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Macrophage expression of IL-10 is a prognostic factor in non-small cell lung cancer

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Running head: "IL-10 and non-small cell lung cancer"

Abstract

Interleukin-10 (IL-10) is expressed in many solid tumours and has an ambivalent role in controlling cancer growth and metastases. To determine whether IL-10 is involved in tumour progression and prognosis of non-small-cell lung cancer (NSCLC), we investigated IL-10 expression in tumour cells and tumour associated macrophages (TAMs) and its associations, if any, with the clinicopathological features.

Paraffin-embedded sections of surgical specimens obtained from 50 patients undergoing surgery for non-small cell lung cancer were immunostained with an antibody against IL-10. TAMs and tumour cells positive to IL-10 were subsequently quantified .

IL-10+ve TAMs were higher in patients with stages II, III and IV and in those with lymph node metastases as compared to patients with stage I non-small cell lung cancer. High IL-10 expression by TAMs was a significant independent predictor for advanced tumour stage and, thus, was associated with worse overall patient survival. Conversely, IL-10+ve tumour cells were not different between stages II, III and IV and stage I non-small-cell lung cancer.

In conclusion, IL-10 expression by TAMs, but not by tumour cells, may have a role in the progression and prognosis of non-small cell lung cancer. These results may be useful in developing novel approaches in anticancer treatments.

Keywords: non-small cell lung cancer, interleukin-10, tumour associated macrophages, prognosis, survival, immunohistochemistry.

Introduction

Lung cancer is the leading cause of cancer death among men and women in the United States and throughout the developed world [1, 2]. On average, the five-year survival rate for lung cancer is only about 15 percent, whereas for stage I lung cancer it exceeds 60 percent [3]. Efforts to improve the poor prognosis of patients with NSCLC depend, in part, upon a better understanding of the biology of the cancer. In recent years, several observations have focused on the prognostic factors of NSCLC, particularly on the possible different expression of molecular factors in stage I (early stage) and stages II, III, IV (late stage) disease [4]. This view of investigation may lead to the identification of patients who are most likely to benefit by specific therapeutic strategies.

Interleukin-10 (IL-10) is an immunosuppressive cytokine produced by a number of cells including normal, neoplastic cells and tumour associated macrophages (TAMs), and it has been implicated in controlling tumour growth and metastasis of different human cancers [5, 6]. IL-10 is produced by a variety of tumour cells and its immunomodulatory effects have yielded controversial results on tumour growth and progression. NSCLC patients with IL-10 expression within tumour cells showed a poorer prognosis than those without IL-10 expression [7]. In contrast, in patients with stage I NSCLC, the lack of IL-10 expression was associated with a worse outcome [8].

As noted above, the cytokine IL-10 has also been localized in TAMs. In human glioblastomas, the cells of microglia/macrophage lineage have been identified as the main source of IL-10, the amount of which showed a positive correlation with the degree of cancer malignancy [9, 10]. TAMs have been proposed as a particular phagocyte population that is committed to produce high levels of IL-10, exhibits little cytotoxicity for tumour cells and promotes tumour-cell proliferation [11]. Furthermore, IL-10 may inhibit the tumoricidal

capacity of macrophages by suppressing the production of many pro-inflammatory molecules responsible for cancer cell killing [12].

Although there is emerging evidence that IL-10 might be involved in the growth and progression of a variety of tumors, to date, data on its expression in TAMs of NSCLC patients are lacking.

In order to investigate whether IL-10 is involved in NSCLC, the present study examined the expression of IL-10 in TAMs of patients with stages I-IV NSCLC, and its relationship with both the clinicopathological factors and the overall patient survival. Given the complexity of the mechanisms implicated in the prognosis of NSCLC, it was decided to examine whether IL-10+ve TAMs could be an independent predictive factor for NSCLC progression.

Methods

Subjects characteristics

The study group comprised 50 patients with primary NSCLC. Lung specimens were obtained at surgery (n=47), biopsy (n=1), or at autopsy (n=2). Patients had not received chemotherapy or radiation before surgery. Data collected included age, gender, smoking history, histopathologic diagnosis, grade of tumour differentiation, size of the tumour and for the 47 cases undergoing surgery, pathologic tumour-node-metastasis (p TNM) stage.

