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Impaired tumor-infiltrating T cells in patients with COPD impacts lung cancer response to PD-1 blockade

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At the Glance Commentary:

Scientific knowledge on the subject: The immune system is strongly involved in the establishment of chronic inflammation in chronic obstructive pulmonary disease (COPD) and in the control of tumor burden in lung cancer. However, despite the strong epidemiological link between these two diseases, the impact of COPD associated chronic inflammation on the immune contexture of lung cancer remains poorly defined.

What this study adds to the field: Here, we report that COPD disrupts the immune microenvironment of non-small cell lung cancer (NSCLC), and we identify CD8 tumor infiltrating lymphocytes (CD8 TILs) as the most affected population. Indeed, we observed higher exhaustion of CD8 TILs, identified by PD-1/TIM-3 coexpression, in NSCLC patients with coexisting moderate to severe COPD. In agreement, the prognostic value of intra-tumor $CD8^+$ T cells that has been found favorable in most cancer types and particularly in NSCLC, has no impact on the survival of patients with coexisting COPD. Together, our data point out COPD patients as a potential NSCLC patient population to treat with immune-checkpoint blockers. In accordance with this hypothesis, data obtained in a cohort of 39 nivolumab treated patients might suggest a higher efficacy of anti-PD-1 treatment in NSCLC patients with a coexisting COPD.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

Abstract

Rationale: Patients with chronic obstructive pulmonary disease (COPD) have a higher prevalence of lung cancer. The chronic inflammation associated with COPD probably promotes the earliest stages of carcinogenesis. However, once tumors have progressed to malignancy, the impact of COPD on the tumor immune microenvironment remains poorly defined, and its effects on immune-checkpoint blockers' efficacy are still unknown.

Objectives: To study the impact of COPD on the immune contexture of non-small cell lung cancer (NSCLC).

Methods: We performed in depth immune profiling of lung tumors by immunohistochemistry and we determined its impact on patients' survival (n=435). Tumor-infiltrating T lymphocyte (TILs) exhaustion by flow cytometry (n=50) was also investigated. The effectiveness of an anti-PD-1 treatment (nivolumab) was evaluated in 39 advanced-stage NSCLC patients. All data were analyzed according to patients' COPD status.

Measurments and Main Results: Remarkably, COPD severity is positively correlated with the coexpression of PD-1/TIM-3 by CD8 T cells. In agreement, we observed a loss of CD8 T cell-associated favorable clinical outcome in $COPD^+$ patients. Interestingly, a negative prognostic value of PD-L1 expression by tumor cells was observed only in highly CD8 T cell-infiltrated tumors of $COPD^+$ patients. Finally, data obtained on 39 advanced-stage NSCLC patients treated by an anti-PD-1 antibody showed longer progression free survival in $COPD^+$ patients, and also that the association between the severity of smoking and the response to nivolumab was preferentially observed in $COPD^+$ patients.

Conclusions: COPD is associated with an increased sensitivity of CD8 TILs to immune escape mechanisms developed by tumors, thus suggesting a higher sensitivity to PD-1 blockade in patients with COPD.

Keywords: NSCLC, tumor immunology, CD8 TILs, anti-PD-1.

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Introduction

Despite abundant evidence that the immune system plays a central role in controlling tumor burden (1–3), it may also have a dark side linked to the maintenance of deleterious inflammation. For instance, patients with inflammatory bowel disease (IBD) (4) or chronic pancreatitis (5) showed increased risk of developing colorectal and pancreatic cancer, respectively. Similarly, chronic obstructive pulmonary disease (COPD) is considered to be an important risk factor for lung cancer (6, 7). This inflammatory condition is linked to a more pronounced destructive inflammation of the lung, compared with non-COPD smokers, characterized by a strong release of TNF-α and CXCL-8 by epithelial cells and alveolar macrophages leading to the recruitment of inflammatory monocytes and neutrophils (8). The presence of B cells in lymphoid follicles has been reported in the airways and parenchyma of patients with COPD (9), illustrating the involvement of adaptive immunity in COPD pathophysiology. This chronic inflammation may promote the earliest stages of carcinogenesis (8) through an increased expression of genes involved in cell proliferation and survival, including NF-κB and STAT3, which are activated by cytokines such as IL-6 and TNF-α.

