ARTICLE

Blood cytokine concentrations in pediatric patients with anaplastic lymphoma kinase-positive anaplastic large cell lymphoma

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ABSTRACT

Patients with anaplastic lymphoma kinase-positive anaplastic large cell lymphoma often present with B-symptoms or hemophagocytosis and generate an anti-tumor immune response. Specific serum cytokine levels or profiles may reflect the tumor burden, non-specific immune stimulation by the tumor or differences in the strength of the patients' anti-lymphoma immunity. We systematically correlated pre-treatment concentrations of 25 cytokines with clinical and biological characteristics in a well-characterized cohort of 119 uniformly treated pediatric patients with anaplastic large cell lymphoma. Fifteen patients with anaplastic large cell lymphoma in remission and 11 patients with low-stage B-cell lymphoma served as controls. Concentrations of interleukin-9, interleukin-10, interleukin-17a, hepatocyte growth factor, soluble interleukin-2 receptor, and soluble CD30 were significantly higher in initial sera of patients than in the sera of subjects from both control groups, indicating an anaplastic large cell lymphoma-type cytokine signature. The levels of interleukin-6, interferon-γ, interferon γ-induced protein, and soluble interleukin-2 receptor correlated with the stage, initial general condition, minimal disseminated disease, anaplastic lymphoma kinase-antibody titers, and the risk of relapse among patients with anaplastic lymphoma kinase-positive anaplastic large cell lymphoma. Only interleukin-6 showed an independent prognostic value in multivariate analyses. Pre-treatment cytokine profiles in patients with anaplastic large cell lymphoma reflect a tumor signature as well as tumor burden and also differences in the strength of the patients' immune response.

Introduction

Patients with anaplastic lymphoma kinase (ALK)-positive anaplastic large cell lymphoma (ALCL) often present with B symptoms or a macrophage activation syndrome indicating an inflammatory or immune reaction.[1] In addition, ALK-positive ALCL elicits a specific host immune response, as indicated by the production of anti-ALK-autoantibodies,[2] and a cellular immune response against ALK.[3-6] In some aspects, the immune response in ALCL is comparable to that occurring in patients with Hodgkin lymphoma, in whom elevated serum cytokine levels have been described at the time of diagnosis.[7,8]

Elevated levels of serum cytokines as immune mediators, such as interleukin (IL)-6, IL-8, IL-10, IL-17a, and IL-22, have also been shown in small series of ALK-positive ALCL patients.[9,10] *In vitro* production of IL-6, IL-8 and interferon (IFN)-γ by an ALK-positive ALCL cell line HSC-M1 has been reported.[11] Soluble CD30 (sCD30) and the soluble IL-2 receptor (sIL-2R) can be shed from ALCL cells and their levels were

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found to be elevated in seven ALK-positive ALCL patients.[12] sCD30 levels correlated with an inferior survival in a series of adult ALCL patients with unknown ALK-status.[13,14] IL-9 has been described as part of an autocrine signaling pathway including JAK3 with a possible role in the pathogenesis of ALK-positive ALCL.[15] IL-22 expressed in three ALCL cell lines contributes to STAT3 activation and tumorigenicity of ALK-positive ALCL.[16]

While above-mentioned reports highlight ALCL as a cytokine-active lymphoma with hints towards an ALCL-type cytokine signature, the type and pattern of cytokine expression among ALK-positive ALCL patients has not been analyzed systematically. Pretreatment serum cytokine levels may reflect the biological activity of the tumor as well as host immune characteristics. Correlations of initial cytokine concentrations and patterns with patients' characteristics, antibody titers against ALK as a surrogate measure of an autologous immune response against ALK, as well as outcomes might allow the definition of cytokine profiles that are associated with disease activity, tumor burden and the patients' specific immune response.

We, therefore, investigated whether pretreatment cytokine concentrations in patients with ALK-positive ALCL correlate with biological and clinical characteristics and ALK-antibody titers in a uniformly treated, large cohort of 119 children and adolescents with ALK-positive ALCL.

Methods

Eligibility

NPM-ALK-positive ALCL patients treated in the Berlin-Frankfurt-Muenster group study NHL-BFM95 or German patients enrolled in the European intergroup trial ALCL99 between August 1998 and December 2008 were potentially eligible for inclusion in this study after giving written informed consent to participation. Both studies were approved by the institutional ethics committee of the primary investigator of the NHL-BFM study group (AR). Patients with completely resected stage I disease were excluded because their treatment was different.

Patients were included if there were pretreatment serum or plasma samples available and had detectable anti-ALK antibody titer levels. Eligibility was confirmed by demonstration of NPM-ALK positivity of the tumor either by *NPM-ALK* polymerase chain reaction, two-color fluorescence *in situ* hybridization for t(2;5) or nuclear and cytoplasmic staining for ALK.

Patients

The inclusion criteria were fulfilled by 119 patients. Staging procedures included bone marrow aspiration cytology and a spinal tap. Bone marrow involvement was defined by cytologically detectable ALCL cells, irrespective of their number. The patients' treatment consisted of a cytoreductive prephase followed by six chemotherapy courses, as described elsewhere.[17]

As control, serum or plasma samples from 15 of those patients in remission without concurrent infection taken before the start of the sixth chemotherapy course were analyzed.

In addition, serum samples taken at the time of diagnosis from 11 age-matched patients with Burkitt lymphoma from risk groups R1 and R2 (stage I – III, lactate dehydrogenase below 500 U/L) included in the B-NHL BFM 04 study served as a second control group.

