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Macrophage expression of interleukin-10 is a prognostic factor in nonsmall cell lung cancer

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ABSTRACT: Interleukin (IL)-10 is expressed in many solid tumours and plays an ambiguous role in controlling cancer growth and metastasis. In order to determine whether IL-10 is involved in tumour progression and prognosis in nonsmall cell lung cancer (NSCLC), IL-10 expression in tumour cells and tumour-associated macrophages (TAMs) and its associations, if any, with clinicopathological features were investigated.

Paraffin-embedded sections of surgical specimens obtained from 50 patients who had undergone surgery for NSCLC were immunostained with an antibody directed against IL-10. TAMs and tumour cells positive for IL-10 were subsequently quantified.

IL-10-positive TAM percentage was higher in patients with stage II, III and IV NSCLC, and in those with lymph node metastases compared with patients with stage I NSCLC. High IL-10 expression by TAMs was a significant independent predictor of advanced tumour stage, and thus was associated with worse overall survival. Conversely, IL-10 expression by tumour cells did not differ between stages II, III and IV and stage I NSCLC.

In conclusion, interleukin-10 expression by tumour-associated macrophages, but not by tumour cells, may play a role in the progression and prognosis of nonsmall cell lung cancer. These results may be useful in the development of novel approaches for anticancer treatments.

KEYWORDS: Immunohistochemistry, interleukin-10, nonsmall cell lung cancer, prognosis, survival, tumour-associated macrophages

Lung cancer is the leading cause of cancer deaths among males and females in the USA and throughout the developed world [1, 2]. On average, the 5-yr survival rate for lung cancer is only ~15%, with a rate >60% for stage I lung cancer [3]. Efforts to improve the poor prognosis of patients with nonsmall cell lung cancer (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, especially the possible different expression of molecular factors in stage I (early stage) and stages II, III and IV (late stage) of the disease [4]. This line of investigation may lead to the identification of patients who are most likely to benefit from specific therapeutic strategies.

Interleukin (IL)-10 is an immunosuppressive cytokine produced by a number of cells, including normal neoplastic cells and tumour-associated macrophages (TAMs), and has been implicated in the control of tumour growth and the 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 as regards tumour growth and progression. NSCLC patients with IL-10 expression within their tumour cells showed a poorer prognosis than those without IL-10 expression [7]. In contrast, the lack of IL-10 expression in patients with stage I NSCLC was associated with a worse outcome [8].

As aforementioned, the cytokine IL-10 has also been localised in TAMs. In human glioblastomas, the cells of microglia/macrophage lineage have been identified as the main source of IL-10, the level of which shows a positive correlation with the degree of cancer malignancy [9, 10]. TAMs have been proposed as a particular phagocyte population that are committed to produce high levels of IL-10, exhibit little cytotoxicity for

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tumour cells and promote tumour cell proliferation [11]. Furthermore, IL-10 may inhibit the tumouricidal 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 tumours, data regarding its expression in TAMs of NSCLC patients are currently lacking.

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

METHODS

Subject characteristics

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

Histological diagnosis and grade of differentiation were determined in accordance with the World Health Organization criteria for lung cancer [13]. The pathological tumour stage was determined according to the revised TNM classification [14]. Smoking data were obtained from the patients' chart, which included cumulative consumption (in pack-yrs), age at which patients started to smoke, duration of smoking (in yrs) and smoking status at diagnosis. With regard to smoking status, patients were classified into two groups: nonsmokers, *i.e.* subjects who had never smoked; and smokers, *i.e.* ex- and current smokers.

Immunohistochemistry

Samples were fixed in 4% formaldehyde in PBS (pH 7.2) and, after dehydration, embedded in paraffin wax and processed for immunohistochemical analysis of IL-10. Sections (5 μm thick) were cut and subsequently hydrated. Endogenous peroxidase activity was blocked by incubating the sections with 3% tris(hydroxymethyl)aminomethane-buffered saline (pH 7.6) for 20 min. Immunohistochemistry was performed using a mouse monoclonal antibody directed against human IL-10 (Biosource International, Camarillo, CA, USA) and the streptavidin–biotin complex method (StreptABComplex/HRP (K0377); Dako, High Wycombe, UK). Immunoreactivity was visualised using diaminobenzidine (D5637; Dako), a peroxidase substrate. The negative control involved omission of the primary antibody.