Once tumors have progressed to malignancy, COPD was shown to worsen the survival of patients with early-stage non-small cell lung cancer (NSCLC) (10) and emphysema was shown to be associated with increased lung cancer mortality (11). Mechanisms governing this prognostic impact, including the role of the immune system, are currently undefined. Although the tumor immune contexture in NSCLC has been extensively characterized, the COPD status of patients has not been taken into account. Nevertheless, a high density of CD8 tumor-infiltrating T lymphocytes (CD8 TILs), together with a concomitant high density of DC-Lamp$^+$ cells that signals the presence of tertiary lymphoid structures (TLS) within tumor tissues, identified patients with the best prognostic outcome (12). However, overexpression of inhibitory receptors by

tumor-infiltrating T cells, also called immune-checkpoints, can keep the immune system under control (13). In NSCLC, their cumulative expression, including programmed cell death-1 (PD-1), T-cell immunoglobulin and mucin domain-containing molecule-3 (TIM-3), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and lymphocyte activation gene-3 (LAG-3), has been described as being a hallmark of dysfunctional T cells and tumor progression (14). Drugs targeting immune-checkpoints, in particular the PD-1/PD-L1 pathway, can unleash anti-tumor immunity and mediate durable cancer regression (15–17). Nevertheless, these new treatments are not efficient in all patients and identifying factors that predict clinical response to these therapies remains a challenge. In melanoma, an association between high PD-L1 expression and clinical response to pembrolizumab had been reported (18). However, patients with PD-L1-negative tumors may also achieve durable responses. In NSCLC, several efforts have also been made recently to more accurately identify patients that would respond to checkpoint therapy. The focus here has largely been on the identification of predictive markers for response to anti-PD-1, such as tumor mutational burden (TMB) (19), PD-L1 expression by tumor cells (20, 21), and gene signature reflecting adaptive immunity (22).

In this context, the present study investigated the potential impact of COPD on the immune microenvironment of NSCLC and, thus, on patients' outcome. Our work reveals that COPD severity is positively correlated with the level of CD8 TIL exhaustion. In agreement, we observed a complete loss of CD8 T cell-associated favorable clinical outcome in $COPD^{+}$ patients. Finally, data obtained on 39 advanced-stage NSCLC patients treated by an anti-PD-1 might suggest a higher sensitivity to this treatment in patients with COPD.

Methods

Patients

A retrospective consecutive cohort of 435 NSCLC untreated patients seen between June 2001 and December 2005 at the department of Thoracic Surgery of Hôtel-Dieu hospital (Paris, France) was used to study by immunohistochemistry the immune composition of the tumor microenvironment. A second cohort of fresh tumor samples, distant non-tumoral lung samples and peripheral blood were obtained from 50 patients with untreated NSCLC who underwent surgery between March 2014 and December 2015. These samples were used to perform flow cytometry experiments. A third cohort of 39 patients with advanced-stage NSCLC receiving an anti-PD-1 antibody (nivolumab) was used to assess the effectiveness of this treatment according to COPD status. Additional details are provided in the online supplements.

COPD assessment

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria were used to assess the presence of COPD and to evaluate the severity of airflow obstruction (23). Additional details are provided in the online supplements.

Immunohistochemistry

Serial section of paraffin-embedded NSCLC tumors were stained as previously described (24). Additional details are provided in the online supplements.

Methods for cell Quantification

Calopix software (Tribvn) was used to count $CD66b^+$ and $CD68^+$ cells in the whole tumor section; CD8 T cells were counted separately in the tumor nests and in tumor stroma. DC-$Lamp^+$

cells were counted manually in the whole tumor section. The proportion of PD-L1^{+} cells among tumor cells was determined manually by at least two independent observers (JB, HO or DD). The positivity threshold was fixed at ≥ 1%. Additional details are provided in the online supplements (Table E1).

