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### ► To cite this version:

Paul Takam Kamga, Marie Mayenga, Louise Sebane, Adrien Costantini, Catherine Julie, et al. Colony stimulating factor-1 (CSF-1) signalling is predictive of response to immune checkpoint inhibitors in advanced non-small cell lung cancer. *Lung Cancer*, 2024, 188, pp.107447. 10.1016/j.lungcan.2023.107447 . hal-04517531

**HAL Id: hal-04517531**

**<https://hal.science/hal-04517531>**

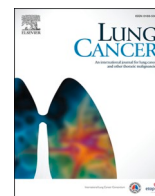
Submitted on 7 Jun 2024

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## Colony stimulating factor-1 (CSF-1) signalling is predictive of response to immune checkpoint inhibitors in advanced non-small cell lung cancer

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### ARTICLE INFO

#### Keywords:

Non-small cell lung cancer

Immunotherapy

Biomarkers

CSF-1

CSF-1R

CD163

IL-34

Chemotherapy

Response

### ABSTRACT

The identification of biomarkers related to treatment in patients with non-small cell lung cancer (NSCLC) treated with immune checkpoint inhibitors (ICIs) represents a significant challenge. The aim of this study was to determine the predictive value of macrophage-related markers assessed in plasma and tissue samples of patients with NSCLC undergoing ICI treatment. This bicentric study included a prospective cohort of 88 patients with advanced NSCLC who received first-line therapy with ICI (either as monotherapy or in combination with chemotherapy) or chemotherapy alone (CT). Samples were collected from the patients at baseline and during follow-up. Plasma levels of CSF-1 and IL-34 were measured using ELISA, while expression levels of the macrophage receptors CD163 and CSF-1-R were evaluated using immunohistochemistry on lung biopsies. At baseline, the median plasma CSF-1 expression was higher in patients who did not respond to immunotherapy compared to those who responded (8898 pg/mL vs. 14031 pg/mL,  $p = 0.0005$ ). Importantly, high CSF-1 levels at the initial assessment were associated with disease progression regardless of the treatment received. Furthermore, high CSF-1 levels were associated with shorter progression-free survival (PFS) and overall survival (OS) in patients receiving ICI therapy, but not in those treated with chemotherapy. There was no correlation between IL-34, CSF-1R, CD163 and therapeutic response. We observed *in vitro* that the activation of lymphocytes mediated by pembrolizumab was hindered by the treatment of PBMC with recombinant CSF-1, suggesting that CSF-1 creates a systemic immunosuppressive state that interferes with ICI treatment. In conclusion, baseline CSF-1 levels represent a potential predictive marker to ICI treatment in NSCLC.

### 1. Introduction

Immune checkpoint inhibitors (ICIs) are the standard first-line therapy for advanced non-small cell lung cancer (NSCLC). They are administered either as monotherapy (in cases of high PD-L1 expression by tumor cells) or in combination with chemotherapy. While ICIs have demonstrated prolonged efficacy in a subgroup of patients, the majority will ultimately experience tumor progression. Thus, the identification of biomarkers associated with treatment resistance and prognosis remains

a significant challenge. [1,2].

The tumor microenvironment (TME) appears to be a major player in oncogenesis and anti-tumor response for its role as a promoter of cancer initiation, growth, invasion, and response to treatment [3]. TME is an heterogeneous milieu containing diverse cells including fibroblasts, mesenchymal stromal cells and immune cells such as T-cells, B-cells, Natural killers and macrophages. Tumor-associated macrophages (TAM) constitute a major component of infiltrating immune population in many cancers such as breast, lung, colon and many other solid cancers

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[4]. TAM infiltration correlates with the tumor stage[4]. However, studies on its prognostic role highlighted contrasting results[5,6], possibly due to the heterogeneity of the macrophagic population, oscillating between two extreme states of polarization defined as M1 and M2. M1 macrophages decrease angiogenesis, increase sensitivity to chemotherapy and decrease tumor viability. Conversely, M2 macrophages increase tumor cell growth and invasion, promote immune evasion and a poor response to ICIs-based immunotherapy[7]. The polarization of TAM into M2 phenotype is triggered by a concerted action of chemokines/cytokines from both tumor cells and surrounding immune cells[4]. A key actor of this polarization is CSF-1R (*Colony Stimulating Factor 1 Receptor*), expressed by monocytes/macrophages and activated by two ligands, Interleukin-34 (IL-34) and CSF-1 (*Colony Stimulating Factor 1*, also called M-CSF, *Monocyte Colony-Stimulating Factor*). In lung cancer, high expression of CSF-1R by TAMs is associated with higher cancer-related mortality[8]. Thus, the activation of the CSF-1/CSF-1R or IL-34/CSF-1R axis could constitute an interesting biomarker to predict the response to treatment in NSCLC.

CSF-1 is physiologically produced by endothelial cells, fibroblasts, osteoblasts, smooth muscle cells and macrophages, but also by tumor cells[9]. In cancers or inflammatory diseases, higher levels of CSF-1 is detectable in human blood, high plasmatic levels being correlated with a worse prognosis[10]. In vitro, CSF-1 recruits bone marrow-derived monocytes and favour their polarization toward an immunosuppressive phenotype with significant production of IL-10 and CCL2/MCP-1 (*C-C Motif Chemokine Ligand 2/ Monocyte chemoattractant protein-1*), which amplifies macrophage recruitment by interacting with CCR2 (*C-C Motif Chemokine Receptor 2*)[11]. CSF-1 expression has been found in lung cancer tissue (NSCLC and small-cell lung cancer), whereas it is not detected in healthy lung, and its expression level seems to be correlated with survival in lung cancer patients[12]. IL-34 has common functions with CSF-1[13]. It promotes the recruitment of macrophages and their polarization into an immunosuppressive phenotype[14,15]. Pre-clinical studies have shown a production of IL-34 by regulatory T-cells (T-regs), suggesting its involvement in immune tolerance[16]. High levels of IL-34 in tumor tissues or in patient's sera are correlated with a worse prognosis and resistance to chemotherapy[17]; Higher serum IL-34 levels have been observed in cholangiocarcinoma compared to healthy controls but its prognostic value in lung cancer is not defined yet[18].

Given the role of CSF-1R/CSF-1 and/or CSF-1R/IL-34 in the recruitment and polarization of immunosuppressive macrophages, their expression levels may be associated with antitumoral immunity and response to anti-tumor treatments. The aim of our study was to assess the influence of the expression levels of TAMs-related on tumor response in patients with advanced NSCLC receiving first-line ICIs therapy.

## 2. Material and methods

### 2.1. Study design

We conducted a translational and exploratory study. All consecutive patients with a previously untreated stage IIIB or IV NSCLC in the Department of Pulmonology and Thoracic Oncology of two centers (APHP – Ambroise Paré Hospital and APHP – Bicêtre Hospital), and receiving a first line treatment by chemotherapy, immunotherapy or a combination of chemotherapy and immunotherapy, between May 2015 and June 2021 were included. All patients were included in the study after signed an informed consent, (CPP IDF n°8). Plasma samples were prospectively and systematically collected from NSCLC patients at various stages of their treatment, including diagnosis (baseline/just before treatment initiation), at the first evaluation (typically 8 weeks after treatment initiation), and, whenever possible, at the time of disease progression. These samples were collected at Ambroise Paré Hospital and stored within the Centre de Ressources Biologiques (CRB) of Ambroise Paré Hospital (ID CRB 2014-A00187-40). Tumor lung biopsies were collected at diagnosis at the Ambroise Paré Hospital and Bicêtre

Hospital and stored as Formalin-fixed paraffin-embedded tissue blocks. PBMC samples were obtained from peripheral blood samples of healthy donors. The exploratory endpoints were progression rate at first evaluation, progression-free survival (PFS) and overall survival (OS) according to plasmatic concentration of the circulating biomarkers, or histologic expression of tissular biomarkers. Tumor response was evaluated every two months using RECIST 1.1 criteria by CT-scan of the brain, chest and abdomen.