In order to determine the phenotype of the cells expressing IL-10, a double immunohistochemical technique was used. Briefly, sections were incubated with the anti-IL-10 monoclonal antibody followed by biotinylated rabbit anti-mouse immunoglobulins (E0413; Dako) and StreptABComplex/AP (K0391; Dako). Slides were incubated with the monoclonal anti-human CD68 antibody (M0814; Dako) followed by the anti-mouse EnVision+^{TM} peroxidase conjugate (K4000; Dako). Sections were developed sequentially with diaminobenzidine and fast red (an alkaline phosphatase substrate; K0699; Dako). IL-10-positive cells were stained red, CD68-positive cells brown and doubly immunostained cells reddish brown. As a negative control procedure, the analyses were repeated omitting one or both of the primary antibodies.

Quantification

Since IL-10-positive immunostaining was located in tumour cells and in tumour macrophages mainly located in the advancing tumour margin, IL-10 expression was quantified 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 blind to the clinical data. The expression of IL-10 in TAMs was quantified using an Olympus BX41 microscope (Olympus Optical Co., Hamburg, Germany) at 600× magnification. At least 20 high-power fields (HPFs) of tumour margin were randomly selected for each section and $\geqslant$100 macrophages were evaluated. Results are expressed as the number of macrophages per HPF and as the percentage of IL-10-positive macrophages.

The expression of IL-10 in tumour cells was quantified at 400× magnification using an Olympus BX41 microscope connected to a video recorder linked to a computerised image analysis system (Image-Proplus software; Media Cybernetics, Inc., Silver Spring, MD, USA). For each slide, 40 microscopic fields, mostly occupied by tumour cells, were randomly selected. In each microscopic field, the tumour cell area was manually delineated, excluding tumour stroma and necrotic areas. The positive tumour cell area was automatically evaluated by the image analysis system, calibrated to select the tissue area immunostained above a threshold considered to represent nonspecific background staining. Results are expressed as the percentage of the total tumour cell area that was IL-10-positive.

Statistical analysis

The percentages of IL-10-positive TAMs and tumour cell area are presented as median (interquartile range). At least three replicate measurements of immunostained slides were performed by the same observer using 10 randomly selected slides in order to assess intra-observer reproducibility [16]. The intraclass correlation coefficient was 0.99.

The pathological stages were grouped into early (stage I) and late (II, III and IV) stages. Differences between groups for IL-10 expression in both TAMs and tumour cells were analysed 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 (with early and late stage as dependent variables) and covariates (age, sex, cumulative cigarette consumption, tumour histology and IL-10 expression in TAMs). For this analysis, the median percentage of IL-10-positive TAMs (16.3%) was chosen as the cut-off point for dividing the patients into the two groups.

The effects of IL-10-positive TAMs and tumour cell area on overall survival were analysed. For the analysis of survival, an *a priori* decision was made to classify IL-10-positive TAM percentages as high or low using the sample median. Survival curves were estimated using the Kaplan–Meier method and were calculated from the date of surgery. The log-rank test was used to compare patients' survival times between groups. Cox's proportional hazards model was used for univariate analysis evaluating the association between survival time and risk factors, and for multivariate analysis modelling the risk of IL-10 expression in TAMs on survival time, with adjustment for clinical and histopathological parameters (age, sex, cumulative cigarette consumption, tumour histology and pathological TNM stage). A p-value of $\leqslant 0.05$ was accepted as statistically significant.

RESULTS

Table 1 shows the characteristics of the subjects examined. Patients (43 males and seven females) had a mean±SEM age of 63.9±1.3 yrs. Of these, 41 (82%) had a smoking history and nine (18%) were nonsmokers. Adenocarcinoma was the most common (68%) lung tumour type followed by squamous cell carcinoma (SCC; 32%). Of the patients who underwent surgery, 24 (51%) were classified as having stage I (early stage) of the disease, and the remaining 23 (49%) as having stages II, III or IV (late stage) of the 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) of the IL-10-positive cells in the tumour margin were macrophages (CD68-positive; fig. 1). Occasional CD68-positive cells coexpressing IL-10 were detected within the tumour stroma and cells (median (interquartile range) 1.9 (0.75–4.10) and 0 (0–2.45)%, respectively) of some subjects. In order to evaluate the data as homogenously as possible, these cells were excluded from further analysis.

The percentage of IL-10-positive TAMs was higher in tumour specimens from patients with stages II, III and IV compared with those with stage I NSCLC (table 2, fig. 2). Interestingly, this difference was more pronounced in subjects with the SCC subtype than in those with the adenocarcinoma subtype. As expected, IL-10-positive TAM percentage was also increased in patients with lymph node metastases (47.6 (14.7–74.4) *versus* 10.9 (2.1–34.8)% for N1/N2/N3 *versus* N0; p=0.0084). In contrast, the total number of macrophages per HPF did not differ between early and late stages of the tumour (table 2).