Methods and the patients' results regarding ALK-antibody titers and minimal disseminated disease at diagnosis were described and published previously.[18-20]

Measurement of cytokine levels

Blood samples were centrifuged and supernatants were immediately frozen and stored at -80°C until analysis. Samples were assessed for the levels of following soluble mediators: IL-1β, IL-2, sIL-2R, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17a, IL-22, IL-23, tumor necrosis factor-α (TNF-α), IFN-γ, monokine induced by γ-interferon (MIG), interferon γ-induced protein 10 (IP-10), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, granulocyte colony-stimulating factor (G-CSF) and soluble CD30 (sCD30). The measurements were performed using FlowCytomix kits (eBioscience, Frankfurt, Germany) according to the manufacturer's instructions. Samples were processed on a FACS Calibur (BD, Heidelberg, Germany) and data were analyzed using the FlowCytomix Software (version 2.4, eBioscience, Frankfurt, Germany).

Statistical methods

Statistical calculations were performed using the R statistical package (R Foundation for Statistical Computing, Vienna, Austria).

Cytokine levels are reported as median values and were compared between different groups according to diagnosis, clinical and biological characteristics using Mann-Whitney U and Kruskal-Wallis tests. The level of statistical significance was 0.05. Event-free survival was defined as the time from diagnosis to relapse, secondary tumor or death from any cause. Estimates of overall and event-free survival were performed using the Kaplan-Meier method. Differences between groups were compared by log-rank test. A multivariate analysis was performed using the proportional hazards method described by Cox on cytokines showing significant differences in univariate analysis and known risk factors[20] with forward selection keeping only significant variables ($P<0.05$) in the model.

Results

Patients' characteristics

The median age of the 119 ALCL patients at diagnosis was 12.0 years (range, 0.3 – 17.8) and 58% (69 patients) were male. The median follow-up was 6.6 years. The 3-year event-free survival rate of the 119 ALCL patients was 66.4 ± 4.3% and their 3-year overall survival rate was 86.5 ± 3.1%. Detailed clinical data of the patients and controls are shown in *Online Supplementary Table S1*.

Pretreatment cytokine levels in patients with anaplastic lymphoma kinase-positive anaplastic large cell lymphoma

Concentrations of IL-9, IL-10, IL-17a, HGF, sIL-2R, and sCD30 were significantly higher in ALCL patients at the time of diagnosis than in the patients in either control group (patients with B-cell non-Hodgkin lymphoma and ALCL patients in remission) (Figure 1).

The median concentrations of all cytokines are shown in *Online Supplementary Table S2*. In 28 ALCL patients, measurements of sIL-2R were above the upper detection limit of 221 869.99 pg/mL. For the analyses, these samples were attributed a value of 221 869.99 pg/mL. IP-10 could not be measured in one patient.

Correlations between cytokine concentrations are shown in *Online Supplementary Table S3*.

Correlation of cytokine levels with clinical and laboratory characteristics

Correlations between cytokine concentrations and various clinical and laboratory characteristics are summarized in Table 1.

Age and sex

There was no significant difference in the median cytokine concentrations between girls and boys. Concentrations of IL-17a (P=0.018) and MIP-1α (214.4 pg/mL *versus* 106.5 pg/mL, P=0.02) were higher in patients aged 0 – 9 years (41/119) than in patients over 9 years old. Levels of IL-2 (P=0.02) and IL-8 (32.1 pg/mL *versus* 79.3 pg/mL, P=0.038) were higher in patients older than 9 years (78/119).

Stage and organ involvement

Patients with Murphy stage III or IV (88/116) had significantly higher concentrations of IL-6 (P=0.002), IL-10 (P=0.02), IFN-γ (P=0.013), IP-10 (P=0.009), MIG (P=0.001), VEGF (P=0.048), HGF (P=0.017), sCD30 (P<0.001), and sIL-2R (P<0.001) compared to patients with a lower stage (*Online Supplementary Figure S1*).

Bone marrow infiltration, defined as blasts detectable in bone marrow smears, was detected in 16 of the 119 (13.4%) ALCL patients. These patients had significantly higher concentrations of IL-6 (30.1 pg/mL *versus* 0 pg/mL, P=0.011), IL-10 (281.2 pg/mL *versus* 0 pg/mL, P=0.003), IL-13 (7/16 *versus* 18/103 elevated, P=0.013), TNF-α (8/16 *versus* 20/103 elevated, P=0.005), IFN-γ (8.2 pg/mL *versus*