### 2.2. Reagents and antibodies

Kits used for ELISA assays were ab245714 (CSF-1), ab235640 (IL-34) and Interferon- $\gamma$  (IFN $\gamma$ , ab100537), all provided by ABCAM (Cambridge, UK). Antibodies used for lymphocyte stimulation were mouse anti-huCD3 (16-0037-85), mouse anti-huCD28 (16-0289-85) (all from ThermoFischer Scientific, France), used in combination with IL-2 (10453-IL-010, R&D System/Bio-Techne, France). The antibodies used for Flow cytometric analysis of circulating lymphocytes and macrophages, including mouse anti-huCD45- BV421 (HI30, 563879), mouse anti-huCD3-PE-Cy7 (SK7, 557851) and mouse anti-huCD4- PE-Cy7<sup>TM7</sup> (SK3, 348809) were all from BD Biosciences (Paris, France). Mouse antiCD16-FITC (MA517719), was from Invitrogen (France). Antibodies used for IHC are anti-huCD163 (Novastra) and anti-CSF-1R (ab52864, ABCAM, Cambridge, UK). Phytohemagglutinin (00-4977-03) for lymphocyte activation was from ThermoFischer Scientific (Paris, France). CarboxyFluorescein Succinimidyl Ester (CFSE) for lymphocyte staining was from ThermoFischer Scientific (C34554, France). MTS colorimetric (ab197010) assay was acquired from ABCAM (Cambridge, UK). Pexidartinib (PLX3397) was from Selleck Chemicals GmbH (Germany). Pembrolizumab (Pembro) was a kind gift of the Pharmacy Unit of the University Hospital Ambroise Paré (Boulogne-Billancourt, France).

### 2.3. ELISA technique

ELISA assays to evaluate CSF-1, IL-34, IFN $\gamma$  concentration in plasma samples were performed using the manufacturer's instructions as previously described[1]. Briefly, standards protein and plasma samples diluted at least at 1:2 ratio in sample buffers were added in ELISA well plates, probed with streptavidin conjugated primary antibodies and HRP conjugate secondary antibody. The assays were revealed through the addition of HRP substrate. The plates were analyzed using a spectrophotometric plate reader (Bio rad PR 3100 EIA PhD software), set at a wavelength of 450 nm. Protein concentrations were calculated from their optical density using the standard curve. Samples, standards and negative controls were tested in duplicate.

### 2.4. Immunohistochemistry (IHC)

Treatment-naïve tumor sections (3  $\mu$ m) were probed with anti-CD163 and anti-CSF-1R antibodies through an automated technique as previously described (LEICA, BOND-III)[19]. Slides were manually read with optical microscope by a thoracic pathologist (CJ) who was blinded of the clinical outcome of the patients. Percentage of positive cells were obtained by cell counting. Macrophage membrane labeling for CD163 and CSF-1R was expressed according to the number of positive cells in the peritumoral stroma classifying the samples in two groups, 0–25 % (low expression), and > 25 % (high expression) as previously described [19–22]. Epithelial cells on the same slide were considered as negative and positive controls, respectively.

### 2.5. Cell cultures and co-cultures

Human NSCLC lines (A549 and H596) were obtained from ATCC. They were maintained in complete DMEM/F-12 medium (DMEM/F-12 supplemented with 10 % foetal bovine serum (FBS), 1 % L-Glutamine (L-

Glu) and 1 % Penicillin/Streptomycin (P/S)). Primary NSCLCs were isolated from immunohistochemically confirmed lung cancer samples obtained from surgical procedures. The resected lung cancer tissues were collected and rinsed in RPMI medium. The tissues were then cut into small pieces and rinsed with sterile phosphate-buffered saline (PBS). The samples were digested in Trypsin-EDTA for 1 h at 37 °C. After enzymatic digestion, the samples were washed and cultured in complete DMEM/F12 supplemented with Primocin as an antibiotic instead of P/S. At passage 2, the cells were used for culture and co-culture experiments. The NSCLC cells were grown as a confluent monolayer in 48-well plates for co-culture experiments. Peripheral blood mononuclear cells (PBMC) were isolated from human blood by density gradient centrifugation using Lymphoprep (Stemcells Technologies). Isolated PBMC were maintained in complete IMDM medium. Macrophage differentiation was achieved by treating isolated PBMCs for one week with recombinant CSF-1 (40 ng/mL) and conditioned medium from A549 cell culture. The conditioned medium was collected in 48h A549 culture, filtered (22 μM) and dilute (1:2) with a fresh complete DMEM medium. For immune regulatory assays, PBMC were activated with 10 μg/mL phytohemagglutinin (PHA) and cultured in the presence of CSF-1 or on an established NSCLC or macrophage monolayer, to evaluate the contribution of CSF-1, macrophage or NSCLC to lymphocyte activation. The addition of pembrolizumab (20 nM corresponding to 3 μg/mL), or the CSF-1R inhibitor Pexidartinib (PLX3397; 0.5 μM) in the culture/co-culture medium was performed to understand the contribution of CSF-1 to ICI-mediated activation of lymphocytes. The immune response was assessed by three methods, including i) the MTS proliferation assay to quantify PBMC or lymphocyte proliferation after 72 h of cell culture; ii) the production of IFN $\gamma$  24 h following cell activation; iii) and flow cytometry analysis of the percentage of proliferating CFSE-stained CD8 + cells after 5 days of cell culture following cell activation.

2.6. MTS proliferation assay

MTS proliferation assay performed as previously described[23], was used to evaluate PBMC and or Lymphocyte/ proliferation. Briefly, after 72 h of cell culture, 10 μL of MTS reagent (ABCAM, ab197010) was added into each well and keeping in incubator for 2 h. Metabolically active, viable cells converted MTS into a colored formazan. The result was then measured at 490 nm in a spectrophotometric microplate reader (Bio rad PR 3100 EIA PhD software). The viability was expressed as the percentage of optical density of treated cells compared to optical density of cells treated with the specific vehicle.

2.7. CFSE staining and flow cytometry

To assess immunomodulatory properties of CSF-1, Pembro or NSCLC lines, PBMC were stained before each experiment with 1 μM carboxy-fluorescein succinimidyl ester (CFSE; Life Technologies). The T-cell proliferation was assessed (after 5 days of cell culture) by flow cytometry on viable CD45<sup>pos</sup>CD3<sup>pos</sup> CD8<sup>pos</sup> cells (Fig. S3) by using FlowJo software (TreeStar) as relative CFSE dilution of treated cells compared to cell treated with specific vehicle as previously described[24].

2.8. Statistical analysis

All statistical analyses were performed using both XLSTAT2021 and GraphPad Prism version 5 software (La Jolla, CA, USA). The comparison between two groups or more was performed using the Student's t-test and one-way ANOVA, respectively, for data with Gaussian distribution. For data with non-Gaussian distribution, we used the Mann-Whitney test or the Kruskal-Wallis test, respectively. Receiver operating characteristic curve (ROC) was used to define the threshold value for each protein with regard to patient's progression using the maximum Youden index (ie, Specificity + Sensitivity-1). Associations between variables were tested using the Pearson Chi-square or the Fisher analyses. Survival

curves were determined according to the CSF-1, IL-34, CD-163 and CSF-1-R expression levels, using the Kaplan Meier method followed by the Log Rank test. Logistic regression and COX proportional hazards model were used for multivariate analyses. Differences were considered as significant for p-value < 0.05.

