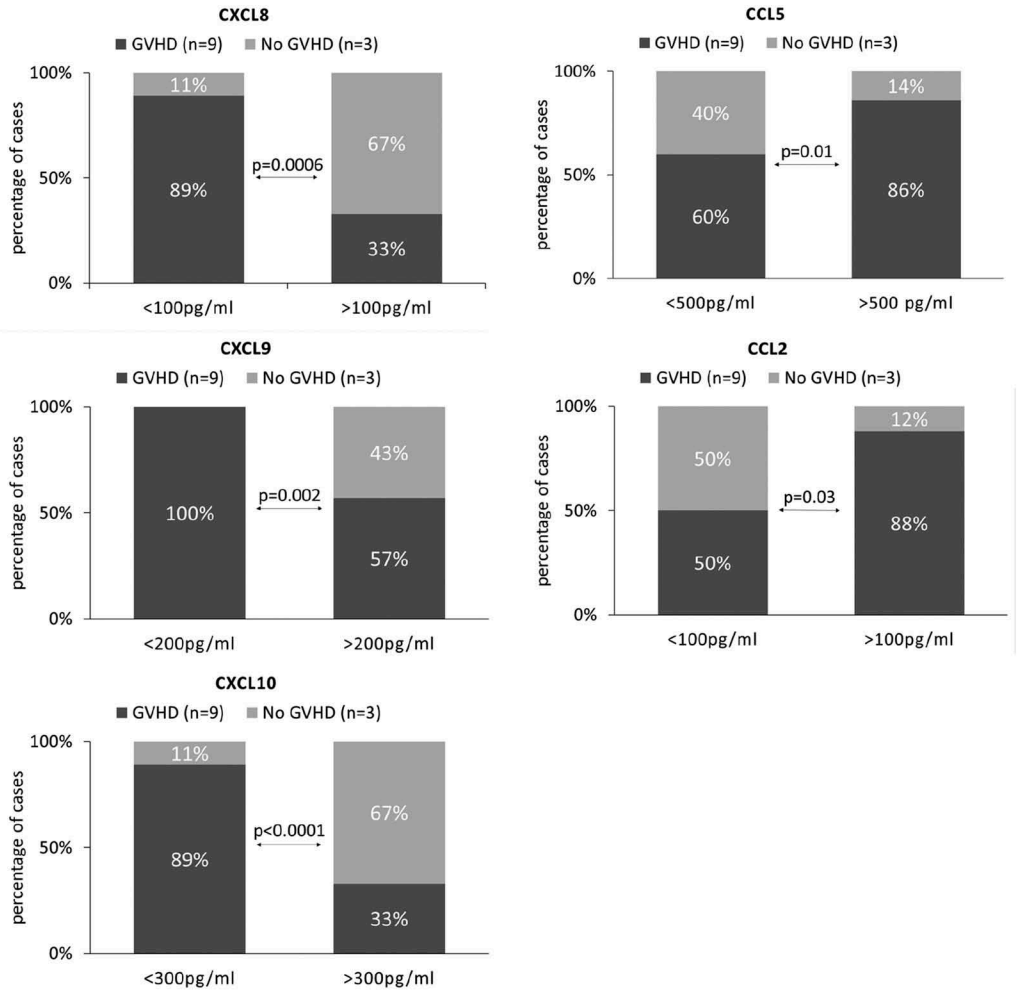


Figure 5. Predictivity of chemokine-release in serum on the occurrence of GVHD.

regulating tumor cell proliferation, cancer invasiveness, and metastasis and antitumor-response by recruitment of regulatory effector T-cells and antitumor-CTL (Chow and Luster 2014; Nagarsheth et al. 2017). Therefore, the presence of chemokines, which determine recruitment of different immune cell subsets is one of the key factors in an efficient antitumor-immune-response (Nagarsheth et al. 2017; Thaiss et al. 2011).