When multivariate logistic regression analysis was performed, patients with higher percentages of IL-10-positive TAMs showed an increased risk of late-stage disease (table 3).

The percentage of IL-10-positive tumour cells did not differ between stages II, III and IV and stage I NSCLC (table 2). No correlations were observed with either tumour histological type or differentiation.

IL-10 expression in TAMs, but not in tumour cells, was significantly associated with overall patient survival. Patients with higher percentages of IL-10-positive TAMs exhibited a shorter survival time than those with lower percentages of IL-10-positive TAMs (p=0.014 (log-rank test); fig. 3). However, TAM IL-10 expression did not prove to be an independent prognostic factor for survival when disease stage was used as the stratification variable in the Cox's multivariate regression analysis.

Finally, IL-10 expression in TAMs and in tumour cells was increased in smokers compared with nonsmokers (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, did

TABLE 1 Demographic and clinical characteristics of the study population

Males/females	43/7
Age yrs	63.9±1.3
Smoking history	
Smokers	41
Nonsmokers	9
Cumulative consumption pack-yrs	44.1±3.6
Duration of smoking yrs	41±1.5
Age at which patients started to smoke yrs	19.3±1.2
Histology	
Adenocarcinoma	34
Squamous cell carcinoma	16
TNM stage[#]	
I	24
II/III/IV	23
Lymph node metastases[#]	
N0	23
N1/N2/N3	24
Tumour grade (cell differentiation)[¶]	
G1/G2	29
G3	20

Data are presented as n or mean±SEM. TNM: tumour, node, metastasis. [#]: data available for 47 patients; [¶]: tumour grade missing for one patient.

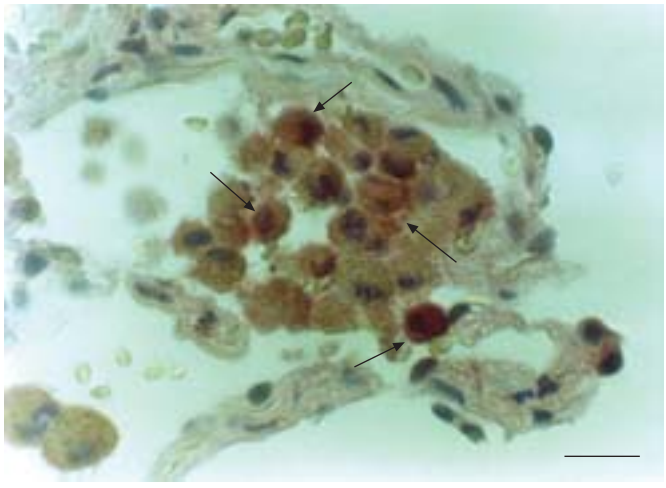

FIGURE 1. Double immunostaining showing interleukin (IL)-10/CD68-positive cells in the alveolar spaces of the advancing tumour margin. IL-10-positive cells are stained red and CD68-positive cells brown. Arrows indicate examples of reddish brown doubly immunostained cells. Scale bar=20 μm.

TABLE 2 Interleukin (IL)-10 expression in tumour-associated macrophages (TAMs) and tumour cells of patients with early- and late-stage nonsmall cell lung cancer

	Late stage	Early stage
Subjects n	23	24
IL-10-positive TAMs %	50.9 (16.1–73.4)#	9.6 (1.4–29.3)
TAMs cells·HPF^{-1}	10.9 (7.4–15.2)	10.3 (5.8–12.2)
IL-10-positive tumour cell area %	6.5 (1.7–21.8)	3.8 (0.9–13.6)
Total tumour cell area $\times 10^3$ μm^2	56 (48–59)	58 (48–61)

Data are presented as median (interquartile range), unless otherwise stated. HPF: high-power field. #: p=0.0014.

not differ between smokers and nonsmokers. The percentage of IL-10-positive tumour cells showed a positive correlation with duration of smoking in years (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 the present study is the role of TAMs expressing IL-10 in the prognosis of NSCLC patients: the experiments presented indicate that expression of IL-10 is increased in TAMs of patients with stages II, III and IV NSCLC compared with those with stage I NSCLC. In addition, as expected, IL-10-positive TAM percentage was higher in patients with lymph node metastases than in those without lymph node metastases. Furthermore, higher IL-10 expression by TAMs was associated with shorter overall survival.

