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CC-Chemokine Ligand 18 Is an Independent Prognostic Marker in Lymph Node-positive Non-small Cell Lung Cancer

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Abstract. *Background/Aim: Tumor-associated macrophages (TAMs) are key players in the immune response in non-small cell lung cancer (NSCLC) and the main producers of CC-chemokine ligand 18 (CCL18). Our study aimed to analyze the clinical significance of CCL18 expression by TAMs in NSCLC. Material and Methods: Tissue multi-arrays from 243 non-selected patients with NSCLC were constructed. Immunohistochemical double staining for CD68 and CCL18 was performed and the number of $CD68^+$, as well as $CCL18^+/CD68^+$ macrophages determined. Results: Comparison of early to advanced lung adenocarcinoma showed significantly more frequent $CD68^+$ as well as CD68/CCL18 double-positive macrophages in advanced disease ($p=0.03$ and $p=0.04$). Multivariate analysis revealed a higher proportion of double-positive macrophages to be an independent prognosticator in lymph node-positive NSCLC (hazard ratio(HR)=0.6, 95% confidence interval(CI)=0.35-0.86, $p=0.009$). Conclusion: In advanced lung adenocarcinoma, infiltration of $CCL18^+$ TAMs was increased and higher expression of CCL18 by TAMs was associated with a favorable prognosis in lymph-node positive NSCLC.*

The significance of the immune system for tumor growth and progression is widely acknowledged and although non-small-cell lung cancer (NSCLC) has traditionally been believed to be non-immunogenic, recent advances in immune-specific approaches have shown encouraging results (1, 2). The tumor microenvironment consists of a variety of immune cells that interact directly and through cytokines and chemokines in a paracrine fashion and thus conduct the local immune response to malignant tumors (2). Common immune cells that are involved in these interactions include tumor-infiltrating lymphocytes, dendritic cells and tumor-associated macrophages (TAMs). TAMs are found in most neoplastic tissues and can make up a large fraction of the tumor mass (3).

TAMs are thought to orchestrate the host's anti-tumoral immune response. Depending on the tumor type, stage of disease and cross-talk between immune and tumor cells, this response can result in pro-tumoral effects by suppressing anticancer immunity and tumor-promoting pro-inflammatory activity, as well as antineoplastic cytotoxicity (4).

Different functional states of TAMs have been described as the M1 and M2 phenotypes. In the tumor microenvironment, mostly TAMs with M2 phenotype are found, which carry out immunosuppressive functions and are involved in the subversion of adaptive immunity by malignant tumors. Each state of polarized macrophages is associated with a distinct pattern of expression of cytokines which are responsible for the observed response (5, 6).

CC-chemokine ligand 18 (CCL18) has been shown to be highly expressed in histopathological tissue sections, as well as in serum and other body fluids, in different tumor types (7-11). In the tumor microenvironment, it is mostly expressed by the M2-macrophages and is involved in the generation of the immune response, contributing to the immune escape of tumor cells by induction of regulatory T-cells (12-14). In order to further assess the local function and prognostic value of TAMs and CCL18 in NSCLC, the expression of cluster of differentiation 68 (CD68) as a marker for TAMs and CCL18 were analyzed by immunochemistry in a well-characterized NSCLC cohort and correlated with clinical and survival data.

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Key Words: CCL18, tumor-associated macrophages, TAMs, non-small-cell lung cancer, NSCLC, tumor microenvironment, immune response, prognostic factors.

Materials and Methods

Patient cohort. A total of 243 non-selected patients with NSCLC who underwent surgery at the Department of Thoracic Surgery, Medical Center – University of Freiburg were included in this study. The patients were treated between 1/1990 and 12/2007 with curative intent. All patients had tumor-free resection margins. Clinicopathological and survival data were collected. The study was approved by the Ethics Committee of the University Medical Center Freiburg (EK 10/12).