Flow cytometry

Multiple stainings on isolated mononuclear cells from Tumor, NT and Blood were performed using various antibodies (see Table E2 and Figure E1), as previously described (24) Additional details are provided in the online supplements.

Genomic DNA extraction and Illumina-based whole-exome sequencing.

Genomic DNA from tumors was isolated from formalin-fixed paraffin-embedded blocks using Maxwell® 16 FFPE Tissue LEV DNA Purification Kit (Promega), according to the manufacturer's instructions. DNA whole exome sequence (WES) data were sequenced on the Novaseq 6000 platform (25). Additional details are provided in the online supplements.

Statistics

Categorical data were compared using Chi-square tests or Fisher exact tests, as appropriate, while they were compared according to COPD stages using exact Cochran-Armitage trend test. For log-rank tests, the prognostic value of continuous variables was assessed by a quartile stratification. For Cox proportional-hazard models, immune cell densities were log-transformed. Multivariate analysis for OS was adjusted for age, gender, vascular emboli, smoking history and stratified on the tumor stage.

In flow cytometry experiments, according to data distribution, a parametric test (ANOVA, student's t test) or a non-parametric test (Kruskal-Wallis, Mann-Whitney), with appropriate post-hoc comparisons, was used to compare quantitative variables across the different groups. Correlations between quantitative parameters were assessed by using the Spearman test. Additional details are provided in the online supplements.

Results

COPD does not affect immune cell density in NSCLC tumor microenvironment.

We first investigated the impact of COPD on the composition of the tumor immune microenvironment in a retrospective cohort of 435 NSCLC patients. Among them, 45% had COPD, and in the $COPD^+$ group, 29% had a COPD GOLD stage I ($COPD^+$ I), 60% a COPD GOLD stage II ($COPD^+$ II) and 11% a COPD GOLD stage III ($COPD^+$ III) (Table 1 and E3). The mean age, the percentages of male and of smokers were higher for $COPD^+$ than $COPD^-$ patients (Table 1). Coexisting COPD was associated with significant worse survival only for NSCLC stage I patients (Figure E2). Because of the small number of $COPD^+$ III patients, the $COPD^+$ II and $COPD^+$ III groups were merged for most of the subsequent analyses. $COPD^+$ and $COPD^-$ patients did not differ in density of neutrophils ($CD66b^+$ cells), macrophages ($CD68^+$ cells), mature DCs (DC-$Lamp^+$ cells) and CD8 T cells in tumor nests ($CD8_{Tu}$) and in stroma ($CD8_s$), regardless of GOLD stage (Figure E3).

Absence of immune cell prognostic value in NSCLC patients with moderate to severe COPD

To indirectly investigate whether the functionality of immune cells in the tumor microenvironment could be modified by coexisting COPD, we first determined their impact on patient survival according to COPD status and GOLD stage (Figure 1) (Table E4 and E5). In the whole retrospective cohort and in the $COPD^-$ group, univariate Cox-regression analysis showed that $CD8_{Tu}$, CD8s and DC-$Lamp^+$ cell densities were all associated with favorable prognostic value, while neutrophil and macrophage density had no impact on patient survival (Figure 1A and B). Strikingly, $CD8_{Tu}$ cell density did not affect patient survival in $COPD^+$ patients (Figure 1C). Furthermore, $CD8_{Tu}$, $CD8_S$ and DC-$Lamp^+$ cell densities were not significantly associated with improved survival in $COPD^+$ II-III patients (Figure 1D).

Then, patients were stratified by quartiles of $CD8_{Tu}$ cell density. In $COPD^-$ patients, $CD8_{Tu}$ cell density was associated with longer overall survival (OS) as soon as the 2^{nd} quartile was reached (Figure 1E), whereas in $COPD^+$ and $COPD^+$ II-III patients the survival curves for all quartiles merged together (Figure 1F and G). DC-$Lamp^+$ cell and $CD8_S$ cell densities were not associated with significant prognostic value in $COPD^+$ II-III patients (Figure E4). Multivariate Cox-regression analysis adjusted for age, gender, vascular emboli, smoking history and stratified on tumor stages, highlighted the absence of CD8 T cell prognostic value for the stroma and tumor nests in $COPD^+$ patients (Figure 1H-J). Together, these data might suggest that the protective impact of a high adaptive immune cell infiltration in NSCLC is altered in $COPD^+$ patients and identify $CD8_{Tu}$ cells as the most affected population.