Expression of IL-10 was assessed in macrophages located at the advancing tumour margin, as IL-10-positive TAMs within the cancer stroma and cells were occasional and not detected in all of the participating subjects. In addition to this, the structural features of the advancing tumour margin have been observed to be of critical significance for prognosis in various cancers [17–19].

To the best of the current authors' knowledge, the present study is the first to demonstrate that, in NSCLC, TAMs express IL-10 and that its expression correlates with both disease progression and prognosis. Previous studies in human glioblastoma [9, 10] have identified macrophages as the major source of IL-10, which correlated with the extent of malignancy. Large amounts of IL-10 have been measured in macrophages from human ovarian carcinoma and mouse tumours, and it has been hypothesised that delegating macrophages to produce high levels of IL-10 may be a common mechanism used by tumours to interfere with the immune response and promote tumour survival [20]. The mechanism behind the pro-tumour role of TAMs expressing IL-10 might include inhibition of the 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, a study by MANTOVANI *et al.* [11] has expanded this view. TAMs could represent a particular mononuclear phagocyte population able to tune

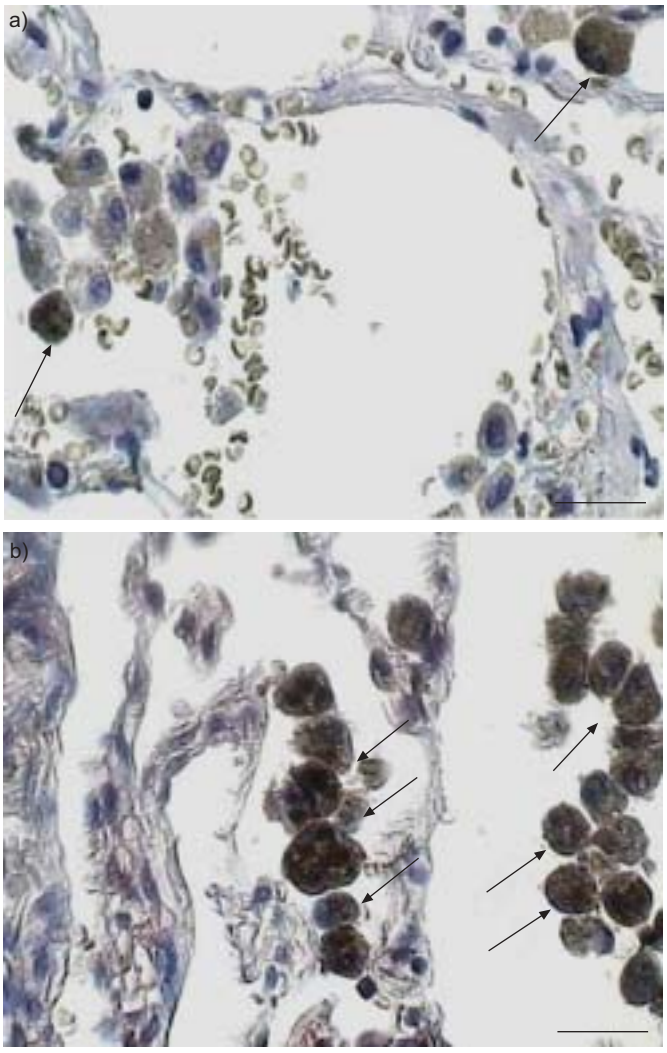

FIGURE 2. Photomicrographs showing examples of interleukin (IL)-10-positive tumour-associated macrophages (arrows) of patients with: a) stage I nonsmall cell lung cancer (NSCLC); and b) stage III NSCLC. Immunostaining was performed using a mouse monoclonal antibody specific for human IL-10. Scale bar=20 μm.

inflammatory responses and adaptive T-helper cell type-1 immunity, to promote angiogenesis and tissue remodelling and repair and, thereby, to favour tumour progression. This phagocyte population is characterised by the production of high IL-10 levels. The present 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-positive TAMs was higher in patients with lymph node metastases than in those without was largely expected, since stage I NSCLC is not associated with lymph node involvement. Nevertheless, this result highlights the findings of a previous study on human gastric carcinoma [23], in which IL-10 mRNA was frequently expressed in tumour tissues of patients with a 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, probably through immunosuppression.