2.9. Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and/or its [supplementary materials](#).

3. Results

3.1. Patient characteristics

Eighty-eight patients were included in this study. Clinical characteristics of patients are presented in Table 1. They were mostly male (68 %), current or former smokers (95 %) and with adenocarcinoma histology (72 %). They had advanced stage disease at diagnosis (97 %) and had a good Performance Status (PS, 0 – 1) at treatment initiation in 85 % of cases. The patients were categorized in two groups according to the first-line treatment they received: the immunotherapy group (ICIs) including the patients receiving ICIs alone or associated with chemotherapy (n = 52) and the chemotherapy group (CT) (n = 36) used as control group. (Table 1; Fig. S1).

3.2. Expression of macrophages associated markers CSF-1, IL34, CD163, CSF-1R and correlation with demographics

To investigate the prognostic value of TAM in NSCLC patients treated with ICIs, we first assessed in tumor biopsies and plasma samples the basal expression of TAM markers including CD163, CSF-1R, CSF-1 and IL34. CD163 and CSF-1R were assessed through IHC of lung biopsies collected before treatment initiation in 52 (ICIs: n = 37; CT alone: n = 15) and 48 (ICIs: n = 35; CT alone: n = 13) patients respectively (Fig. S1;

Table 1 Patients' characteristics.

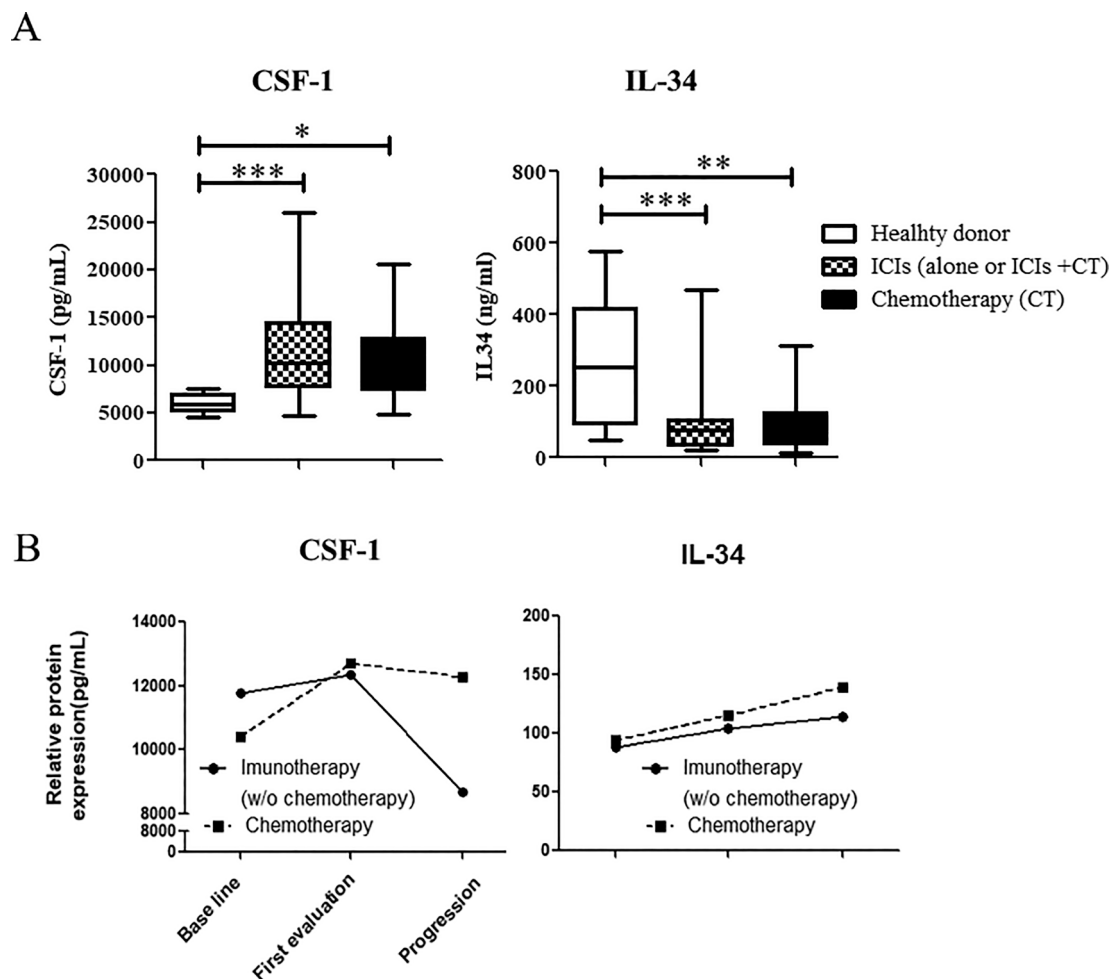
	n = 88	Chemotherapy n = 36	Immunotherapy +/- CT n = 52
Age	67 (62-73)	67 (65-74)	66 (62-71)
≤ 67	60	25	45
> 67	28	11	17
Gender			
Male	60 (68 %)	25 (69 %)	35 (67 %)
Female	28 (32 %)	11 (31 %)	17 (33 %)
Body Mass Index, BMI (IQR1-IQR3) (20-26)	23	21 (19-25)	23 (20-26)
Smoking status			
Current or ex-smoker	81 (92 %)	34 (94 %)	47 (90 %)
Non-smoker	7 (8 %)	2 (6 %)	5 (10 %)
Histology			
Adenocarcinoma	63 (72 %)	24 (66 %)	39 (75 %)
Squamous carcinoma	15 (17 %)	6 (17 %)	9 (17 %)
Others	10 (11 %)	6 (17 %)	4 (8 %)
Performance status			
0-1	72 (82 %)	32 (89 %)	41 (79 %)
2	15 (16 %)	4 (11 %)	11 (21 %)
TNM stage			
IIIB or IIIC	5 (6 %)	2 (6 %)	3 (6 %)
IV	83 (94 %)	34 (94 %)	49 (94 %)
Number of metastatic sites			
0-1	36/83 (43 %)	18/34(54 %)	18/49 (37 %)
2 or more	47/83 (57 %)	16/34 (46 %)	31/49 (63 %)

**S2).** As mentioned earlier (see methods' section), results were expressed as low expression or high expression according to the staining intensity (Fig. S2) [19–22]. In patients treated with ICIs, CD163 levels were high in 30/37 patients (81 %) and low in 7/37 patients (19 %). In patients treated with CT, CD163 levels were high in 14/15 patients/15 (93 %) and low in 1/15 patient (7 %). In patients treated with ICIs, CSF-1R levels were high in 16/35 patients (40 %) of patients. In patients treated with CT, CSF-1R levels were high in 3/13 patients (23 %). We next analyzed the expression of macrophages associated ligands in plasma samples collected from patients at the diagnostic (n = 42 for ICIs patients and n = 34 for CT patients). The median expression levels at treatment initiation for ICIs group were 11396 ± 894.7 pg/mL for CSF-1 and 80.31 ± 22.01 ng/ml for IL-34. The median expression levels for the CT groups were 9975 ± 716.2 pg/mL for CSF-1 and 77.14 ± 19.02 ng/ml for IL-34 (Table S1; Fig. 1A). We found that CSF-1 concentrations were higher in patient samples compared to samples from healthy donors, while IL-34 levels were lower in patient samples compared to healthy donor plasma. (Fig. 1A). We also observed increased levels of plasma CSF-1 and IL-34 in samples from patients at the initial evaluation following treatment with ICIs or CT (Fig. 1B). No correlations were found between expression levels of CD163 or CSF-1R and the plasma levels of CSF-1 or IL34. We also analyzed the association between expression values and demographics including age, gender, performance status (PS), tumor grade, the number of metastatic sites and PD-L1. We found that at baseline, higher levels of CSF-1 were related to performance status 2. Except for PS, no other correlation and/or

association were found for any other parameters (Table S1).