TIL exhaustion, identified by PD-1/TIM-3 co-expression, is correlated with COPD severity

Based on above results, we investigated whether effector functions of TILs were altered in $COPD^+$ patients using a prospective cohort of 50 NSCLC patients (Figure 2 and E5) (Table E6 and E7). Regardless of COPD status, within the tumor tissue (Tumor), the proportion of CTLA-4^+, LAG-3^+, PD-1^+ and TIM-3^+ cells among CD4 (Figure E5A) and CD8 T cells (Figure 2A and B) was consistently higher than in the other anatomical sites (blood and non-tumor distal lung tissue (NT)). A marked increased of $CD4^+$ $FoxP3^+$ regulatory T cells (Treg) frequency among total CD4 T cells in Tumor was also observed (Figure E6A and B). Regarding cytokine secretion, the frequency of CD4 (Figure E5B) and CD8 T cells (Figure 2C and D) positive for Granzyme B, TNF-α, IFN-γ and IL-17 was lower in Tumor than in NT. As shown by the correlation matrix exposed on Figure 2E, among CD8 TILs, PD-1 and TIM-3 expression was strongly positively correlated, as was the frequency of IFN-γ^+ and TNF-α^+ cells. Remarkably, CD8 TILs co-

expressing PD-1 and TIM-3 were restricted to Tumor (Figure 2F), and for this cell subset only, there was a significant inverse correlation with both IFN-γ and TNF-α secretion (Figure 2G). Overall, similar results were observed regarding CD4 TILs (Figure E5C and D). However, less CD4 TILs coexpressed PD-1/TIM-3 (Figure E5D), and the relationship between cytokine secretion and PD-1/TIM-3 coexpression was weaker (Figure E5C and E).

Based on above results, we investigated the link between COPD and TIL exhaustion. In COPD, airflow obstruction severity is inversely correlated with the Forced Expiratory Volume in 1 second expressed as a percentage of normal predicted values (FEV1% predicted) (see Method section). Remarkably, FEV1% predicted was inversely correlated with the proportion of CD8 TILs expressing PD-1 and co-expressing PD-1/TIM-3 in $COPD^+$ patients only (Figure 3A). In agreement, in $COPD^+$ patients, FEV1% predicted was positively correlated with the proportion of CD8 TILs secreting TNF-α and IFN-γ (Figure 3A). Regarding CD4 TILs, only IFN-γ was positively correlated with FEV1% predicted (Figure E7A). Interestingly, frequency of $TIM\text{-}3^+$, $PD\text{-}1^+$ and $TIM\text{-}3^+/PD\text{-}1^+$ cells among CD4 and CD8 TILs was higher in $COPD^+$ II-III patients than in $COPD^-$ patients (Figure 3B) (Figure E7B). The proportion of Treg among CD4 TILs was not different according to patients' COPD status (Figure E7C). Overall, these results demonstrate that COPD severity is strongly correlated with TIL exhaustion, and that this association is more pronounced for CD8 TILs.

Strong correlation between $CD8_{Tu}$ cell density and CD8 TIL exhaustion: a phenomenon exacerbated in $COPD^+$ patients

An association between CD8 TIL exhaustion ($PD\text{-}1^+$ cell frequency) and the immune composition of the tumor microenvironment (density of $CD8^+$ T cells) was recently reported in colorectal cancer (26). We investigated this interrelation in our prospective cohort and then

studied the impact of COPD. First, CD8 TIL exhaustion (based on PD-1 and TIM-3 expression) and cytokine secretion were only linked to $CD8_{Tu}$ cell and $CD8_S$ cell densities (Figure E8A). Regarding CD4 TILs, none of the immune cell densities studied was associated with their exhaustion, and only their cytokine secretion was slightly inversely correlated with $CD8_S$ cell density (Figure E7D). Due to the strong association between CD8 TIL exhaustion and their density in the tumor nests, we then focused our analysis on CD8 TILs. Importantly, $CD8_{Tu}$ cell density and CD8 TIL exhaustion were more strongly associated in $COPD^+$ patients than in $COPD^-$ patients (Figure 3C) and (Figure E8B and C).