TABLE 3 Logistic regression analysis of the association between tumour stage groups and clinicopathological features[#]

	Adjusted regression coefficient	SEM	Chi-squared	p-value	OR (95% CI)
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)
Cigarette consumption	-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-positive TAMs	1.936	0.682	8.044	0.0046	6.928 (1.818–26.398)

The dependent variable was being in the early- or late-stage group. The independent variables included sex (0=female; 1=male), age (continuous variable, in yrs), cumulative cigarette consumption (continuous variable, in pack-yrs), histology (0=adenocarcinoma; 1=squamous cell carcinoma) and interleukin (IL)-10-positive tumour-associated macrophage (TAM) percentage (0=low (<16.3%); 1=high (⩾16.3%)). OR: odds ratio; CI: confidence interval. [#]: n=47.

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

At variance with TAMs expressing IL-10, the percentage of IL-10-positive tumour cells did not differ between patients with late- and early-stage NSCLC and did not correlate with either clinicopathological factors or overall 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 of HATANAKA *et al.* [7], who suggested that IL-10 expression by the tumour is an indicator of poor prognosis in NSCLC patients. In the present study, IL-10 expression was analysed at the protein level whereas the mRNA level was analysed by HATANAKA *et al.* [7]. The difference between the present results and those by HATANAKA *et al.* [7] are probably due to the fact that the mRNA measurements were performed globally on tissues including tumour cells, stromal cells and TAMs. Indeed, by performing an immunohistochemical analysis in the present study, it was possible to identify TAMs as the cells that 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 remains controversial and merits further study.

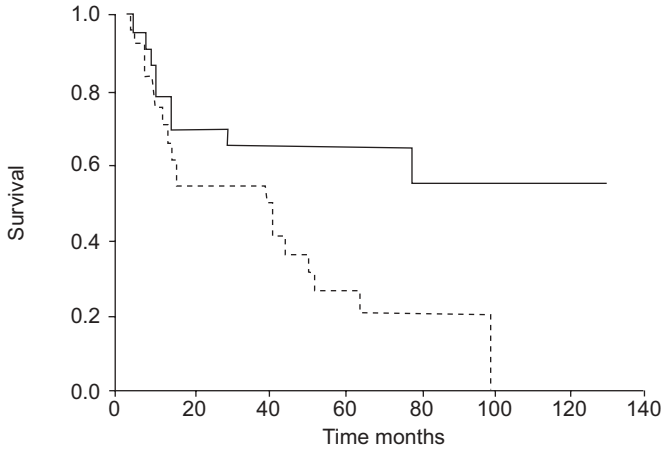

FIGURE 3. Survival curves of patients with high (-----; n=24) and low percentages (———; n=23) of interleukin (IL)-10-positive tumour-associated macrophages (TAMs). The median percentage of IL-10-positive TAMs (16.3%) was chosen as the cut-off point for dividing the patients into the two groups. Patients with high IL-10 expression showed worse overall survival than those with low IL-10 expression.

With regard to the smoking status of the patients, expression of IL-10 was increased in both TAMs and tumour cells of smokers compared with nonsmokers. The study by LIM *et al.* [27] showed a positive influence of cigarette smoke on IL-10 production by alveolar macrophages obtained by bronchoalveolar lavage. The present study confirms 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 duration of smoking (in yrs) and the age at which patients started to smoke. In this context, one study [28] has shown that normal bronchial epithelium of both smokers and nonsmokers constitutively expresses IL-10. Others have reported that primary human bronchial epithelial cells of light smokers or nonsmokers do not express IL-10 [29]. Indeed, these findings need to be interpreted with caution since it has recently been demonstrated, using microarray analysis, that many genes are differently expressed in lung adenocarcinoma of smokers compared to nonsmokers [30]. Finally, the present result of the more prominent difference in the percentage of IL-10-positive TAMs between early- and late-stage NSCLC in patients with the SCC subtype may also be explained by tobacco use, since SCC is the histological subtype more related to tobacco use.

The possibility that tumour cells of different stages can affect IL-10 expression by TAMs could be a source of potential bias in

the current study. The present data do not permit the exclusion of a role for tumour stage in the expression of IL-10 by TAMs. Despite this limitation and awareness of the fact that correlations do not imply a cause–effect relationship, the significant correlations between the increased percentage of IL-10-positive TAMs and both the late stages and worse overall survival indicate that TAMs expressing this cytokine are probably involved in the progression of the disease.

In conclusion, the current authors have shown that interleukin-10 expression in tumour-associated macrophages correlates with disease progression and prognosis in patients with nonsmall cell lung cancer. These findings may be useful in understanding the mechanisms involved in nonsmall cell lung cancer and, hopefully, in developing alternative therapeutic regimens for lung cancer patients.

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