### 3.3. Correlation with patient's outcomes in the ICIs group

Fifty-two (52) patients were treated with ICIs with or without chemotherapy. Objective Response Rate (ORR) was 38 % (n = 20, considered as responders), whereas stable disease rate was 25 % (n = 13) and 19 patients (36 %) experienced tumor progression as best response. Patients with stable disease and tumor progression were classified as non-responders. To categorize patients into two groups based on CSF-1 and IL-34, we determined their optimal cut-off values for predicting the efficacy of therapy and patient progression using ROC curves. The optimal cut-off values (threshold) for each cytokine at different treatment steps (baseline and first evaluation) are presented in Table 2. Patients with expression levels below the threshold were classified as low-expressing patients, while those with expression values above the threshold were categorized as high-expressing patients (Table 2).Plasmatic CSF-1 levels at treatment initiation were significantly higher in non-responders compared to responders (12865 ± 1110 vs 8868 ± 680.4 pg/mL; p = 0.006) (Fig. 2A; Table S2). Concordantly, plasmatic CSF-1 levels at treatment initiation were significantly higher in patients presenting a progression at the first assessment compared to non-progressing patients (14031 ± 1450 pg/mL vs 8898 ± 695 pg/mL; p = 0.0005) (Fig. 2B; Table S2). This was not the case for CSF-1R, CD163 or IL34. Subsequently, we observed a correlation between elevated CSF-1 levels at baseline and ORR (RR = 7.00; 95 % CI



**Fig. 1. Expression of TAM markers in NSCLC patients.** A) Plasma levels of CSF-1 and IL-34 at baseline in patients treated with immunotherapy or chemotherapy alone or in healthy donors (n = 5). B) Plasma levels of CSF-1 and IL-34 in NSCLC at baseline (initiation), first evaluation and progression. Data are expressed as mean ± SD of 35 and 31 ICIs patients and 27 and 28 CT patients for CSF-1 and IL-34 respectively.

**Table 2**  
Receiver operating characteristic curve (ROC) in relation with patient's progression.

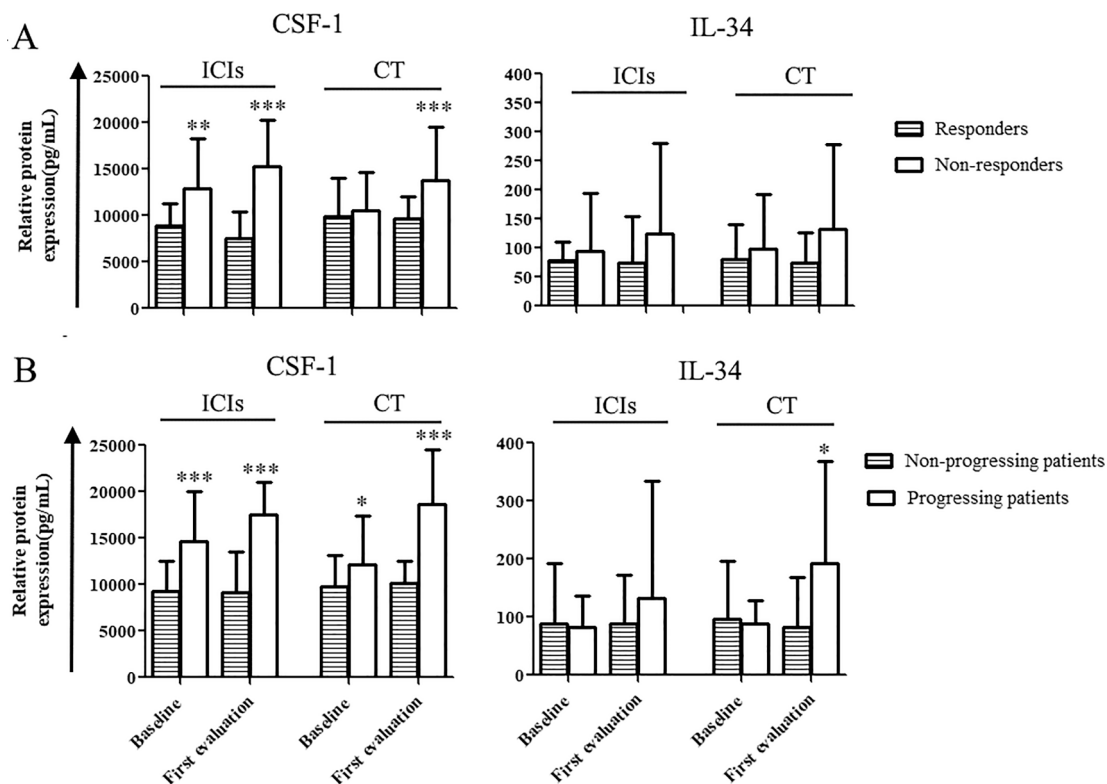
ROC analysis in relation with patient's progression					
Biomarker	AUC	95 % CI	Optimal specificity + sensitivity	Optimal threshold	p-value
Immunotherapy (ICIs alone or ICIs + CT)					
CSF-1 baseline	0.818	0.672–0.963	1.628	12545.93 pg/mL	P < 0.001
CSF-1 first evaluation	0.938	0.855–1.000	1.813	9042.22 pg/mL	P < 0.001
IL-34 baseline	0.625	0.419–0.831	1.321	89.375 pg/mL	P = 0.234
IL-34 first evaluation	0.496	0.261–0.732	1.222	30.57 pg/mL	P = 0.975
Chemotherapy (CT)					
CSF-1 baseline	0.618	0.385–0.851	1.386	9679.024 pg/mL	P = 0.319
CSF-1 first evaluation	0.901	0.708–1.000	1.875	14313.171 pg/mL	P < 0.001
IL-34 baseline	0.610	0.340–0.880	1.400	28.125 pg/mL	P = 0.425
IL-34 first evaluation	0.762	0.534–0.989	1.548	82.143 pg/mL	P = 0.024

(1.007—48.68); p = 0.004) as well as patient progression (RR = 3.73; 95 % CI (1.337—10.43); p < 0.001) (Table S2). No correlation was identified between CSF-1R, CD163, or IL34 and patient progression (Table S2). This pattern persisted when the analysis included patients receiving ICIs only or those treated with the combination schedule only (ICIs + CT) (Table S3). We previously showed that expression levels of plasma cytokines/proteins in samples at the first evaluation could yield predictive/prognostic biomarker for ICIs therapy in NSCLC[19]. Here we found that plasma CSF-1 levels at first assessment were higher in non-responders (compared to responders) (16071 ± 1160 pg/mL vs