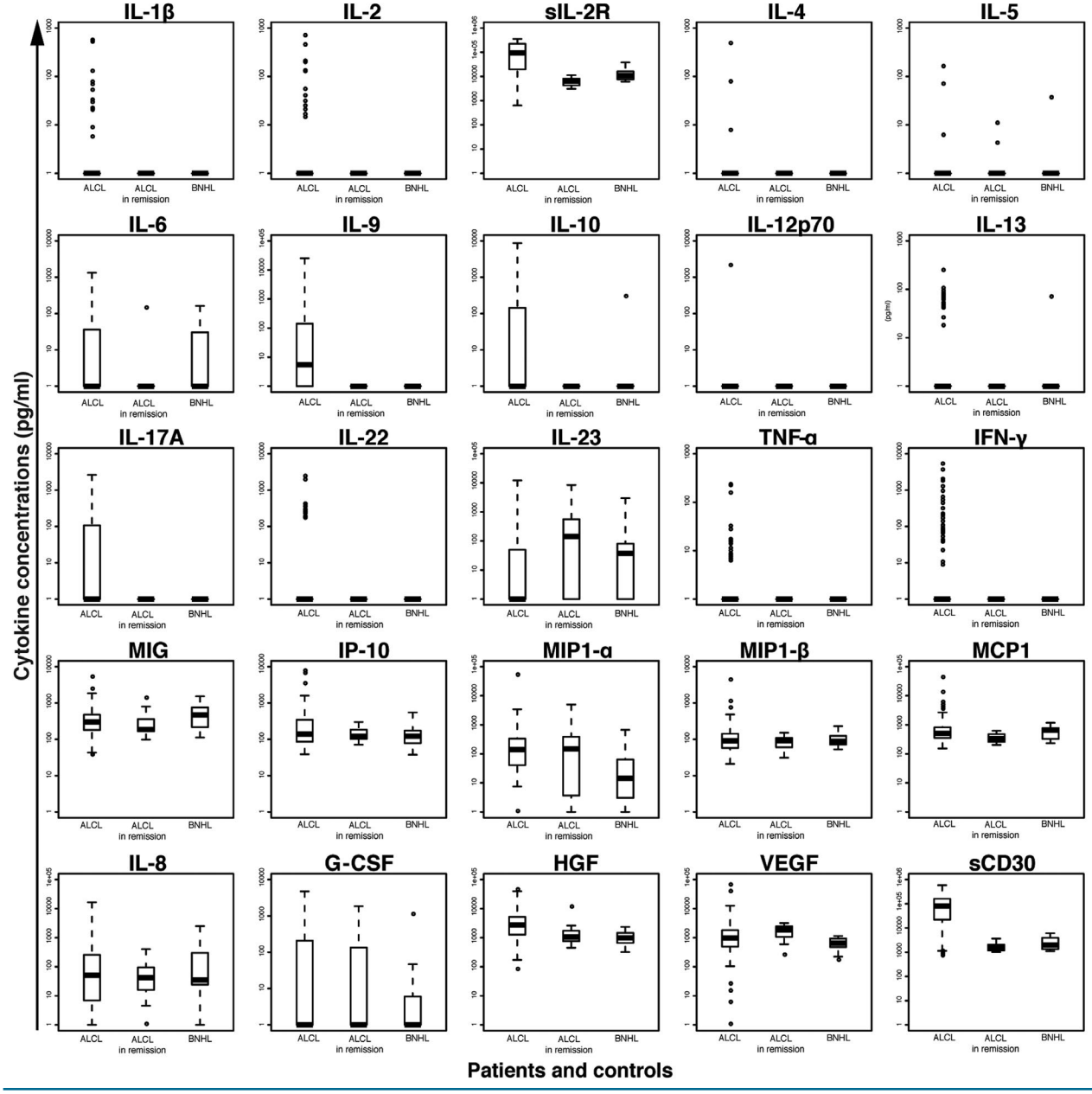

Figure 1. Cytokine levels in patients with anaplastic lymphoma kinase-positive anaplastic large cell lymphoma and controls. Logarithmic representation of cytokine levels in pg/mL for 119 ALCL patients at the time of diagnosis, 15 ALCL patients in remission and 11 patients with B-cell non-Hodgkin lymphoma (B-NHL).

Table 1. Cytokine concentrations and clinical characteristics.