To confirm these results, median $CD8_{Tu}$ cell density was used to separate patients into two groups according to a Low (Figure 3D) or a High (Figure 3E) $CD8_{Tu}$ cell density. In the $CD8_{Tu}^{Low}$ group, the level of CD8 TIL exhaustion did not differ according to COPD status (Figure 3D). In the $CD8_{Tu}^{High}$ group, the frequencies of CD8 TILs expressing TIM-3 and co-expressing PD-1/TIM-3 were significantly higher in $COPD^+$ patients than in $COPD^-$ patients (Figure 3E). Overall, CD8 TIL exhaustion was restricted to highly CD8 T cell infiltrated tumors and this phenomenon was exacerbated in $COPD^+$ patients.

PD-L1 expression by malignant cells is associated with shorter survival only in $CD8_{Tu}^{High}$ $COPD^+$ patients

The strong impact of immunosuppression on tumor burden is based on TIL exhaustion, but also on concomitant mechanisms that malignant cells develop to avoid immune surveillance. The most-studied mechanism is probably PD-L1 expression by malignant cells (Figure E9A-D). No difference of PD-L1 expression by tumor cells was observed according to patients' COPD status and Gold stages (retrospective cohort) (Figure E9F). Consistent with previous studies, we

found that high CD8 T cell density is associated with high PD-L1 expression by tumor cells (Figure E9G) (27, 28). The frequency of tumor cells expressing PD-L1 was also higher, but to a lesser extent, in neutrophil^{High}, macrophage^{High} and DC-Lamp^{High} groups. Additionally, among these highly infiltrated groups, PD-L1 expression was similar between COPD^{-} and COPD^{+} patients (Figure E9G).

We then investigated whether coexisting COPD modified the prognostic value of PD-L1 expression by tumor cells. Whatever the group of patients considered, PD-L1 expression was not associated with significant prognostic value (Figure 4A and B). Since PD-L1 expression was strongly linked to CD8 T cell density (Figure E9G), we then deciphered the prognostic value of PD-L1 according to a High/Low $CD8_{Tu}$ cell density, and also to patient COPD status. For $CD8_{Tu}^{Low}$ groups, PD-L1 expression was not associated with significant prognostic value in COPD^{-} or in COPD^{+} patients (Figure 4C and D). Interestingly, for $CD8_{Tu}^{High}$ groups, PD-L1 expression did not affect survival for COPD^{-} patients (Figure 4E), but was associated with a reduced OS for COPD^{+} patients (Figure 4F). Moreover, in COPD^{-} patients, the prognostic value of $CD8_{Tu}$ and of $CD8_{S}$ cell density was similar whether tumor cells expressed PD-L1 or not (Figure 4G). Remarkably, for COPD^{+} patients, $CD8_{Tu}$ and $CD8_{S}$ cell densities were associated with extended OS for those with PD-L1^{-} tumors, while these prognostic values were not observed in the PD-L1^{+} groups (Figure 4H). Finally, these results were confirmed in subgroups of patients defined by a cut-off of PD-L1^{+} tumor cell frequency ≥5% (Figure E10A and B) and ≥10% (Figure E10C and D).