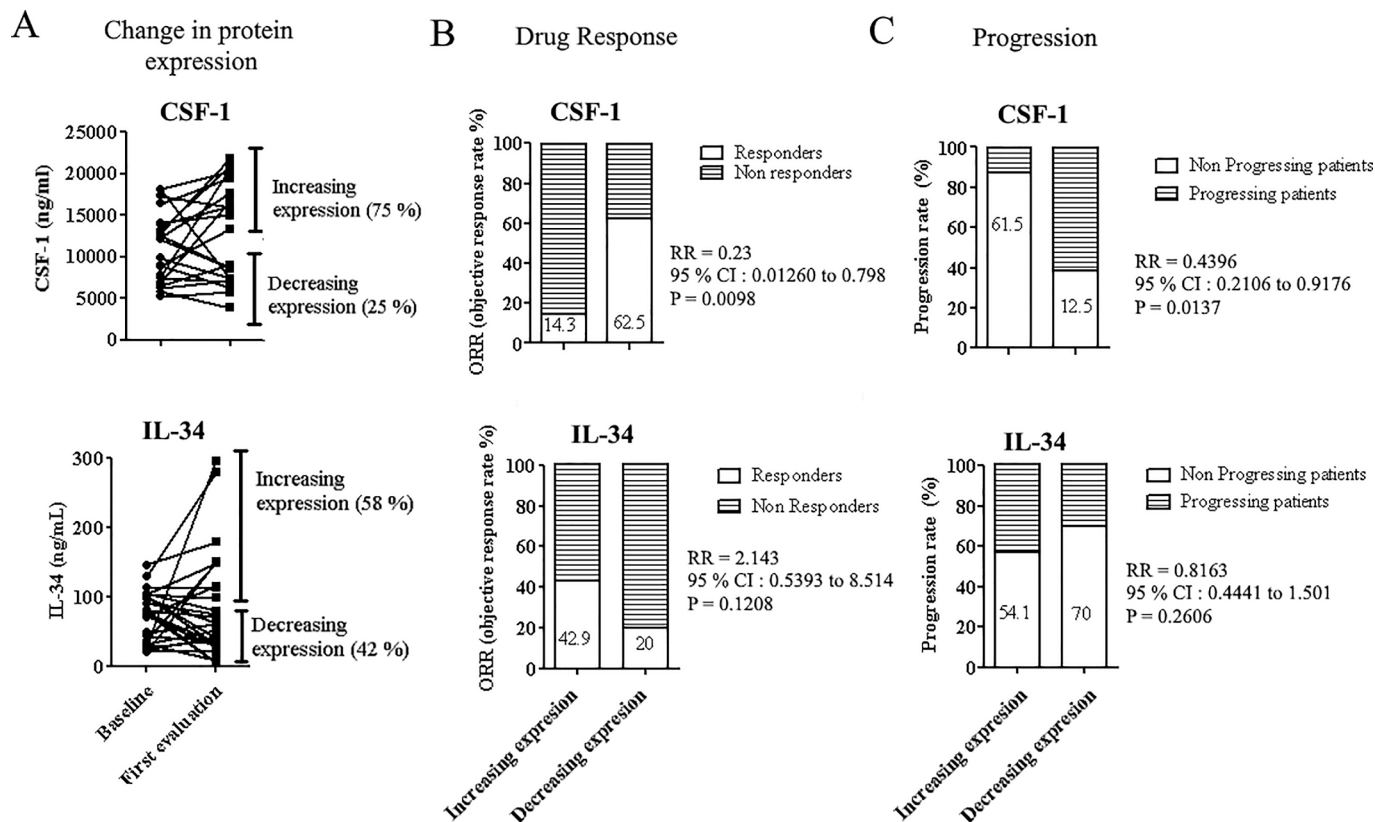
7379 ± 875 pg/mL; p = 0.0002) and progressing patients compared to non-progressing patients (17350 ± 1211 pg/mL vs 7694 ± 1015 pg/mL; p < 0.001) (Fig. 2B). We confirmed that high levels of CSF-1 at first evaluation were related with ORR (RR = 11.25; CI 95 % (1.646—76.89); p < 0.001) and patient's progression (RR = 3.75; CI 95 % (1.620—8.681); p < 0.001). Once again no relation were found between ORR, progression, and the expression levels of CSF-1R, CD163 or IL34 at the first evaluation (Table S2). As mentioned earlier, both CSF-1 and IL-34 expressions were increased in patients at the first evaluation (Fig. 1B). Considering each patient individually, 75 % and 58 % of patients exhibited increased expression levels of CSF-1 and IL-34, respectively (Fig. 3A). Interestingly, we observed a significant association between increased levels of CSF-1, poor ORR (RR = 0.23; 95 % CI (0.013—0.798); p < 0.001), and a high progression rate (RR = 0.44; 95 % CI (0.211—0.918); p = 0.014) (Fig. 3B-C). Multivariate analyses using logistic regression revealed that CSF-1 level at the first evaluation was an independent predictive factor for drug response, while patient's progression was related to CSF-1 levels both at baseline and at the first evaluation. (Table S4).

3.4. Correlations with patients' outcomes in patients receiving CT

Table 2 provides the AUC (Area under the curve) and cut-off values for CSF-1 and IL-34 concerning the objective response rate (ORR) and progression rate of patients treated with chemotherapy alone. These cut-off value were used to define high and low expressing patients. For patients treated with chemotherapy alone, initial sampling revealed no significant association between ORR and the expression levels of all Macrophage-related factors, including CSF-1R, CD163, CSF-1, and IL-34, both at baseline and at the first evaluation (Tables 2 and S2; Fig. 2). In contrast, patients' progression rate was related to the expression levels of CSF-1 (RR = 1.473; 95 % CI (0.9445—2.298); p = 0.040) and IL-34 (RR = 2.43; 95 % CI (1.376—4.287); p = 0.031)



**Fig. 2.** CSF-1 and IL34 expression according to drug response and disease progression. Plasma samples were collected from NSCLC patients at baseline (initiation) and at the first evaluation. ELISA assays were used to study CSF-1 and IL-34 expression levels in plasma samples. Then, expression values were classified according to drug response (A) and disease progression (B). Data are expressed as mean ± SD. \*p < 0.05; \*\*p < 0.01 \*\*\*p < 0.001.