Histological diagnosis and grade of differentiation were determined in accordance with the World Health Organization (WHO) criteria for lung cancer [13]. The pathologic tumour stage (p stage) was determined according to the revised TNM classification [14]. Smoking data were obtained from the patient's chart which included: pack-years, age at starting smoking, years of smoking and smoking status at diagnosis. With regards to smoking status, we classified patients into two groups: "non-smokers", i.e., subjects who had never smoked, and "smokers", i.e., ex-smokers and current-smokers.

Immunohistochemistry

Samples were fixed in 4% formaldehyde in phosphate buffer saline (PBS) at pH 7.2 and, after dehydration, embedded in paraffin wax and processed for immunohistochemical analysis of IL-10. Sections (5μm thick) were cut and subsequently hydrated. Endogenous peroxidase activity was blocked by incubating the sections with 3% tris buffer saline (TBS) for 20 min. Immunohistochemistry was performed using a mouse monoclonal antibody against human IL-10 (anti-IL-10; AHC9102; Biosource Int., Camarillo, CA, USA), with the streptavidin-biotin-complex method (StreptABComplex/HRP; K0377; DAKO, Ltd., High Wycombe, UK) and immunoreactivity was visualized with diaminobenzidine (D5637; DAKO). Negative control was performed by omission of the primary antibody.

To determine the phenotype of the cells expressing IL-10, a double immunohistochemistry technique was used. Briefly, sections were incubated with the anti-IL-10 monoclonal antibody followed by biotinylated rabbit anti-mouse immunoglobulins (E0413; DAKO Ltd., High Wycombe UK) and steptABComplex/AP (K0391; DAKO). Slides were incubated with the monoclonal anti-human CD68 antibody (M0814; DAKO), followed by the anti-mouse EnVision+™ peroxidase conjugated (K4000; DAKO). Sections were developed sequentially with diaminobenzidine (a peroxidase substrate) (D5637; DAKO), and fast-red (an alkaline-phospathase substrate) (K0699; DAKO). IL-10+ve cells stained red, CD68+ve cells stained brown and double immunostained cells stained reddish brown. As a negative control procedure, the analyses were repeated omitting one or both of the primary antibodies.

Quantification

Because IL-10 positive immunostaining was located in tumour cells and in tumour macrophages mainly located in the advancing tumour margin, we quantified IL-10 expression in these two compartments. The advancing tumour margin was defined as the transition zone between the periphery of the tumour and normal lung tissue [15]. Slides were coded and microscopic analysis was carried out blindy to the clinical data. The expression of IL-10 in TAMs was quantified using an Olympus BX41 microscope (Olympus Optical Co., Hamburg, Germany) at a magnification of 600x. At least 20 high-power fields (hpf) of tumour margin were randomly selected for each section and a number of $\geq$ 100 macrophages were evaluated. Results were expressed as the number of macrophages per hpf and as the percentage of IL-10+ve macrophages.

The expression of IL-10 in tumour cells was quantified at a magnification of 400x using an Olympus BX41 microscope connected to a video recorder linked to a computerised image analysis system (Software: Image-Proplus; Media Cybernetics, Inc., Silver Spring, MD, USA). For each slide, we randomly selected 40 microscopic fields mostly occupied by tumour cells.

In each microscopic field, the tumour cell area was manually delineated excluding tumour stroma and necrosis area. Positive tumour cell area was automatically evaluated by the image analysis system, calibrated to select the tissue area immunostained above a threshold considered as non-specific background. Results were expressed as percentage of IL-10+ve tumour cell area/total tumour cell area.

Statistical Analysis

The percentages of IL-10+ve TAMs and tumour cell area were expressed as median with interquartile ranges shown in parentheses. At least three replicate measurements of immunostained slides were performed by the same observer in 10 randomly selected slides to assess the intra-observer reproducibility [16]. The intraclass correlation coefficient was 0.99.