CXCL9 and **CXCL10** are binding to CXC-chemokine receptor 3 on effector $CD8^+$ T cells, T_H1 cells, and NK cells, thereby regulating their recruitment into tumors (Nagarsheth et al. 2017). **CXCL10**, which is an important factor in NK cell-recruitment and NK cell-mediated lysis of tumor cells has been shown to be strongly produced in the tumor compared to the adjacent tissue, suggesting that an immune response is occurring in the tumor (Degos et al. 2019). In various malignancies, **CXCL9** and **CXCL10** were reported to be produced predominantly by activated $CD103^+$ dendritic cells, (tumor-associated) macrophages and tumor cells (Lieber et al. 2018; Spranger et al. 2017). Their release by tumor cells can be impaired by PGE2 or increased by administration of L. monocytogenes-based anticancer vaccines (Bronger et al. 2012; Guirnalda et al. 2013).

CXCL8 is produced by tumor cells and subsets of T_{reg} cells in the human cancer microenvironment, which promotes neutrophil migration into tumors (Kryczek et al. 2016; Waugh and Wilson 2008). Moreover, CXCL8 is targeting vascular endothelial cells and tumor cells and thus supports angiogenesis, tumor progression, and metastasis (De Larco et al. 2004; Lee et al. 2012, Nagarsheth et al. 2017). It has been shown to reflect responses related to tumors or treatment of diverse malignancies (Sanmamed et al. 2014). For example, in pts with renal cell carcinoma or leukemoid reactions, high serum levels of **CXCL8** and also **CCL2** were reported (Åström et al. 2018). In CML **CXCL8** was found to be upregulated upon Bcr–Abl expression and downregulated upon inhibitor treatment suggesting a potential role as a biomarker and in the pathogenesis of the disease (Hantschel et al. 2008).

CCL2 is secreted by tumor cells, B-ALL, AML cells and cells in the tumor stroma like fibroblasts and CCL2 expression correlates with the numbers of tumor-associated macrophages in many tumors (Kitamura et al. 2015; Ma et al. 2019; Nagarsheth et al. 2017; Yoshimura 2018). It is associated with poor patient prognosis in some cancers, including breast cancer, where it is an important factor promoting lung metastasis or glioblastoma or is known to recruit regulatory T cells and myeloid-derived suppressor cells to promote tumor growth (Wu et al. 2018; Chang et al. 2012, 2016).

CCL5 is secreted by tumor cells in various types of cancer and hematological malignancies like Multiple Myeloma (Aldinucci and Colombatti 2014). In mesenchymal glioblastoma, tumor cells express high levels of **CCL5** as an autocrine signal to increase cell survival (Pan et al. 2017). In subtypes of aggressive breast cancer (Triple-negative breast cancer) **CXCL8, CCL2** and **CCL5** act as pro-metastatic factors in the tumor microenvironment (Liubomirski et al. 2019).

Taken together, CCL2, CCL5, and CXCL8 can mediate a crosstalk between tumor- and immune cells and can support antitumor reactions (e.g. attracting antitumor cells) or pro-tumor reactions- (e.g. mediating tumor metastasis or progression).

Depending on the tumor type, chemokines can be favorable or unfavorable prognostic indicators. **CXCL9** and **CXCL10** are associated with a more favorable prognosis in ovarian cancer, but unfavorable in pancreatic and renal cancer and **CXCL9** is also favorable in endometrial and breast cancer (Vilgelm and Richmond 2019).

Among others, **CCL5** and **CXCL10** have already been included in cancer-vaccine strategies in murine models (Kang et al. 2011; Li et al. 2007).

In both, preclinical and clinical trials against various tumor types, local or systemic administration of **IL-12** has been established with varying response and toxic side effects of IL-12 depending on dose and model (Floros and Tarhini 2015; Kilinc et al. 2006; Mahvi et al. 2007; Smyth et al. 2000). In one preclinical trial for a possible future immunotherapy a lentivector was used to induce **IL-12**-expression in AML-blasts in vitro (Huang et al. 2016).