	Sex			Age			General condition				B symptoms			Leukocytosis			LDH			CRP			BM involvement			Stage			Histology		
	Male	Female		≤ 9	> 9		1	2	3		no	yes		≤ 10/nL	> 10/nL		≤ 240	> 240		≤ 4 mg/L	> 4 mg/L		Negative	Positive		I + II	III + IV		n. common	common	
	N = 69	N = 50	*P*	N = 41	N = 78	*P*	N = 81	N = 24	N= 14	*P*	N = 56	N = 63	*P*	N = 72	N =47	*P*	N = 62	N = 57	*P*	N = 55	N = 64	*P*	N= 103	N = 16	*P*	N= 28	N= 88	*P*	N = 37	N= 50	*P*
IL-1	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.0	0.013	0.0	0.0	n.s.	0.0	0.0	0.043	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
IL-2	0.0	0.0	n.s.	0.0	0.0	0.020	0.0	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
sIL-2R	94163.8	91444.0	n.s.	82570.5	102780.3	n.s.	63245.2	121650.4	221870.0	<0.001	28242.1	145441.8	<0.001	95534.4	85983.0	n.s.	30878.7	130878.5	<0.001	25329.4	125883.3	<0.001	82290.2	190649.2	0.01	17857.6	118455.5	<0.001	85557.6	89737.8	n.s.
IL-4	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
IL-5	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
IL-6	0.0	0.0	n.s.	7	0.0	n.s.	0.0	17.1	74.1	<0.001	0.0	14.9	<0.001	0.0	14.9	0.010	0.0	11.0	n.s.	0.0	15.2	<0.001	0.0	30.1	0.011	0.0	13.7	0.002	13.2	0.0	0.047
IL-9	4.5	6.0	n.s.	5.1	2.2	n.s.	0.0	8.0	6.0	n.s.	8.2	4.5	n.s.	2.2	5.1	n.s.	2.2	5.1	n.s.	16.5	2.2	n.s.	4.5	2.6	n.s.	8.2	2.2	n.s.	0.0	32.0	n.s.
IL-10	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	13.2	280.0	<0.001	0.0	37.4	<0.001	0.0	10.3	n.s.	0.0	13.2	n.s.	0.0	6.6	0.044	0.0	236.5	0.003	0.0	6.2	0.020	0.0	0.0	n.s.
IL-12p70	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.0	0.024	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
IL-13	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.013	0.0	0.0	n.s.	0.0	0.0	n.s.
IL-17A	0.0	0.0	n.s.	0.0	0.0	0.018	0.0	0.0	65.9	<0.001	0.0	0.0	n.s.	0.0	69.3	<0.001	0.0	0.0	n.s.	0.0	0.0	0.002	0.0	26.2	0.044	0.0	0.0	n.s.	0.0	0.0	0.018
IL-22	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
IL-23	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
TNF	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	3.7	0.005	0.0	0.0	n.s.	0.0	0.0	n.s.
IFN	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	0.0	0.040	0.0	0.0	0.04	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	8.2	0.002	0.0	0.0	0.013	0.0	0.0	n.s.
MIG	286.1	330.6	n.s.	276.4	336.2	n.s.	276.6	338.1	404.5	n.s.	272.3	342.8	0.046	341.1	276.6	n.s.	230.3	377.2	<0.001	278.8	333.5	n.s.	276.4	441.0	0.006	187.7	344.2	<0.001	296.1	339.3	n.s.
IP10	130.3	139.0	n.s.	137.3	138.9	n.s.	118.3	131.9	314.9	0.025	115.5	187.2	0.008	136.9	153.7	n.s.	117.7	251.7	0.003	118.0	183.8	n.s.	118.3	343.9	0.002	102.9	183.8	0.009	130.3	193.2	n.s.
MIP1-α	164.0	121.2	n.s.	214.4	106.5	0.020	134.5	135.8	150.4	n.s.	179.2	116.7	n.s.	126.1	158.3	n.s.	136.9	148.0	n.s.	139.3	134.4	n.s.	148.0	98.9	n.s.	214.2	125.6	n.s.	201.7	132.8	n.s.
MIP1-β	96.3	83.5	n.s.	81.2	96.7	n.s.	86.3	95.1	104.9	n.s.	90.2	89.2	n.s.	94.8	85.7	n.s.	84.2	96.4	n.s.	98.6	85.4	n.s.	90.7	75.7	n.s.	83.5	93.6	n.s.	96.3	94.1	n.s.
MCP1	514.0	501.4	n.s.	450.9	535.5	n.s.	481.7	557.3	744.8	n.s.	450.1	533.5	n.s.	487.5	530.9	n.s.	501.4	511.8	n.s.	460.9	551.9	n.s.	499.6	548.7	n.s.	367.4	535.5	n.s.	583.7	466.4	n.s.
IL-8	40.0	71.1	n.s.	32.1	79.3	0.038	40.0	68.9	100.3	n.s.	50.9	45.0	n.s.	50.9	41.6	n.s.	38.7	64.9	n.s.	64.8	43.6	n.s.	49.5	49.3	n.s.	45.5	43.6	n.s.	40.6	94.8	n.s.
G-CSF	0.0	0.0	n.s.	6.7	0.0	n.s.	0.0	0.0	0.0	n.s.	38.0	0.0	0.035	0.0	0.0	n.s.	0.0	0.0	n.s.	1.1	0.0	n.s.	0.0	0.0	n.s.	48.1	0.0	n.s.	0.0	0.6	n.s.
HGF	2751.1	2488.7	n.s.	2751.1	2488.7	n.s.	2000.9	3146.6	10545.8	<0.001	2013.3	3086.7	0.002	2184.7	3636.6	0.003	2675.9	2811.8	n.s.	2159.4	3111.9	0.006	2678.2	4789.9	n.s.	1677.0	2955.0	0.017	2685.2	3021.0	n.s.
VEGF	1113.5	887.5	n.s.	904.5	1032.5	n.s.	719.2	1254.1	1639.7	0.006	803.9	1213.6	n.s.	873.1	1278.0	n.s.	816.0	1213.6	n.s.	790.2	1239.0	n.s.	1085.4	732.3	n.s.	691.6	1146.1	0.048	933.2	1031.2	n.s.
sCD30	63697.1	105707.4	n.s.	79788.9	81300.3	n.s.	63367.9	140824.1	75364.2	n.s.	35553.0	121979.7	0.002	81300.3	79788.9	n.s.	53811.2	125200.9	0.008	46643.1	123590.3	<0.001	84067.9	75992.3	n.s.	11771.9	107003.4	<0.001	125200.9	78485.7	n.s.

Median values of cytokine levels in pg/mL according to clinical parameters of 119 ALCL patients at the time of diagnosis. *P* values are given when significant differences were found. General condition was grouped as: 1 – no or slight impairment; 2 – moderate or strong impairment; 3 – bedridden and severely ill. Bone marrow (BM) involvement was considered positive if blasts were seen on smears, irrespective of cell numbers. IP-10 could not be measured in one patient. LDH: lactate dehydrogenase, CRP: C-reactive protein.

0 pg/mL, *P*=0.002), IP-10 (343.9 pg/mL *versus* 118.3 pg/mL, *P*=0.002), MIG (441.0 pg/mL *versus* 276.4 pg/mL, *P*=0.006), and sIL-2R (190649.2 pg/mL *versus* 82290.3 pg/mL, *P*=0.01) compared to patients without bone marrow involvement.

The central nervous system was involved in two of the 116 patients who had a lumbar puncture. Both patients had high concentrations of IL-6 (170.9 pg/mL, 513.5 pg/mL) and IL-17a (2623.3 pg/mL, 1468.3 pg/mL).