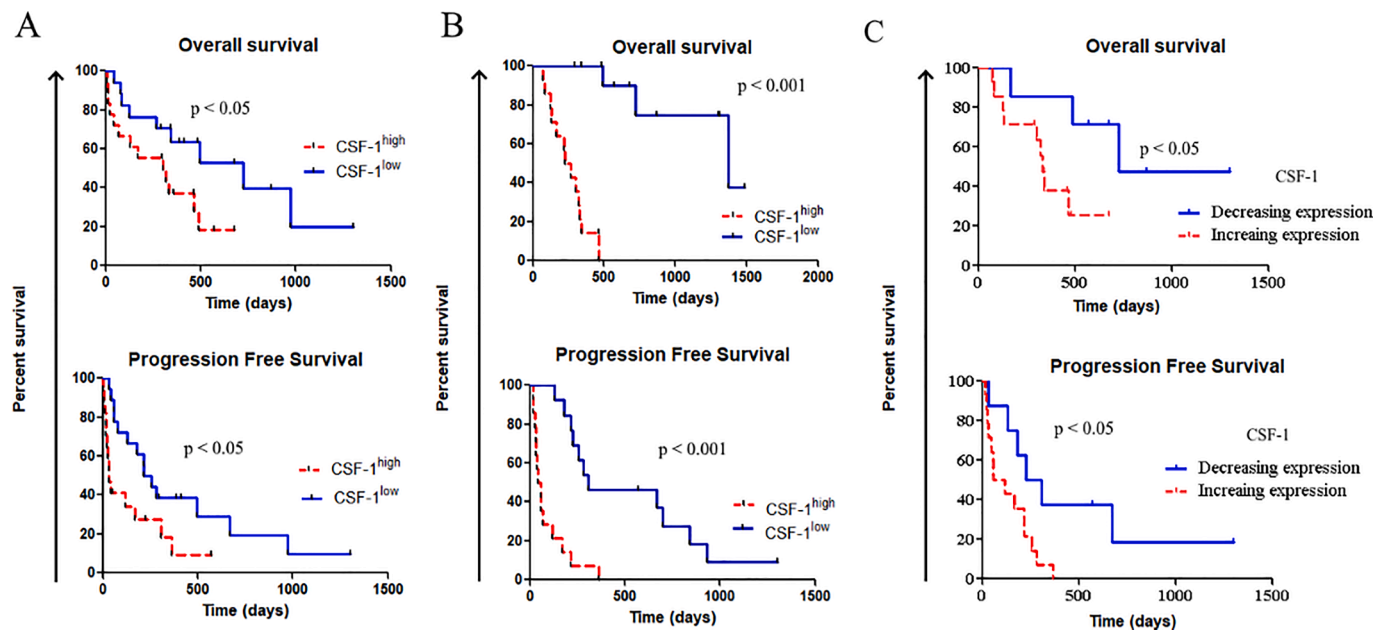


**Fig. 3. Influence of CSF-1 and IL-34 evolution on drug response and disease progression.** A) Evolution of CSF-1 and IL-34 levels at the first evaluation. B) Drug response and C) Disease progression in patients according to changes in CSF-1 and IL-34 levels observed at the first evaluation.

(Tables 2 and S2). No correlations were found for CSF-1R and CD163 (Table S2).

When considering expression levels at the first evaluation, CSF-1 was higher in progressing patients compared to non-progressing patients

(18319 ± 2075 pg/ml vs. 10180 ± 530.3 pg/ml;  $p < 0.001$ ) (Fig. 2). Association tests also revealed a significant relationship between progression rate and high levels of CSF-1 (RR = 7.58; 95 % CI (1.207—47.57);  $<0.001$ ) and IL-34 (RR = 1.80; 95 % CI



**Fig. 4. ICI treated patient's survival according to CSF-1 expression levels.** Plasma samples were collected from NSCLC patients at A) baseline (initiation) and at B) the first evaluation. Elisa assays were employed to measure CSF-1 expression levels in plasma samples. Subsequently, expression values were categorized as high or low based on the threshold value defined by ROC analysis or according to C) change in expression levels between baseline and the first evaluation. Kaplan-Meier analysis, followed by the log-rank test, was used to study patient survival.

(0.936—3.460);  $p = 0.025$ ) at the first evaluation. Once again, no relationship was found between progression or ORR and the expression levels of CSF-1R and CD163, both at baseline and at the first evaluation (refer to Table S2).

### 3.5. Correlation with patient's survival for ICIs therapy

Based on cut-off value as assessed through ROC, in patients treated with ICIs, high CSF-1 concentrations at initiation were associated with poorer progression-free survival (median survival of 33 days in the high CSF-1 group versus 259 days in the low CSF-1 group (HR = 0.199 CI 95 % (0.08453—0.4689),  $p < 0.001$ ) (Fig. 4A; Tables 2 and S5) and poorer overall survival with a median of 234.5 days in the high CSF-1 group vs 725 days in the low CSF-1 group (HR = 0.272; CI 95 % (0.108—0.688),  $p = 0.006$ ). High CSF-1 concentrations at first evaluation were correlated with shorter PFS (median 56 days vs. 487.5 days (HR = 0.137; CI 95 % (0.052—0.359),  $p < 0.001$ ) (Fig. 4B; Table S4) and poorer OS with a median of 268 days in the high CSF-1 group vs 1369 days in the low CSF-1 group (HR = 0.088; CI 95 % (0.029 to 0.268),  $p < 0.0001$ ) (Fig. 4A; Tables 2 and S5). Except for CSF-1, there was no correlation between patients' survival and the expression levels of all other markers (Table S5). It is noteworthy that Patients treated with ICIs alone, as well as those receiving a combination of ICIs and chemotherapy (ICI + CT), demonstrated similar survival patterns compared to the entire ICIs population (with or without CT). Notably, higher levels of CSF-1 were related to shorter survivals (PFS and OS) (Table S6). As mentioned earlier, patients displaying elevated levels of CSF-1 exhibited a poor ORR and a high progression rate. Consequently, we investigated the influence of increased levels of CSF-1 on patients' survival, revealing a correlation with shorter OS and PFS (Fig. 4C). When analyzing patients treated with CT, elevated CSF-1 concentrations at the first assessment, but not at baseline, were associated with shorter survival. This suggests that only baseline CSF-1 was a fully predictive factor for ICIs, being related to ORR, progression rate, and OS (Table S5). Regardless of the type of therapy (ICIs or CT), there was no correlation between patient survivals and the expression levels of all other macrophage-related markers, except for CSF-1 (Fig. 4A; Table S4). The patients' survival in ICIs groups was also shorter for those who presented a low expression of PD-L1 and PS > 1. Therefore, we implemented multivariate analysis through COX proportional hazards model, demonstrating that shorter survivals (OS and PFS) were found in ICIs patient with low levels of CSF-1 both at the baseline and at the first evaluation. (Table S7). Considering patients treated with CT, High CSF-1 concentrations at the first assessment and not at baseline was associated with survival, suggesting that only baseline CSF-1 was fully predictive of ICIs response (Progression rate and Survival) (Table S5).

### 3.6. CSF-1 suppressed lymphocyte proliferation and interfere with immunotherapy

Our data support that peripheral blood CSF-1 could favor the emergence of drug resistance in NSCLC patients treated with ICIs, suggesting that CSF-1 levels is not only related to the regulation of local peritumoral TAMs, but to a systemic change in the immune system. To understand the source of enriched CSF-1 levels in NSCLC patients, we analyzed the supernatant of NSCLC cell lines or NSCLC primary cells. We found that both cell lines (A549, H596) and primary cells secreted CSF-1 as revealed by ELISA assays (Figure S4A).

Then, we analyzed the influence of CSF-1 on immune properties of PBMC in the presence and absence of Pembrolizumab (ICI). We started by treating activated PBMC with recombinant CSF-1. PBMC was activated either with an antibody cocktail (Anti-CD3, 0.5  $\mu\text{g}/\text{mL}$ ; anti-CD28, 0.5  $\mu\text{g}/\text{mL}$  and rIL2, 20 IU/mL) or phytohemagglutinin (PHA, 10  $\mu\text{M}$ ). The two strategies were able to activate T-cells proliferation and induce the production of IFN $\gamma$  (Figures 5, S3; S4B-C). However, CSF-1 inhibited T-cell proliferation only when PBMC was treated with PHA and not with