Since overexpression of certain chemokines was observed in many types of cancer and found to be associated with tumor-progression trials to antagonize these chemokines have become popular in the last years. In inflammatory diseases and murine melanoma models neutralizing antibodies for **CXCL8** were already implied with promising results (Huang et al. 2002). In one study a clinical-stage monoclonal antibody that neutralizes **CXCL8** (HuMax-IL8) has been used as a potential therapeutic option for Triple-negative breast cancer where it reverted mesenchymalization in tumor models and significantly decreased the recruitment of polymorphonuclear myeloid-derived suppressor cells at the tumor site (Dominguez et al. 2017).

In mouse models of human liver cancer where **CCL2**-overexpression was detected, blocking of the CCL2-CXCR2-signalling has been shown to suppress tumor growth (Li

et al. 2017). Moreover, in prostate cancer neutralizing antibodies for **CCL2** were found to leed to tumor-regression in vivo (Loberg et al. 2007). One study demonstrated that by suppressing **CCL2**-expression in a leukemic cell line, cell proliferation can be decreased by arresting cells in the G1 phase (Wu et al. 2018).

In a murine trial of myeloma with administration of a **CXCL10**-Ig fusion protein, not only an enhancement of antitumor immunity but also a suppression of tumor growth was described (Barash et al. 2014). In another study, the combination of CXCL10-loaded nanoparticles and DC/tumor cell fusion vaccine against hepatocellular carcinoma (HCC) effectively inhibited tumor cell proliferation in mice (Hu et al. 2017). As **CXCL9**- and **CXCL10**-mediated tumor infiltration by T-cells is suppressed due to DNA-methylation of the encoding genes, treatment with epigenetic modulating drugs can increase chemokine release and subsequently also effector T cell tumor-infiltration (Peng et al. 2015).

Not only chemokines but also their receptors have recently become a target of immunotherapeutic strategies against cancer. In this context inhibition of the chemokine receptor CXCR5 which binds **CCL5** among others has been shown to inhibit tumor-cell-growth in colorectal cancer (Halama et al. 2016).

CXCL9 and **CXCL10** play a critical role in the recruitment of T and NK cells into tumors of various origins and it has been demonstrated that blockade of either them or their receptor CXCR3, impairs recruitment of adoptively transferred T cells into melanoma tumors (Chheda et al. 2016; Mikucki et al. 2015; Stoll et al. 2018).

In murine models, antagonism of the **CXCL9**- and **CXCL10**-receptor CXCR3 was described to stop lung metastasis of breast cancer (Walser et al. 2006). In preclinical and clinical trials, several antagonists of CXCR4 like AMD3100 have already been implied in pts with relapsed or refractory AML as a chemosentisizer or for stem-cell-mobilization with varying results (Duarte et al. 2019; Rashidi and Uy 2015). Furthermore, smoldering type adult T-cell leukemia/lymphoma has been reported to be effectively treated with the anti-CC chemokine receptor 4 monoclonal antibody mogamulizumab (Nishikawa et al. 2019).

In detail, we analyzed the release of CXCL8, CCL5, CXCL9, CCL2, CXCL10 and IL-12, chemokines that are involved in a variety of biological processes with relevance for hematological disorders, by CBA to further characterize their release in leukemic serum-samples and their role in the clinical course of the disease.

Chemokine-release in serum correlates with clinical features

In healthy individuals, chemokines control leukocyte trafficking during homeostasis as well as during inflammation. Homeostatic chemokines regulate migration and activation of progenitor cells during organ development and angiogenesis, they direct immature lymphocytes to maturation sites in lymphoid organs and regulate neovascularization and wound healing, whereas inflammatory chemokines recruit leukocytes in response to certain events (Rosenkilde and Schwartz 2004).