Tissue preparation and tissue multi-arrays (TMAs). Gross specimens were formalin fixed (24 to 48 hours in 4% buffered formalin) and paraffin embedded at the Institute of Surgical Pathology, Medical Center – University of Freiburg, using routine procedures. All cases were microscopically reclassified by three experienced lung pathologists (AC, CK, GK) with respect to the current WHO classification (15). This included immunohistochemical analyses as previously described (16). Furthermore, Union for International Cancer Control (UICC) classification of all cases was adjusted to the TNM classification (7th edition) (17). TMAs with a core diameter of 2 mm were constructed. With respect to tumor heterogeneity of all NSCLCs, three cores, whenever possible from three different paraffin blocks for each patient, were used to build the TMA. For comparison with non-tumorous tissue, a TMA with non-neoplastic lung tissue (including alveolar and bronchial tissue) of 29 patients included in the cohort was built.

Immunohistochemistry and scoring. Three-micrometer-thick TMA sections were dewaxed and rehydrated in graded alcohol and subsequently subjected to antigen retrieval with citrate buffer (pH 6.1) for 30 minutes at 95˚C. Incubation for retrieval of CCL18 (monoclonal mouse anti-human MAB394, 1:25; R&D Systems, Minneapolis, MN, USA) was 30 minutes at room temperature. Visualization was performed using DAKO K5005 kit (Agilent, Santa Clara, CA, USA). Afterwards, second primary antibody directed against CD68 (monoclonal mouse anti-human 1PG-M1, 1:2000; DAKO) was applied for 30 minutes at room temperature. DAKO Real Flex kit served for visualization (Figure 1).

Prior to scoring, all slides were digitized using a Panoramic Scan (3D Histech, Budapest, Hungary). Of all TMA cores, the percentage area of vital tumor was assessed and within this, all single $CD68^+$ as well as all $CCL18^+/CD68^+$ macrophages were counted. From these figures, the actual numbers of $CD68^+$ and $CCL18^+/CD68^+$ macrophages per mm^2 within vital tumor tissue were calculated. Mean values of all three available TMA cores for each patient were used for statistical analyses.

Statistics. Statistical analyses were performed using the SPSS software suite 21 (IBM Corp., Armonk, NY, USA). As the distributions of CCL18 and CD68 values were not normal, non-parametric tests (Mann–Whitney *U*-test; Kruskal–Wallis test) were applied to investigate for differences between subgroups. Non-normally distributed data are shown as medians with interquartile range (IQR), normally distributed data as mean with standard deviation (SD). Survival analysis was carried out using Kaplan–Meier plots and log-rank tests as well as Cox regression models for multivariate analysis. Results were considered significant for *p*-values of less than 0.05.

Results

Patient characteristics. A total of 243 patients were included in the study. The mean age at the time of surgery was 64 (±9.6) years. Sixty-nine patients were female and 174 were male. All patients suffered from histologically-proven NSCLC, of which 84 were classified as adenocarcinoma, 115 as squamous cell carcinoma, 18 as large cell carcinoma and 26 as large cell neuroendocrine carcinoma. Clinical and histopathological characteristics are summarized in Table I.

Expression of CD68 and CCL18 in pathological sections of NSCLC. $CD68^+$ and $CCL18^+/CD68^+$ macrophages per mm^2 within vital tumor tissue were determined. Considering all sections, a median of 59 (range=20-95) $CD68^+$ macrophages per mm^2 and 2.6 (range=1-5) $CCL18^+/CD68^+$ macrophages per mm^2 were detected. The median fraction of $CCL18^+/CD68^+$ out of the total $CD68^+$ macrophages was 5.8% (range=2-14%). There were no significant differences in $CD68^+$ or $CD68^+/CCL18^+$ macrophages when comparing the different tumor entities nor by T-and N-stage. Moreover, when comparing the respective expression patterns according to gender, no differences were observed (p=0.873 and p=0.826).

Infiltration of $CD68^+$ cells is associated with poorer differentiation of lung adenocarcinoma. The frequency as well as proportion of $CD68^+$ and $CCL18^+/CD68^+$ macrophages were tested for correlation with different histopathological and clinical parameters. In adenocarcinoma, a higher grading was significantly associated with a higher median density of $CD68^+$ macrophages, especially in well-differentiated [8.7 (range=7-49)/mm^2] as opposed to moderate and poorly differentiated carcinomas [65 (24-98)/mm^2 and 68 (27-113)/mm^2, p=0.04]. No respective differences were seen for $CD68^+/CCL18^+$ macrophages. Furthermore, the proportion of $CCL18^+/CD68^+$ macrophages did not correlate with tumor grading (p=0.57). No correlation between TAM infiltration, CCL18 expression and tumor differentiation was observed for any of the other tumor entities.