The pathological stages were grouped into early stage (stage I) and late stages (II, III, IV). Differences between groups for IL-10 expression in both TAMs and tumour cells were analyzed using the Mann-Whitney U-test. Spearman's rank correlation test was used to examine the association between IL-10 expression and tumour characteristics. Multivariate logistic regression was performed to examine the relationships between the pathological stage (being early and late stage the dependent variable) and covariates (age, sex, pack-years, tumour histology and IL-10 expression in TAMs). For this analysis, the median value of IL-10+ve TAMs (16.3 %) was chosen as the cut-off to divide patients into two groups.

The effects of IL-10+ve TAMs and tumour cell area on overall survival were analysed. We made an a priori decision to classify IL-10+ve TAMs values as high or low, using the sample median for the analysis of survival. Survival curves were estimated using the Kaplan-Mayer method and were calculated from the date of surgery. The Log-rank test was used to compare patient's survival time between groups. Cox proportional hazards model was used for univariate analysis to evaluate the association between survival time and risk factors and for multivariate analysis to model the risk of IL-10 expression in TAMs on survival time, with

adjustment for clinical and histopathological parameters (age, sex, pack-years, tumour histology and the pathologic tumour-node-metastasis stage).

All statistical analyses were carried out using the Stat View software (version 5.0.1; Abacus Concepts, Berkely, CA). Probability values of $p \leq 0.05$ were accepted as significant.

Results

Table 1 shows the characteristics of the subjects examined. Patients (43 males and 7 females) had a mean age of 63.9 ± 1.3 years. Forty-one patients (82%) had a smoking history, and 9 (18%) were non-smokers. Adenocarcinoma was the most common lung tumour type (68%) followed by squamous cell carcinoma (SCC) (32%). Twenty-four patients (51%) were classified as stage I (early stage) disease, and the remaining twenty-three (49%) as stages II, III or IV (late stage) disease.

IL-10 positive immunostaining was observed in tumour cells and in macrophages located in the advancing tumour margin. Double labelling confirmed that 94.7% (median value) of the IL-10+ve cells in the tumour margin were macrophages (CD68+ve) (Figure 1). Occasional CD68+ve cells co-expressing IL-10 were detected within tumour stroma and cells [median (interquartile range): 1.9 (0.75-4.10) % and 0 (0-2.45) %, respectively] only of some subjects. In order to evaluate data as homogenous as possible, these cells were excluded from further analysis.

The percentage of IL-10+ve TAMs was higher in tumour specimens of patients with stages II, III and IV as compared to those with stage I (Table 2, Figure 2). Interestingly, this difference was more pronounced in subjects with the SCC subtype than in those with the adenocarcinoma one. As expected, IL-10+ve TAMs were also increased in patients with lymph node metastases [47.6 (14.7-74.4) % *versus* 10.9 (2.1-34.8) % in N_1-N_2-N_3 *versus* N_0, $p = 0.0084$]. In contrast, the total number of macrophages per hpf was not different between early and late stages of the tumour (Table 2).

When multivariate logistic regression analysis was performed, patients with higher percentages of IL-10+ve TAMs had an increased risk for late stage disease (Table 3).

The percentage of IL-10+ve tumour cells was not different between stages II, III and IV and stage I NSCLC (Table 2). No correlations were observed with either tumour histological type or tumour differentiation.

IL-10 expression in TAMs, but not in tumour cells, was significantly associated with the overall patient survival. Patients with higher percentages of IL-10+ve TAMs had a shorter survival time than patients with lower percentages of IL-10+ve TAMs ($p = 0.014$ Log-rank test) (Figure 3). However, TAM IL-10 expression did not prove an independent prognostic factor for survival, when disease stage was considered as the stratification variable in the Cox multivariate regression analysis.