In our study, in general, all chemokines were detected in higher and IL-12 in low amounts in serum samples. As all samples were taken in active stages of the disease it can be concluded that there is an overweight of 'tumor-growth promoting' factors which might function as promoters of leukemogenesis and suppressors of normal hematopoiesis.

Several studies have demonstrated an overexpression of serum-**CXCL8** in pts with newly-diagnosed AML and MDS compared to healthy controls (Hantschel et al. 2008; Kittang et al.

2016; Kornblau et al. 2010). Increased levels of **CXCL10** were also described in untreated AML/MDS as well as in the course of the disease even after the achievement of complete remission (Olsnes et al. 2009). Furthermore, **IL-12**-levels were found to be elevated in newly-diagnosed AML compared to healthy controls, where **IL-12**-release was below detection (Kleiner et al. 2013; Kornblau et al. 2010). In former studies, higher or similar **CCL2**-levels were seen in untreated AML compared to healthy controls, but lower levels in monocytoid AML (Kornblau et al. 2010; Macanas-Pirard et al. 2017; Mazur et al. 2007). By correlating chemokine-release with cytogenetic risk groups only a decreased **CCL2**-expression has been observed in cases with intermediate and unfavorable cytogenetic risk (Kornblau et al. 2010).

It has also been shown that serum-levels of **CCL2** are significantly higher in AML-pts with M0-, M1-, or M2- compared to M4- and M5-subtypes (Mazur et al. 2007). Others have observed a decreased **CCL5**-release in younger AML-/MDS-pts and an increased **CCL5**-release in older pts in comparison to healthy controls (Fredly et al. 2012; Kittang et al. 2016; Kornblau et al. 2010).

Comparing different stages of the disease, no differences in serum-chemokine-levels were described in newly-diagnosed and relapsed pts (Kornblau et al. 2010).

CXCL8-expression differed between subgroups with significantly lower expression in acute promyelocytic leukemia (APL) and highest expression in non-APL-AML patients with FLT3-internal tandem duplication (Kuett et al. 2015). For CXCL9, CXCL10, and IL-12, no association with any AML-subtype was described until now.

Our results confirm previous findings that an increased **CXCL8**-release is found in newly-diagnosed AML. **CCL2**, which is known to be a proangiogenetic factor, was found to be significantly increased released at relapse compared to the first dgn. Moreover, CCL5 was increased in persisting disease compared to the first dgn. This could point to a shift of functionality of CCL2 and −5 chemokines to chemokines promoting tumor-associated macrophages or regulatory T-cells in the progressive/therapy-refractory stages of the disease. No comparison with healthy subjects was performed in our study since the aim was to evaluate and define predictive cut-off values. Higher blast counts have been described to be associated with the release of certain chemokines (Kuett et al. 2015). Nevertheless, our data did not prove any significant differences in blast counts between different stages of the disease in correlation with the amounts of released chemokines. Comparing 'undifferentiated' and 'differentiated' AML-subtypes or allocation to cytogenetic-risk-groups in our study, no significant differences in chemokine levels could be detected, which might be due to a limited number of pts in each group. IL-12 was detected in low levels in most pts independent of stage, cytogenetic risk and FAB-subtype. By defining cut-off values of all cases pooled a higher release of CXCL8, CXCL9, CXCL10 and a lower release of CCL2 and CCL5 correlated with a lower age (<50 years) and lower blast proportions (<80%) in the PB thus pointing to a better prognosis. We could not deduce predictive cut-off values to differentiate cases with more or less favorable FAB-types, cytogenetic risk groups or stages of the disease.

In summary, little differences were detected in chemokine-release and association with pts' diagnostic features.

Chemokine-release is a prognostic marker for the clinical response to immunotherapy and GVHD

Another focus of this report was to evaluate possible correlations between chemokine-release in serum with pts' clinical response to immunotherapy.