$CD68^+$ and $CCL18^+/CD68^+$ cells are more frequent in clinically advanced lung adenocarcinoma. Comparison of the different clinical stages (UICC I-IV) showed significant differences regarding $CD68^+$ (p=0.02), but not $CD68^+/CCL18^+$ cells per mm^2 (p=0.15). Notably, when comparing early (UICC I/II) to advanced (UICC III/IV) lung adenocarcinoma, $CD68^+$ (p=0.03) as well as $CD68^+/CCL18^+$ macrophages were significantly more frequent in advanced disease (p=0.04). However, the proportion of $CCL18^+$ macrophages did not differ in early- *versus* late-stage lung adenocarcinoma (p=0.71). In none of the other examined tumor entities were corresponding differences observed. The data are summarized in Table II.

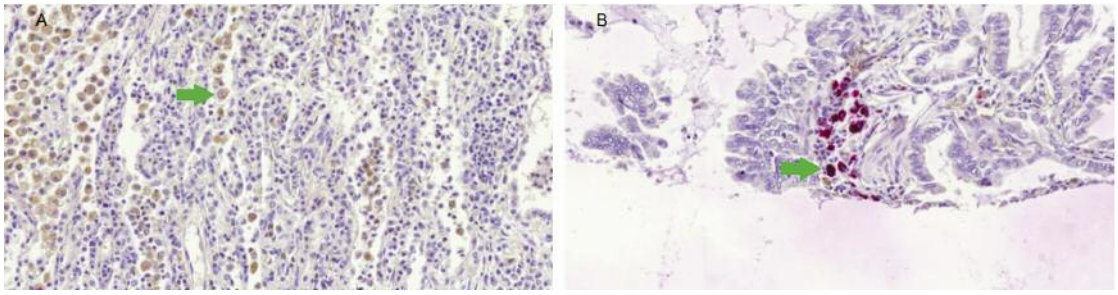

Figure 1. *Immunohistochemistry showing CD68+ cells as brownish (A) and CCL18+ cells as reddish stain (B).*

Table I. *Patient characteristics.*

Total patients	243
Age, years	
Mean±SD	64 (±9.6)
Gender, n (%)	
Female	69 (28%)
Male	174 (72%)
Histology, n (%)	
Adenocarcinoma	84 (35%)
Squamous cell carcinoma	115 (47%)
Large cell carcinoma	18 (7%)
LCNEC	26 (10%)
Clinical stage, n (%)	
I	87 (36%)
II	62 (26%)
III	81 (34%)
IV	9 (4%)
Lymph node status, n (%)	
Positive	102 (43%)
Negative	137 (57%)
Pleural infiltration	
Yes	69 (30%)
No	174 (70%)

LCNEC: Large cell neuroendocrine carcinoma; SD: standard deviation. In the case of missing values, status was either unknown or not attributable.