Finally, IL-10 expression in TAMs and in tumour cells was increased in smokers as compared with non-smokers [33.6 (7.9-67.1) % *versus* 11.4 (0.3-17.4) %, $p = 0.040$ and 5.7 (1.2-22.4) % *versus* 0.7 (0.02-6.6) %, $p = 0.021$, respectively]. The total number of macrophages per hpf as well as the total tumour cell area were not different between smokers and non-smokers. The percentage of IL-10+ve tumour cells showed a positive correlation with years of smoking ($r = 0.33$, $p = 0.034$) and a negative correlation with the age at which patients started to smoke ($r = -0.55$, $p = 0.0005$).

Discussion

The main finding of this study is the role of TAMs expressing IL-10 in the prognosis of NSCLC patients. The experiments presented here indicate that the expression of IL-10 is increased in TAMs of patients with stages II, III, IV as compared to those with stage I NSCLC. In addition, as expected, IL-10+ve TAMs were higher in patients with lymph node metastases compared to those without lymph node metastases. Furthermore, higher IL-10 expression by TAMs was associated with shorter overall patient survival.

We assessed the expression of IL-10 in macrophages located at the advancing tumour margin, because IL-10+ve TAMs within cancer stroma and cells were occasional and not detected in all the studied subjects. Besides that, the structural features of the advancing tumour margin have been observed to have a critical significance for prognosis in various cancers (17-19).

To best of the current authors' knowledge, this is the first study demonstrating that, in NSLC, TAMs express IL-10 and that its expression correlates with both disease progression and prognosis. Previous studies have shown that, in human glioblastoma, macrophages were identified as the major source of IL-10, which was correlated with the extent of malignancy [9, 10]. High amounts of IL-10 have been measured in macrophages from human ovarian carcinoma and mouse tumours and it has been hypothesized that delegating macrophages to produce high levels of IL-10 may be a common mechanism used by tumours to interfere with immune response and promote tumour survival [20]. The mechanism behind the pro-tumour role of TAMs expressing IL-10 might include inhibition of tumour cytotoxicity exerted by human monocytes and alveolar macrophages through the inhibition of the production of many, if not all, pro-inflammatory cytotoxic molecules responsible for tumour cell killing [12, 21, 22]. Recently, one study has expanded this view [11]. TAMs could represent a particular

mononuclear phagocyte population able to tune inflammatory responses and adaptive Th1 immunity, to promote angiogenesis, tissue remodelling and repair and therefore to favour tumour progression. This phagocyte population is characterized by the production of high IL-10 levels. Our finding of a higher expression of IL-10 in TAMs of late stage NSCLC further supports this hypothesis.

The observation that the percentage of IL-10+ve TAMs was higher in patients with lymph node metastases than in those without, was largely expected, since stage I is not associated to lymph node involvement. Nevertheless, this result highlights the findings of a previous study on human gastric carcinoma, where IL-10 mRNA was frequently expressed in tumour tissues of patients with high degree of stage or lymph node metastases [23]. Indeed, the local expression of the immunosuppressive cytokine IL-10 may contribute to the progression of the tumour, likely, through immunosuppression.

Although a different patient survival rate has been shown in lung tumours with high TAMs density as compared to those with low TAMs density [24], data on the prognostic value of IL-10 in TAMs are lacking. Compelling evidence indicates that, in animal models, the lack of IL-10 expression in tumour macrophages was associated with prolonged survival and increased frequency of tumour rejection [25, 26]. In the present study, we have shown that a higher percentage of IL-10+ve TAMs is associated with worse overall survival in humans. This result was easily predictable, since IL-10 staining in TAMs was positively associated to the stage of the disease, and survival is directly connected to the stage of the disease. Indeed, in the multivariate analysis, the percentage of IL-10+ve TAMs proved an independent prognostic factor for the disease stage, but not for survival.