Certain chemokines have also been proven to act as possible prognostic indicators. For example, high production of CXCL8 is supposed to be associated with poor prognosis in AML-subsets (Kuett et al. 2015; Schinke et al. 2015). In MDS CXCL10 was identified as an independent prognostic indicator, as increased CXCL10-levels in serum were associated with pts' shorter overall survival (Pardanani and Tefferi 2012).

In previous studies, pts who attained remission were more likely to release higher CCL5-levels and higher levels of CCL5 and lower levels of CXCL8 were associated with longer survival in AML (Kornblau et al. 2010). In a subgroup of pts with FLT3-tandem duplication, high CXCL8-expression was also considered as prognostically unfavorable (Kuett et al. 2015). So far, no association between CCL2-, CXCL9- and IL-12 levels and the clinical outcome and survival of pts were found.

Our data did not show significant differences of serum-chemokine release in 'responders' vs. 'non-responders' to immunotherapy probably due to only a few cases available in each group.

Serum-CXCL9 was shown to be elevated in early chronic GVHD but not in pts with chronic GVHD for more than 3 months (Kitko et al. 2014). Also, CXCL8 as a possible chemoattractant for effector cells to the various target organs of GVHD, has been identified as one of the four potential biomarkers (IL-2Rα, TNFR1, CXCL8, HGF) for GVHD concerning development of GVHD, clinical outcome, response to treatment and mortality (Levine et al. 2012; Paczesny et al. 2009). As chemokines are known as mediators of GVHD, strategies employed neutralizing antibodies against chemokine receptors (anti-CXCR3, anti-CXCR5) to reduce the infiltration of $CD8^+$ T-cells into GVHD target issues (He et al. 2008; Murai et al. 1999).

In pts with skin-only chronic GVHD and also delayed-acute GVHD, the serum levels of CXCL9 and CXCL10 were found significantly elevated compared with SCT-pts who did not develop chronic or delayed-acute GVHD whereas in pts without involvement of the skin, no significant differences in serum concentrations were identified (Croudace et al. 2012). In acute GVHD in contrast, only one of those three chemokines – CXCL10 – was found in increased amounts in serum (Piper et al. 2007).

We did not find significant correlations of chemokine releases with GVHD-subtypes (we only analyzed acute GVHD vs. noGVHD), what might be owed to only few pts in each group. In our pts cohort, we could define cut-off values that enabled a separation in cases with vs. without GVHD. Pts had a 50–100% chance of occurrence of GVHD if less than 100 pg/m CXCL8, 200 pg/ml CXCL9, 300 pg/ml CXCL10 and more than 100 pg/ml CCL2 or 500 pg/ml CCL5 were found in serum.

Despite only few cases available, our clinical data point to a higher probability of response to immunotherapy with higher serum values of CXCL8, −9, −10 and CCL2 and lower values of CCL5 in serum of 'responders' to immunotherapy, thus pointing to induced antileukemic-activity in these patients.

To monitor and explain the involvement of chemokines in response to immunotherapy more accurately further tests in serum taken directly before and in the course of immunotherapy might be needed.

Summary and conclusion

It was shown that chemokines can mediate a crosstalk between tumor- and immune cells and can support antitumor- or pro-tumor reactions in various tumors.

In summary, in our study, no clear associations of chemokine-release-patterns in AML-pts' serum with diagnostic subtypes were detected, although higher release of CXCL8,-9,-10 and a lower release of CCL2 and −5 correlated with more favorable subtypes. Interestingly higher values of CCL2 and −5 were found in persisting disease or relapse compared to first dgn pointing possibly to a change of the 'disease activity' detectable with chemokine levels.

Since human tumors are weakly immunogenic chemokines are also a useful tool to induce and modify tumor microenvironment and to promote antitumor adaptive immune responses. They have already been explored in several studies as vaccine-adjuvants to improve cell-based vaccines (Dell'Agnola and Biragyn 2007; Kumamoto et al. 2002; Schiavo et al. 2006). Correlations of chemokine-levels in serum might therefore help to refine vaccination-strategies.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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