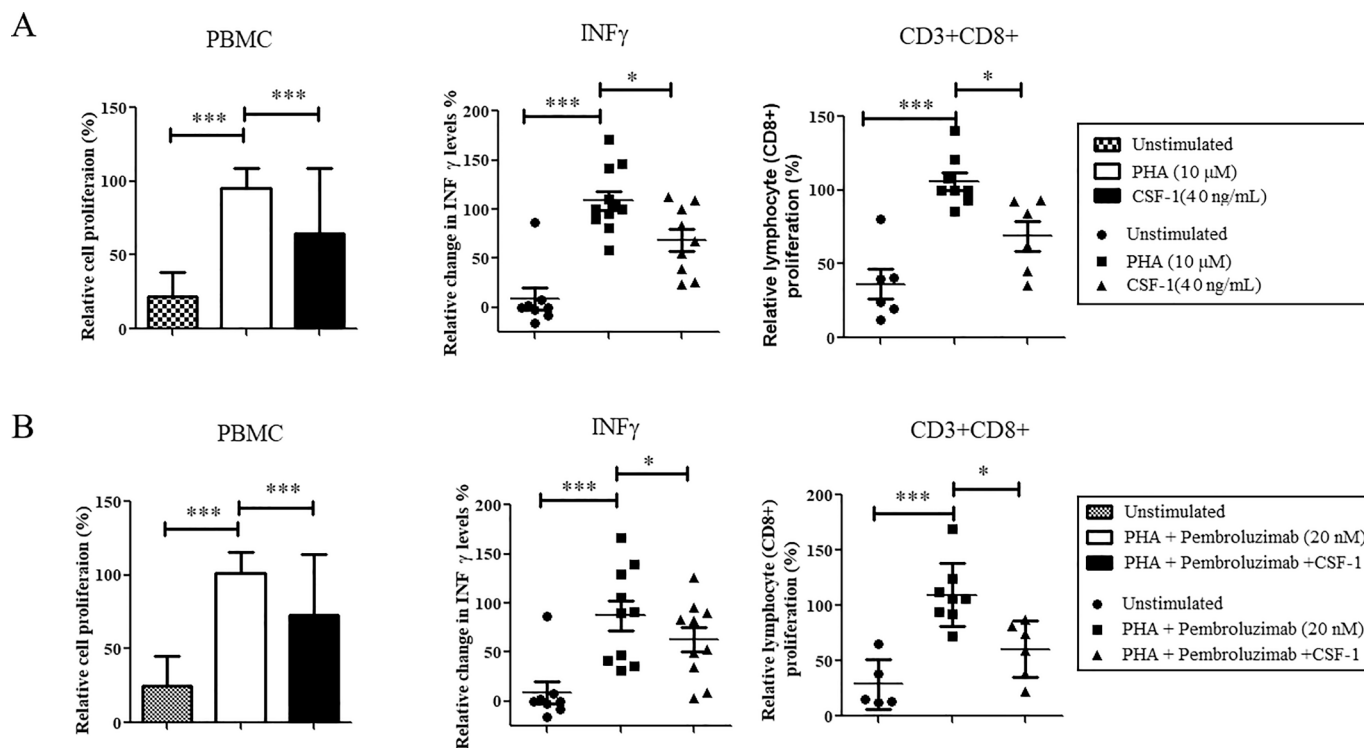
the antibody cocktail (Figures 5, S3; S4B-C). Indeed, T-lymphocytes are not reported as CSF-1R expressing cells, therefore, CSF-1 cannot directly target T-cell but required the involvement of immunosuppressive populations such as monocytes/macrophages. Consistently, Ceupens et al. have shown that the use of PHA to activate T cells requires the involvement of monocytes [25]. Therefore, we also used PHA-mediated activation of the PBMC (contains both lymphocyte and monocyte) as a surrogate to analyze lymphocyte activation in the presence of monocytes [25,26]. In this condition, we observed that CSF-1 treatment reduced lymphocyte proliferation and IFN $\gamma$  production (Fig. 5). This suggests that CSF-1 stimulates immunosuppressive properties of CSF-1R-positive cells (monocytes), which in turn suppress PHA-mediated CD8 + T-cells proliferation and reduce levels of IFN $\gamma$ . Therefore, we successfully used PLX3397, a CSF-1R inhibitor, to demonstrate that the inhibition of CSF-1R signaling reinstated CD8 + T-cells proliferation (Fig. 6A). To translate this finding into NSCLC context, we co-cultured PBMC on Macrophages primed with NSCLC-conditioned medium. Interestingly, CSF-1R receptor inhibition was potent to enhance CD8 + lymphocyte proliferation in the presence of Macrophages primed with NSCLC-conditioned medium, providing further evidence that targeting CSF-1/CSF-1R signaling could potentially enhance the activity of immune checkpoint inhibitors (ICIs), even in the presence of tumor-associated macrophages (TAMs) (Fig. 6B, S4D-E). Activated PBMC cultured on NSCLC monolayer also underwent reduced proliferation of CD8 + T-cells, but the effect was not sensitive to CSF-1R inhibition (Fig. S4F). This may be attributed to the fact that the primary effect of NSCLC-derived CSF-1 is to bestow immunosuppressive properties upon monocytes and/or macrophages, rather than directly targeting T-cell activation.

## 4. Discussion

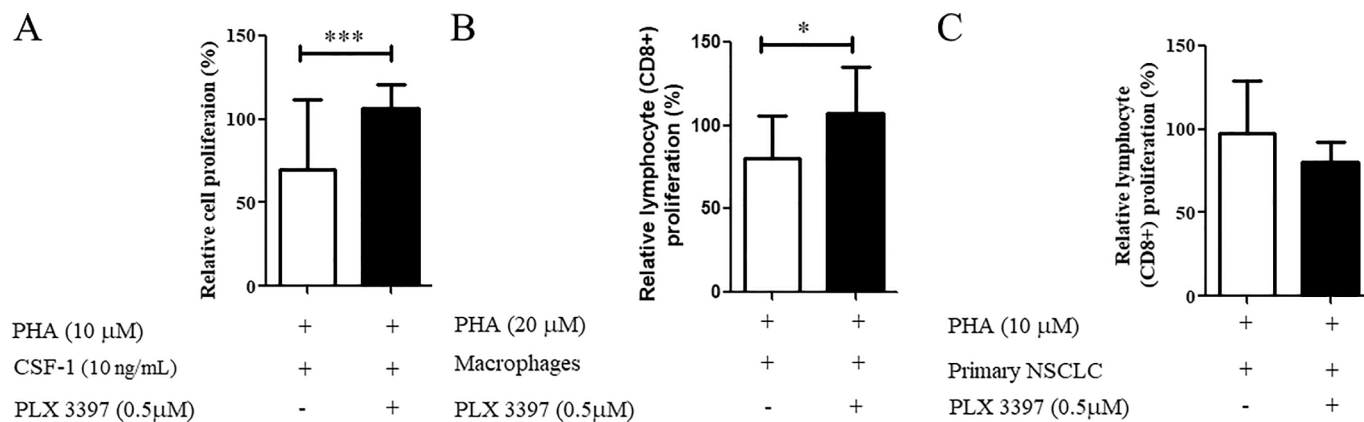
The current study investigated the prognostic value of TAMs related markers in immunotherapy for advanced NSCLC patients receiving first-line immunotherapy, associated or not with chemotherapy. We reported that high levels of peripheral (plasmatic) CSF-1 concentrations are positively correlated with disease's progression and shorter survival with ICI therapy. These results were not influenced by the fact that ICI treatment was administered alone or associated with chemotherapy.