Correlations of cytokine concentrations with other organ involvement (skin, mediastinum, liver, lung and spleen) are shown in *Online Supplementary Table S4*.

Patients in a poorer general condition (24/119) at the time of diagnosis (4 or 5 on a subjective scale from 1 to 5) had significantly higher concentrations of IL-1β (*P*=0.013), IL-6 (*P*<0.001), IL-10 (*P*<0.001), IL-12p70 (*P*=0.024), IL-17a (*P*<0.001), HGF (*P*<0.001), sIL-2R (*P*<0.001), VEGF (*P*=0.006), IFN-γ (*P*=0.039), and IP-10 (*P*=0.025) (*Online Supplementary Figure S2*).

B symptoms, leukocytosis, C-reactive protein, and lactate dehydrogenase levels

In comparison to patients without B symptoms, patients with B symptoms (63/119) had significantly higher concentrations of IL-6 (*P*=0.001), IL-10 (*P*<0.001), IL 12p70 (*P*=0.024), IFN-γ (*P*=0.04), MIG (*P*=0.046), HGF (*P*=0.002), sIL-2R (*P*<0.001), sCD30 (*P*=0.002), and IP-10 (*P*=0.008), and lower median concentrations of G-CSF (*P*=0.035). Likewise, patients with CRP values above 4 mg/L (64/119) had significantly higher concentrations of sIL-2R (*P*<0.001), IL-6 (*P*<0.001), IL-10 (*P*=0.044), IL-17a (*P*=0.002), HGF (*P*=0.006), and sCD30 (*P*<0.001) than patients with a lower CRP.

Patients with lactate dehydrogenase values above 240 U/L (57/119) had significantly higher concentrations levels of sIL-2R (*P*<0.001), IP-10 (*P*=0.003), MIG (*P*<0.001), and sCD30 (*P*=0.008). Patients with leukocytosis (white blood cells: >10 x 10^9/L, 47/119) had significantly higher concentrations of IL-1β (*P*=0.043), IL-17a (*P*<0.001), IL-6 (*P*=0.01), and HGF (*P*=0.003).

Histological subtype

Histological subtype was analyzed in 87 ALCL patients. The 37 patients with non-common histological subtype had significantly higher concentrations of IL-6 (13.2 pg/mL *versus* 0 pg/mL, *P*=0.047) and IL-17a (*P*=0.018) as compared to patients with a common histology.

Correlations of cytokine levels with biological characteristics

Minimal disseminated disease

Minimal disseminated disease, defined as a positive polymerase chain reaction for *NPM-ALK* transcripts in bone marrow or peripheral blood, was detected at diagno-