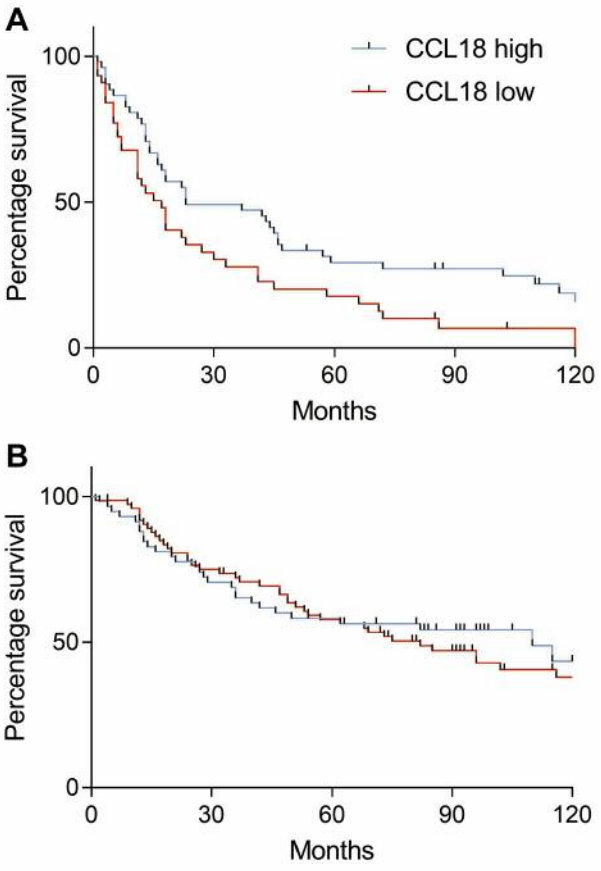

Figure 2 *Survival analysis according to the proportion of CCL18+/CD68+ cells in (A) lymph node-positive and (B) lymph node-negative non-small cell lung cancer (NSCLC). In lymph node-positive disease, a higher CCL18 expression was associated with a significantly better overall survival (p=0.03). Multivariate analysis confirmed CCL18 as an independent prognosticator in NSCLC with lymph node metastasis (hazard ratio=0.6, 95% confidence interval=0.35-0.86, p=0.009).*

The fraction of CCL18 and CD68 double-positive cells in pathologicaI sections is an independent prognosticator in lymph node-positive NSCLC. CCL18 has been shown to be a prognosticator in various tumor types. We compared survival according to high and low expression of $CD68^+$ and $CD68^+/CCL18^+$ macrophages, as well as the proportion of $CCL18^+$ macrophages for the whole cohort.

In patients with tumor-positive lymph nodes, a high fraction of $CCL18^+$ macrophages was linked to a better overall survival (p=0.03) (Figure 2A). In lymph node-negative patients, no such correlation was observed (p=0.62) (Figure 2B).

Multivariate analysis including the variables grading, gender and clinical tumor stage confirmed a high proportion of $CCL18^+$ macrophages to be an independent prognosticator in late-stage NSCLC (hazard ratio=0.6, 95% confidence

Table II. *Frequency of CD68+ and CCL18+ macrophages per mm2 according to clinical tumor stage in lung adenocarcinoma.*

Parameter	UICC stage	Median	IQR	*p*-Value
$CD68^+$ per mm^2	I/II	49.6	14-91	
	III/IV	74.8	29-129	0.03
$CCL18^+/CD68^+$ per mm^2	I/II	2.8	1-5	0.04
	III/IV	4.2	2-10	
$CCL18^+/CD68^+$ relative to total $CD68^+$ cells, %	I/II	6.2	2-16	
	III/IV	7.2	2-6	0.71

UICC: Union for International Cancer Control, IQR: interquartile range.

Table III. *Univariate and multivariate analyses of variables related to overall survival in patients with lymph node-positive disease.*

		Univariate analysis			Multivariate analysis		
Variable	Subgroup	HR	95% CI	*p*-Value	aHR	95% CI	*p*-Value
Sex	Female *vs.* male	0.6	0.38-0.98	0.05	0.5	0.29-0.79	0.004
Grading	Poor *vs.* mod.+ well	1.8	1.2-2.6	0.004	1.7	1.14-2.5	0.008
UICC Stage	Advanced *vs.* early	1.6	1-2.5	0.03	1.8	1.08-2.94	0.02
CCL18 fraction	High *vs.* low	0.6	0.41-0.97	0.03	0.6	0.35-0.86	0.009

HR: Hazard ratio, aHR: adjusted HR, CI: confidence interval, mod.: moderate, UICC: Union for International Cancer Control.

interval=0.35-0.86, p=0.009) (Table III). Interestingly, neither the total number of $CD68^+$ cells nor $CD68^+/CCL18^+$ cells showed predictive value in the studied cohort.