At variance with TAMs expressing IL-10, IL-10+ve tumour cells did not differ between patients with late and early stages NSCLC and did not correlate either with clinicopathological factors or with the overall patient survival, suggesting that IL-10

expression in this compartment is not involved in the progression of the disease. These findings are in contrast with a previous report by Hatanaka et al. [7], who suggested that IL-10 expression by the tumour is an indicator of poor prognosis in NSCLC patients. We have analyzed IL-10 expression at the protein level as compared with the mRNA level for Hatanaka et al. The difference and the tendency as compared to their strong correlations are likely due to the fact that mRNA measurements are global on tissues including tumour cells, stromal cells and TAMs. Indeed, by performing an immunohistochemical analysis, we were able to identify TAMs as the cells which contribute to the expression that correlates with higher tumour stage, progression and survival. Other authors have shown that the lack of IL-10 protein expression by the tumour was associated with a worse survival rate in patients with stage I NSCLC [8]. These conflicting results imply that the role of IL-10 expression by tumour cells in the progression and prognosis of lung cancer is still controversial and deserves further studies.

With regards to the smoking status of the patients, the expression of IL-10 was increased both in TAMs and in tumour cells of smokers as compared with non-smokers. The study by Lim S. et al. has shown a positive influence of cigarette smoke on IL-10 production by alveolar macrophages obtained by bronchoalveolar lavage [27]. We confirm these results demonstrating that cigarette smoke increased the production of IL-10 by tumour macrophages. Among smokers, the expression of IL-10 in tumour cells correlated with both the years of smoking and the age at which patients started to smoke. In this context, one study has shown that normal bronchial epithelium of both smokers and non-smokers constitutively expresses IL-10 [28]. Others reported that primary human bronchial epithelial cells of light or non-smokers do not express IL-10 [29]. Indeed, these findings need to be interpreted with caution because, using microarray analysis, it has been recently demonstrated that many genes are differently expressed in lung adenocarcinoma of smokers as compared to non-smokers

[30]. Finally, our result of the more prominent difference of the percentage of IL-10+ve TAMs between early and late stages in patients with the SCC subtype may also be explained by tobacco use, as SCC is the histological subtype more related to tobacco use.

The possibility that tumour cells with different stages can affect IL-10 expression by TAMs could be a potential bias of the current study. Our data do not allow to exclude a role for tumour stage in the expression of IL-10 by TAMs. Despite this limitation, and even if we are well aware that correlations do not imply a cause-effect relationship, the significant correlations between the increased number of IL-10+ve TAMs and both the late stages and the worse overall survival, indicate that TAMs expressing this cytokine are likely involved in the progression of the disease.

In conclusion, we have shown that IL-10 expression in TAMs correlates with the disease progression and prognosis in patients with NSCLC. These findings may be useful in the understanding the mechanisms involved in NSCLC and hopefully, in developing alternative therapeutic regimens for lung cancer patients.

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Conflict of interest statement

None declared any financial or personal relationships with other people or organizations that could inappropriately influence his/her work.

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Table 1: Demographic and clinical data of the study population[*]

Clinical parameters	
Sex (male/female)	43/7
Age (yrs)	63.9 ± 1.3
Smoking history	
- smoking status (n. of subjects)	
smokers	41
non-smokers	9
- pack-years	44.1 ± 3.6
- years smoked	41 ± 1.5
- starting age	19.3 ± 1.2
Histology (n. of subjects)	
- adenocarcinoma	34
- squamous cell carcinoma	16
TNM stage[‡] (n. of subjects)	
- I	24
- II, III, IV	23
Limph node metastases[‡] (n. of subjects)	
- N_0	23
- N_1, N_2, N_3	24
Tumour grade (cell differentiation)[†] (n. of subjects)	
- G_1, G_2	29
- G_3	20

[*]Values are expressed as mean ± SEM or as number of subjects.

[‡]Data were available for 47 patients. [†]Tumour grade is missing for one patient.

Table 2: Interleukin-10 (IL-10) expression in tumour associated macrophages (TAMs) and in tumour cells of patients with early and late stage NSCLC

Compartments	Late stage NSCLC (n=23)	Early stage NSCLC (n=24)
IL-10+ve TAMs (%)	50.9(16.1-73.4) *	9.6(1.4-29.3)
TAMs/hpf (n)	10.9(7.4-15.2)	10.3(5.8-12.2)
IL-10+ve tumour cell area (%)	6.5(1.7-21.8)	3.8(0.9-13.6)
Total tumour cell area (μm^2)	56(48-59) x 10^3	58(48-61) x 10^3

Definition of abbreviation: hpf = high-power field.