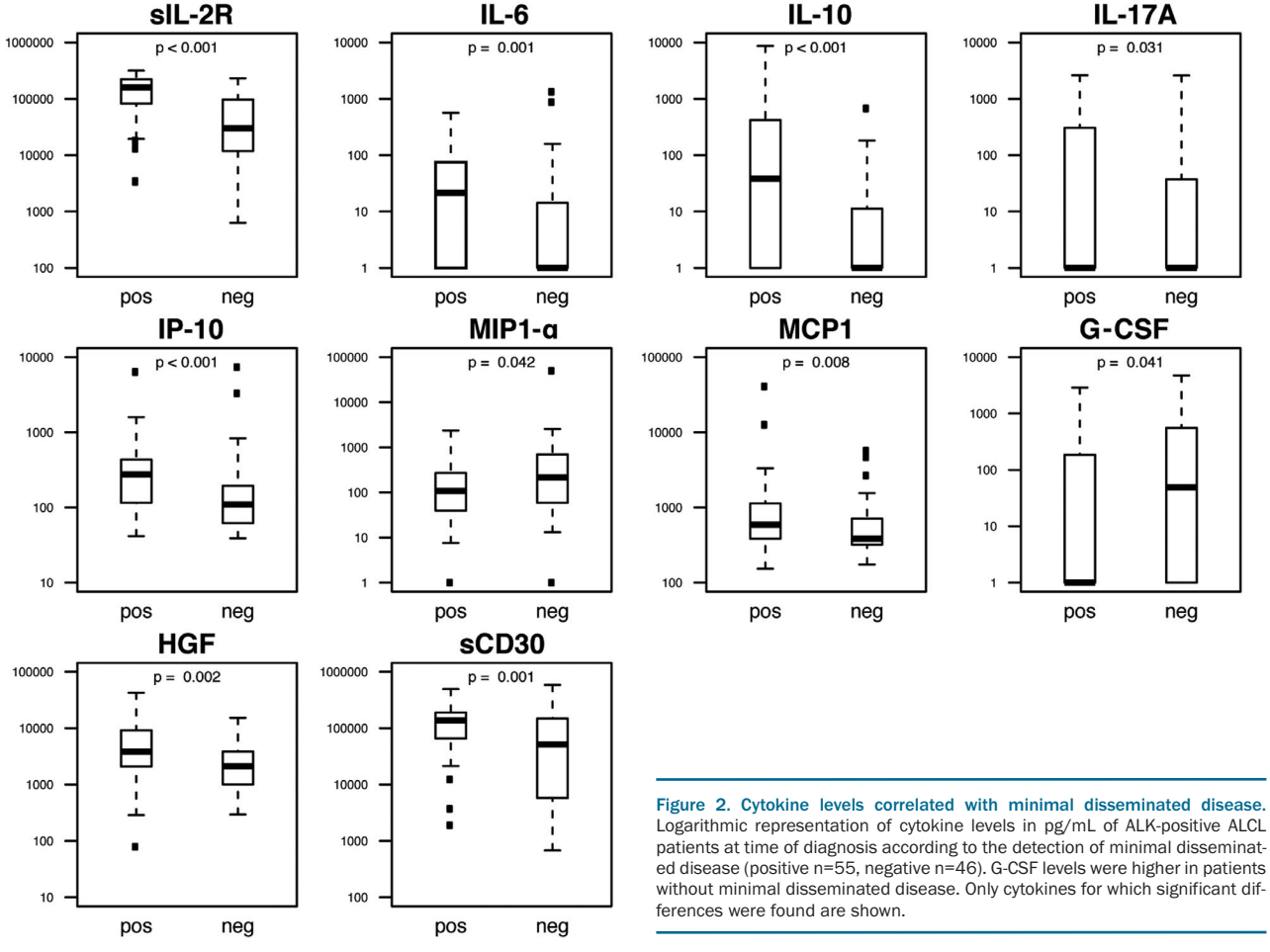

Figure 2. Cytokine levels correlated with minimal disseminated disease. Logarithmic representation of cytokine levels in pg/mL of ALK-positive ALCL patients at time of diagnosis according to the detection of minimal disseminated disease (positive n=55, negative n=46). G-CSF levels were higher in patients without minimal disseminated disease. Only cytokines for which significant differences were found are shown.

sis in 55 of 101 evaluable patients. The presence of minimal disseminated disease, an independent prognostic factor in ALK-positive ALCL, was associated with elevated concentrations of IL-6 (20.7 pg/mL *versus* 0 pg/mL, *P*=0.001), IL-10 (37.4 pg/mL *versus* 0 pg/mL, *P*<0.001), IL-17a (*P*=0.031), MCP-1 (588.8 pg/mL *versus* 383.8 pg/mL, *P*=0.008), HGF (3877.2 pg/mL *versus* 2101.1 pg/mL, *P*=0.002), IP-10 (272.94 pg/mL *versus* 108.3 pg/mL, *P*<0.001), sCD30 (137 449.9 pg/mL *versus* 51 781.1 pg/mL, *P*=0.001), and sIL-2R (159 428.4 pg/mL *versus* 30 087.6 pg/mL, *P*<0.001) (Figure 2). G-CSF (48.1 pg/mL *versus* 0 pg/mL, *P*=0.041) and MIP-1α concentrations (214.2 pg/mL *versus* 106.6 pg/mL, *P*=0.042) were significantly higher in patients without minimal disseminated disease.

Using quantitative polymerase chain reaction, 29 of 90 patients were found to have a normalized copy number >10, where the number of copies of *NPM-ALK* is normalized to 10 000 copies of *ABL*. Concentrations of sIL-2R (*P*<0.001), IL-6 (*P*=0.004), IL-10 (*P*<0.001), IFN-γ (P<0.001), MIG (*P*=0.041) and IP-10 (*P*=0.003) were higher among these patients than among patients with lower normalized copy numbers.

Anti-anaplastic lymphoma kinase-antibody titers

Anti-ALK antibody titers inversely correlate with the risk of relapse and may serve as a surrogate for the strength of the ALK-specific immune response.[19,20] The patients were grouped according to the strength of the antibody titer into those with low (≤1/750), intermediate (>1/750 – <1/60750) and high (≥1/60750) titers. There were 34, 53, and 32 patients in the low, intermediate and high titer groups, respectively.

Patients with a low titer had significantly higher median concentrations of sIL-2R (*P*=0.013), IL-6 (*P*<0.001), IFN-γ (*P*=0.022), and IP-10 (*P*=0.021), but lower concentrations of IL-23 (*P*=0.008) compared to patients with an intermediate or high titer (Figure 3).

Correlation of cytokine levels with outcomes

In univariate analysis, patients with concentrations above the median of IL-6, IL-10, IL-17a, IFN-γ, MCP-1, HGF, IP-10 and sIL-2R had a significantly lower 3-year event-free survival rate compared to patients with levels below the median (Table 2). The greatest difference in event-free survival rates was found between patients with IL-6 concentrations above the detection threshold and patients with no detectable IL-6 [event-free survival: 85.7% (95% confidence interval: 77.5 - 94.8) *versus* 44.6% (95% confidence interval: 33.4 - 59.8), *P*(log-rank)<0.001]. In a stepwise Cox regression analysis, including known risk factors and all cytokines for which findings were significant in the univariate analysis, only IL-6 retained an independent prognostic value with a hazard ratio of 2.9 ± 0.4 (Table 2).