Discussion

Cancer-related immune response is a fundamental element of tumor initiation and progression (2, 18, 19). TAMs are believed to be key players in the tumor microenvironment and to orchestrate the immune response (18). TAMs are predominantly polarized to an M2 state, in which they carry out mostly immunosuppressive functions and are responsible for tissue remodeling and repair (20). They are the main producers of CCL18, which is believed to be involved in the subversion of adaptive immunity and to participate in diverse immune responses in malignant tumors. CCL18 is also known to have chemotactic functions and to participate in the homing process of T-cells and dendritic cells and to differentiate dendritic cells able to prime regulatory T-cells and thus induce immune tolerogenity (12, 13, 21). Furthermore, CCL18 has been shown to promote tumor invasion, migration and epithelial to mesenchymal transition in NSCLC, which are essential hallmarks of metastatic tumor spread (22, 23).

We evaluated TAMs and their expression of CCL18 in a large and well-characterized cohort to further elucidate these processes in NSCLC. As expected, we found a higher density of $CCL18^+$ macrophages in advanced lung adenocarcinoma represented by the clinical stages UICC III and IV. These findings are in line with different previous studies where CCL18 expression in tumor sections was associated with lymphangiogenesis, as well as with lymph node and distant metastasis (23-25). Additionally, analysis of bronchoalveolar lavage in NSCLC revealed an increase of CCL18 concentration to be associated with tumor size and presence of lymph node metastasis (9).

In the present study, survival analysis showed a higher proportion of $CCL18^+$ TAMs to be an independent prognosticator in lymph node-positive NSCLC. Notably, the total number of $CCL18^+/CD68^+$ macrophages was not prognostic for our cohort. In addition, neither CCL18 nor CD68 expression in the tumor sections had any prognostic value in NSCLC without the presence of lymph node metastasis.

Multiple previous studies reported controversial results concerning the prognostic value of CCL18 in different tumor types. On the one hand, in gastric and colorectal cancer, higher CCL18 expression in tissue samples was linked to prolonged patient survival (26, 27). Additionally, in the latter, significance rose with increasing tumor stage, in line with our study. On the other hand, in a study by Pinto and colleagues, colorectal cancer matrices were shown to polarize macrophages towards the anti-inflammatory M2 phenotype and thus to promote cancer cell invasion *via* CCL18 signaling (24). Interestingly,

CCL18 expression in TAMs in those with an advanced tumor stage was higher at the tumor front, where it is thought to promote tumor invasion. In patients suffering from breast cancer, higher CCL18 levels in blood as well as in cancer stroma, were associated with an increased occurrence of metastasis, tumor invasion and reduced patient survival (28).

In a study on lung adenocarcinoma, high serum CCL18 concentrations were associated with a shorter survival time (7). However, it is unlikely that serum CCL18 concentrations are linked directly to CCL18 expression in tumor tissues, as measurement before and after resection of NSCLC showed similar serum CCL18 concentrations and local and systemic CCL18 concentrations in NSCLC need not necessarily be connected and results should not be compared (9).

Furthermore, we found more $CD68^+$ macrophages in poorly differentiated tumors and in clinically advanced tumor stages. Prognostic significance of TAMs has been shown in several other tumor types, including carcinoma, sarcoma, melanoma and lymphoma (29-32). In NSCLC, multiple previous studies have shown contradictory results on the prognostic value of TAM infiltration in tumor sections, as positive, and negative, as well as no prognostic correlation has been reported (33-35). Dai and colleagues suggested that the prognostic value of TAMs in NSCLC depended on the micro-localization represented either by tumor islets or tumor stroma (36). Interestingly, in our study, despite the clear association of TAM infiltration in adenocarcinoma with negative prognosticators such as tumor grading and advanced clinical stage, the number of $CD68^+$ macrophages in histopathological sections did not have any prognostic value. Our current study was not designed to differentiate the micro-localization of the respective cells, but in future studies addressing TAM infiltration as well as CCL18 expression, localization within the tumor should be addressed in order to identify any possible influence on its various functions.

Conclusion

Our study showed an increased infiltration of $CCL18^+$ TAMs in advanced lung adenocarcinoma and identified the proportion of $CCL18^+$ TAMs as being an independent prognosticator in lymph node-positive NSCLC. However, the underlying interactions are complex and not yet fully understood. Nevertheless, CCL18 seems to carry out important functions, especially in lung adenocarcinoma and lymph node-positive NSCLC, and our findings warrant further studies on the underlying mechanisms.

Disclosure

There are no conflicts of interest regarding the material discussed in this article.

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