Data are expressed as median (interquartile ranges: 25^{th}-75^{th} percentile). *p=0.0014.

Table 3: Logistic regression analysis of the association between tumour stage groups and clinicopathological features in 47 patients

Variables*	Adjusted Regression coefficient	Standard error	X^2 Value	P	OR[a]	95% CI[b]
Sex	-0.517	1.219	0.180	0.6714	0.596	0.055-6.508
Age	0.024	0.040	0.368	0.5440	1.024	0.947-1.108
Pack-years	-0.001	0.015	0.002	0.9607	0.999	0.971-1.029
Histology	0.279	0.739	0.142	0.7059	1.322	0.311-5.622
IL-10+ve TAMs	1.936	0.682	8.044	0.0046	6.928	1.818-26.398

*The dependent variable is early stage or late stage group; the independent variables included: sex: 0 = female and 1 = male; age: continuous variable in years; pack-years: continuous variable in numbers; histology: 0 = adenocarcinoma and 1 = squamous cell carcinoma; IL-10 +ve TAMs: 0 = low (< 16.3%) and 1 = high (≥16.3%).

[a]OR, odds ratio, [b]CI, confidence interval.

Figure legends:

Figure 1: Double immunostaining showing IL-10/CD68+ve cells in alveolar spaces of the advancing tumour margin. IL-10+ve cells stained red, CD68+ve cells stained brown. Arrows indicate examples of double immunostained cells stained in reddish brown. Scale bar indicates 20 μm.

Figure 1

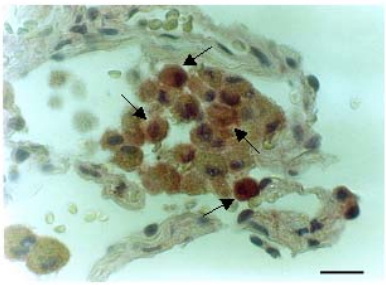


Figure 2: Microphotographs showing examples of IL-10+ve TAMs (arrows) of patients with: **A**) stage I NSCLC and **B**) stage III NSCLC. Immunostaining was performed with a mouse monoclonal antibody specific for human IL-10. Scale bar indicates 20 μm.

Figure 2

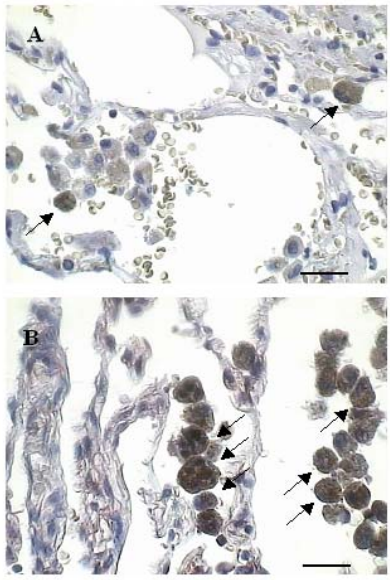
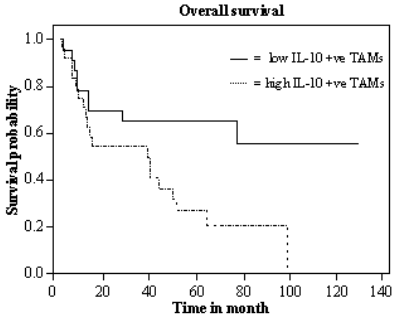


Figure 3: Survival curve of patients with high IL-10+ve TAMs and low IL-10+ve TAMs. The median value of IL-10+ve TAMs (16.3) was chosen as the cut-off to divide the patients into the two groups. The patients with high IL-10 expression (broken line, n = 24) had a worse overall survival than the patients with low IL-10 expression (solid line, n = 23).

Figure 3


Overall survival
= low IL-10 +ve TAMs
= high IL-10 +ve TAMs
Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
Time in month