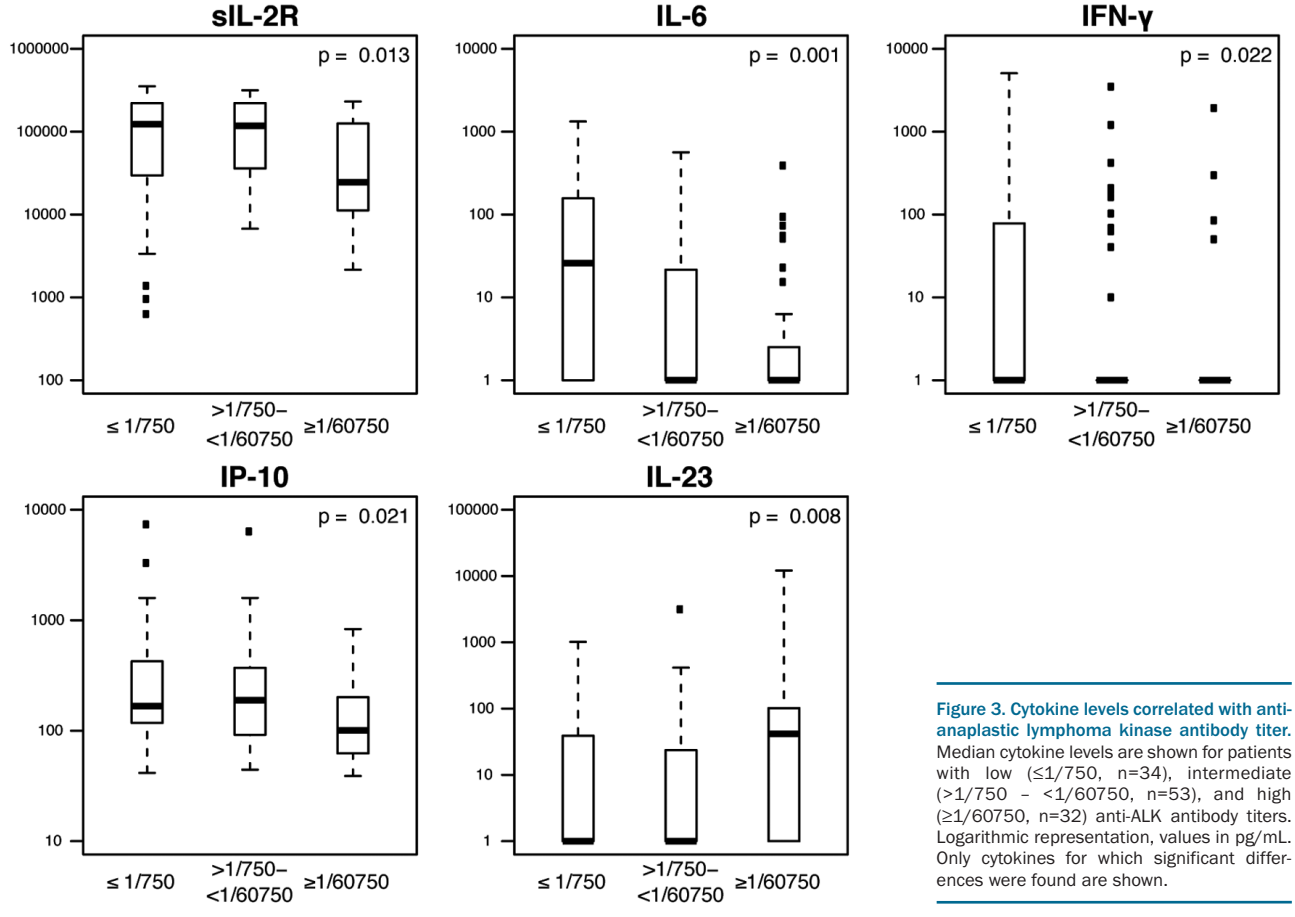

Figure 3. Cytokine levels correlated with anti-anaplastic lymphoma kinase antibody titer. Median cytokine levels are shown for patients with low (≤1/750, n=34), intermediate (>1/750 – <1/60750, n=53), and high (≥1/60750, n=32) anti-ALK antibody titers. Logarithmic representation, values in pg/mL. Only cytokines for which significant differences were found are shown.

Discussion

The aim of this study was to describe pretreatment serum cytokine concentrations and correlate them with clinical and biological characteristics among pediatric patients with NPM-ALK-positive ALCL.

Although blood was collected at 51 different trial sites, serum or plasma was used depending on availability, the time from blood collection to freezing varied by several hours and the storage period differed considerably, the correlations found between cytokines and clinical characteristics such as IL-6 and the patients' general condition (Table 1) are indicative of reliable measurements.

Elevated pretreatment cytokine levels in patients with ALCL compared to either post-treatment samples or other non-Hodgkin-lymphoma patients have been reported in two series of patients. Savan *et al.* found higher levels of IL-22 and IL-8 in nine of 11 untreated ALCL patients compared to post-treatment controls.[9] Mellgren *et al.* recorded higher levels of IL-6, IL-10, MIP-1α, and sIL-2R in six pediatric ALCL patients at time of diagnosis compared to the levels in children with other non-Hodgkin lymphoma.[10]

Our systematic analysis of serum cytokine levels confirmed and extended these findings in a large group of children with untreated NPM-ALK-positive ALCL. IL-9, IL-10, IL-17a, HGF, sIL-2R, and sCD30 levels form a kind

Table 2. Cytokines in univariate and multivariate analyses.