Due to the involvement of Macrophages in immune evasion and resistance to immunotherapy [27], TAMs-related markers such as receptors (CD163, CSF-1R), and secreted cytokines (CSF-1, IL-34, IL-10, etc.), have been extensively studied as predictive biomarkers for cancer immunotherapy [28,29]. In both preclinical and clinical studies, CD163 and/or CSF-1R are commonly employed to stain and assess macrophage infiltration and their polarization into an M2 phenotype [31,32]. CD163 and CSF-1R function as receptors for the "twin ligands" IL-34 and CSF-1, respectively. The expression of these ligands is often analyzed in conjunction with CD163 and CSF-1R to comprehensively evaluate TAM density and infiltration [15]. In lung cancers, both CD163 and CSF-1R were found to be expressed on macrophages as well as cancer cells, and their expressions were positively associated with drug resistance, poor response to therapy, and shorter patient survival [8,30]. This correlation extends to their ligands as well, as studies have demonstrated that elevated expression of IL34 and CSF-1 is linked to tumor progression and poor survival in lung cancer [31]. Our data revealed that most patients (>80 %) presented high expression of CD163, while high CSF-1R staining was found in less than half of the patients. Previous works revealed high expression of CD163 and CSFR-1 in about 50 % and 49 % of patients respectively [8,32]. These discrepancies could be explained by the staining methods (automated quantification versus manual scoring), the stage (stage I rather than stage IV), the type of samples used and the positivity threshold used [12]. These potential biases and the small sample size ( $n = 37$  for ICIs and  $n = 15$  for CT) could explain the lack of correlation between the expression of these two receptors and progression or survival. In addition, even though it is quite





**Fig. 5. CSF-1 suppresses PHA-mediated activation of PBMC.** A-B) PBMC stained or not with CFSE and activated with phytohemagglutinin (PHA, 10 μg/ml) were cultured alone (A) or in the presence of Pembrolizumab (20 nM) (B) or/and CSF-1 (40 ng/mL). Cell supernatants were collected at 24 h for IFN $\gamma$  quantification. MTS proliferation assay were used to quantify lymphocyte/PBMC proliferation at 72 h, while CFSE stained cells were collected, stained with PE-Cy7-conjugated-anti-CD3/BV421conjugated-CD45/APC-conjugated-anti-CD8, and analyzed through flow cytometry. Data are expressed as mean  $\pm$  SD of at least 3 independents experiments. \*p < 0.05; \*\*\*p < 0.001.



**Fig. 6. CSF1-R inhibition interferes with CSF1 mediated immune suppression.** PBMC stained or not with CFSE and activated with phytohemagglutinin (PHA, 10 μg/ml) were cultured alone or in the presence of PLX3397 with either CSF-1 (40 ng/mL) or on Macrophages or primary NSCLC monolayer. MTS proliferation assay were used to quantify lymphocyte/PBMC proliferation at 72 h, while CFSE stained cells were collected, stained with PE-Cy7-conjugated-anti-CD3/BV421conjugated-CD45/APC-conjugated-anti-CD8, and analyzed through flow cytometry. Data are expressed as mean  $\pm$  SD of at least 3 independents experiments. \*p < 0.05; \*\*\*p < 0.001.

established that M2 macrophages are correlated with poor prognosis, recent data support that the binary view of M1 and M2 polarization is more complex and may explain discrepancies observed between studies [12,33,34].

Regarding IL-34 and CSF-1, the majority of studies have primarily focused on the biomarker values of CSF-1 and IL-34, with particular attention given to their expression in the peritumoral stroma as evaluated by IHC. Due to the limited availability of tumor biopsy samples, we opted to assess the expression of CSF-1R ligands (IL-34 and CSF-1) in plasma samples. This strategy respond to a growing need of non invasive

biomarker for ICIs therapy, and supported by previous studies suggesting that the levels of signaling proteins in plasma collected from NSCLC patients, both before any treatment and during the initial treatment evaluation, have the potential to serve as biomarkers [1,19,35]. This has led to growing interests on blood biomarkers since blood is less invasive to collect[1,36]. However, there are currently limited or few validated circulating biomarkers for ICIs therapy in NSCLC. The current data indicates that elevated levels of plasmatic CSF-1 in pretreatment samples (baseline) were significantly associated with poor ORR, high progression rate, and shorter survivals (OS and PFS) in NSCLC patients treated

with ICIs. Although some significant correlations were found between CSF-1 levels and patient progression in the control group (chemotherapy), the relations with ORR and survival were observed only with baseline (pre-treatment) samples in the ICIs groups. This suggests that plasmatic levels of CSF-1 in pre-treatment samples could potentially serve as a predictive biomarker for ICIs therapy in NSCLC.

Numerous studies have focused on investigating circulating cells of the myeloid lineage, although certain cell types, such as macrophages, are primarily found in tissues rather than in the bloodstream[37]. CSF-1 plays a significant role in regulating the immune state of monocytes/macrophages, both in the bloodstream and in tissues. Its expression in the bloodstream is correlated with TAM infiltration. Therefore, assessing CSF-1 levels in plasma samples can enhance the information obtained from the analysis of circulating myeloid cells, providing comprehensive information about peri-tumoral TAM[38].

Similar to the findings of our research, it has been observed in melanoma that CSF-1 is significantly elevated in pretreatment samples from patients and is associated with unfavorable patient outcomes. The proposed mechanism suggests that CSF-1 contributes to the recruitment and polarization of myeloid lineage cells, which can impede the effectiveness of ICI therapy[42]. Consistently, the results of our in vitro assays demonstrate that treatment of PBMC samples with CSF-1 leads to the suppression of effector CD8 + cell activation in the presence of pembrolizumab. To further validate this observation, we utilized Pexidartinib, a CSF-1R inhibitor, to reverse the immunosuppressive effects mediated by CSF-1. This confirmed that elevated levels of CSF-1 as observed in NSCLC contribute to ICIs resistance by triggering activation of CSF-1R on monocytes/macrophage cells.[39]. These observations suggest that reducing CSF-1 levels through the use of a neutralizing antibody could be a potential strategy to delay the emergence of ICI resistance in NSCLC. Because it may be possible to disrupt the immunosuppressive effects mediated by CSF-1, thereby enhancing the efficacy of ICI therapy and prolonging its effectiveness in NSCLC patients. This approach holds promise as a potential therapeutic strategy to counteract resistance mechanisms and improve patient outcomes in the context of ICI treatment. [39].

No association was observed between plasmatic levels of IL-34, the second ligand of the CSF-1R receptor, and ORR, progression rate, or patient survival in the ICIs groups. Previous studies have shown that weak expression of IL-34 as assessed by IHC, is a common characteristic in naive lung cancer tumor samples, while chemotherapy induced increased expression of IL-34[12,17]. However, the prognostic or predictive value of IL-34 in lung cancers remains unclear, as studies have produced conflicting results. This could be due to the use of more heterogeneous cohorts in terms of histology (including both NSCLC and SCLC), disease stages (ranging from stages I to IV), and the inclusion of both naive and treated samples[12,17,40,41]. To gain clarity on the role of IL-34 as a prognostic and/or predictive biomarker in NSCLC, it is crucial for future studies to address the aforementioned issues. These include considerations such as histology, disease stages (ranging from early to advanced), treatment modalities, and patient characteristics.

In summary, our study provides evidence that elevated levels of circulating CSF-1 are associated with immune evasion, resistance to ICIs, and independently correlated with poor treatment response and patients' survival in NSCLC treated with ICIs. These findings underscore the significance of CSF-1 as a potential biomarker for assessing treatment response and prognosis in NSCLC patients undergoing ICI therapy.

This work was funded by grants from La Ligue Contre Le Cancer 2021 and Legs Poix 2019.

## 5. Disclosure

Etienne Giroux Leprieur declare to have received honoraria/personal fees (advisory boards) from AstraZeneca, Bristol-Myers-Squibb MSD, and Roche.

All the authors have accepted and validated the final version of this

manuscript.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lungcan.2023.107447>.

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