			Univariate		Multivariate	
	Patients	Events	HR	*P*	HR	*P*
Minimal disseminated disease	101		6.0 ± 0.4	<0.001	6.6 ± 0.5	< 0.001
Positive	55	31				
Negative	46	6				
AntiALK antibody titer	119		3.7 ± 0.3	< 0.001	3.6 ± 0.4	< 0.001
≤ 1/750	34	21				
> 1/750	85	21				
Clinical risk (HR)	119		6.5 ± 0.5	< 0.001	4.5 ± 0.5	0.005
Standard risk	42	5				
High risk	77	37				
B symptoms	119		2.3 ± 0.3	0.01		
Positive	63	29				
Negative	56	13				
Histology	87		2.2 ± 0.4	0.038		
Non-common	37	17				
Common	50	13				
sIL-2R	119		2.7 ± 0.3	0.002		
> median	59	28				
≤ median	60	14				
IL-6	119		5.0 ± 0.4	< 0.001	2.9 ± 0.4	0.007
> 0 pg/mL	56	32				
= 0 pg/mL	63	10				
IL-10	119		2.9 ± 0.3	0.001		
> 0 pg/mL	53	27				
= 0 pg/mL	66	15				
IL-17a	119		2.5 ± 0.3	0.004		
> 0 pg/mL	43	22				
= 0 pg/mL	76	20				
IFN-γ	119		3.5 ± 0.3	< 0.001		
> 0 pg/mL	29	18				
= 0 pg/mL	90	24				
IP-10	118		2.3 ± 0.3	0.008		
> median	59	27				
≤ median	59	15				
MCP1	119		2.6 ± 0.3	0.003		
> median	59	29				
≤ median	60	13				
HGF	119		2.5 ± 0.3	0.004		
> median	59	28				
≤ median	60	14				
sCD30	119		2.0 ± 0.3	0.028		
> median	60	27				
≤ median	59	15				

Stepwise regression was used to test whether cytokines have additional prognostic value having taken into account the known risk factors minimal disseminated disease, anti-ALK-antibody titer and clinical risk group.[37] HR, hazard ratio.

of cytokine-signature for ALK-positive ALCL when compared with those of both remission samples and samples from age-matched children with low-stage B-cell non-Hodgkin lymphoma as separate controls.

The concentrations of sIL-2R and sCD30 were expectedly higher in ALCL patients than in controls since ALCL cells, by definition, express CD30 and show strong staining for CD25, the α-subunit of the IL-2 receptor.[12-14,21] Both molecules can be shed by the tumor cells.[12,22] The detection of IL-9 would be in accordance with the previously described autocrine IL-9/JAK3 signaling in ALCL.[15] ALCL cells have been described to resemble a Th17 phenotype and to produce IL-17.[9,23] Cumulatively, these data suggest that these elevated serum cytokines might be produced by the lymphoma.

Within the cohort of patients with ALK-positive ALCL, high levels of IL-6, IFN-γ, IP-10, and sIL-2R correlated with high stage, initial poor general condition, minimal disseminated disease, low ALK-antibody titers, and lower event-free survival at 3 years. The concentrations of sIL-2R and IL-6 correlate with the extent of disease, relapse risk and survival in different tumor types including Hodgkin lymphoma and peripheral T-cell lymphoma.[24-27] The levels of sIL-2R, sCD30 and IL-6 have been described as independent prognostic markers in both Hodgkin lymphoma patients and patients with peripheral T-cell lymphoma.[8,27,28] Several strong independent biological prognostic parameters are available in patients with ALK-positive ALCL.[29] It is not, therefore, unexpected that only IL-6 retained an independent prognostic value for event-free survival in our cohort of ALK-positive ALCL patients in a multivariate analysis including the established risk factors, minimal disseminated disease and anti-ALK antibody titers.[18-20]

sIL-2R was described as a marker of disease activity in a cohort of nine ALK-negative and ALK-positive ALCL patients evaluated at different time points.[12] As for sIL-2R, higher levels of sCD30 were associated with higher stage, presence of minimal disseminated disease and other clinical characteristics in our cohort. These findings support a role of sCD30 and sIL-2R as markers of tumor burden.[25]

The cumulative observations that IL-23 levels correlated directly with the anti-ALK antibody titers in our study and that this cytokine has been shown to be produced by activated dendritic cells,[30] is involved in Th17 effector functions[31] and has a role in autoimmunity[32] might suggest that IL-23 could support the production of autoantibodies.

Elevated concentrations of IL-10 were correlated with minimal disseminated disease positivity, disease stage and significantly lower event-free survival at 3 years in univariate analyses and could hint toward an immune evasion of the tumor. ALK-positive ALCL express PD-L1,[33] involved in suppression of the immune response, and IL-10-secretion in ALK-positive ALCL is induced via STAT3 signaling.[34] Elevated concentrations of IL-10 may reflect immune evasion of the tumor and suppression of cytotoxic T-cell functions.

We also investigated whether a Th-subset-specific serum cytokine pattern could be identified in ALCL patients. Although some patients showed a pattern of elevated IFN-γ, IP-10 and MIG (these latter two both produced upon stimulation with IFN-γ[35]) and levels of IL-17 and IL-23 might hint towards the activation of Th17 cells, the majority of ALCL patients did not show a conclusive pattern. The concept of a certain Th response linked to a disease has been questioned by the discovery of a plethora of newly described subsets and the plasticity of those cell types.[36] In addition, a multitude of host factors, tumor dissemination and individual tumor characteristics could influence the cytokine expression pattern.

In summary, our findings suggest that expression of IL-9, IL-10, IL-17a, HGF, sIL-2R, and sCD30 form a cytokine signature typical of ALK-positive ALCL. The levels of IL-6, IFN-γ, IP-10, and sIL-2R correlated with lymphoma dissemination, other poor prognostic factors and the risk of relapse among pediatric patients with ALK-positive ALCL. Our data underline the role of immune mediators in explaining part of the typical clinical presentation of ALCL patients with B symptoms and further signs of systemic inflammation. IL-6, as a classical cytokine marker of inflammation, was also an independent prognostic parameter. More work is needed to elucidate the role of the cellular immune response to ALK-positive ALCL and to understand the role of mediators in the tumor microenvironment in patients.

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