Anti-PD-1 antibody (nivolumab) efficacy in advanced-stage NSCLC patients according to coexisting COPD and to smoke exposure

We investigated the impact of COPD on the effectiveness of an anti-PD-1 antibody from a cohort of 39 patients with advanced-stage NSCLC receiving nivolumab (Table E8). The percentage of smokers and the number of pack-years were higher for $COPD^+$ than $COPD^-$ patients, while no significant differences were observed between the two groups of patients regarding NSCLC subtypes, the duration of follow-up, the age and the gender (Table E8). At the completion of the study, a significantly longer progression-free survival (PFS) (Figure 5A and B) and a higher percentage of patients still alive (Table E8 and E9) (Figure 5C) were observed in the $COPD^+$ group. However, we did not observe any impact of COPD severity, assessed using the FEV1 % predicted, on nivolumab efficacy (Table E9).

In addition, it was previously shown that the efficacy of pembrolizumab, another anti-PD-1 antibody, was greater in patients with a smoking-associated mutational signature or with a higher nonsynonymous mutation burden in tumor (19). Consequently, we investigated whether the increased PFS seen with nivolumab in $COPD^+$ patients was linked to the higher smoke exposure observed in this group (Table E8). In the whole cohort, a smoke exposure >30 pack-years was associated with a better PFS and OS (Table E9), while in non-COPD patients, smoke exposure >30 pack-years was not associated significantly with a better PFS or OS (Table E9) (Figure 5D and F). Remarkably, in the $COPD^+$ group, a smoke exposure >30 pack-years was associated with a better PFS and also with a dramatic improvement of OS (Table E9) (Figure 5E and G). Regarding PFS, these results were confirmed when smoke exposure was assessed using number of pack-years as a continuous variable (Table E9).

A strong relationship between tobacco smoke exposure and the number of somatic mutations was previously reported in NSCLC (29). Consequently, we tried to investigate whether the number of nonsynonymous mutations per megabase (TMB) differ according to patients'COPD status, by performing whole exome sequencing (WES) experiments. Among the

22 patients for whom enough DNA was available (Figure 5C), we did not observe any difference regarding the TMB in $COPD^+$ patients compared to non-COPD patients (Figure E11A). Remarkably, the number of pack-years was significantly correlated with the TMB only in non-COPD patients (Figure E11C-E). Additionally, it has been shown that *TP53* and/or *KRAS*-mutated tumors, two mutations strongly associated with tobacco smoke exposure (30, 31) , had a better response to PD-1 blockade (32). Among the 31 patients who had a molecular interrogation of their tumor before starting anti-PD-1 treatment (Figure 5C), using next-generation sequencing (NGS), we did not detect a differential distribution of *TP53* and/or *KRAS*-mutated tumors according to patients' COPD status (Figure E11B).

Our preliminary data might suggest a differential link between smoking history and response to nivolumab, but also between TMB and smoking history, in $COPD^+$ patients vs non-COPD patients. Consequently, we investigated whether COPD and tobacco had a synergistic impact on CD8 TIL exhaustion and on immune cell prognostic value. First, in the prospective cohort, the number of pack-years was positively correlated with the proportion of CD8 TILs co-expressing PD-1/TIM-3 in $COPD^+$ patients only (Figure E12A). Moreover, a higher CD8 TILs exhaustion was observed in $COPD^+$ group compared with $COPD^-$ group, in patients with a number of pack-years > 60 (Figure E12B). Secondly, in our retrospective cohort of 435 NSCLC patients, we investigated whether immune cell prognostic value (CD8Tu, CD8S and DC-$Lamp^+$ cells) was impacted by a strong smoke exposure (>30 pack-years). In heavy smokers, immune cell prognostic value was stronger in non-COPD patients (Figure E12C), than the one observed in Figure 1B. Conversely, in $COPD^+$ patients, the $CD8_S$ cell prognostic value was not significant in heavy smokers, and was completely absent for $CD8_{Tu}$ cells (HR:1.01, p=0.948) (Figure E12D). Altogether, NSCLC patients with COPD, a group characterized by a complete loss of CD8 T cell-

associated favorable clinical outcome in heavy smokers probably due to their marked exhaustion, also showed a longer PFS after nivolumab treatment.

Discussion

Our main objective was to evaluate the potential impact of COPD on the immune contexture of NSCLC. First, immune cell densities did not differ according to the COPD status of the patients. Immune cell recruitment into the malignant lesion is probably driven by tumor cells according to their immunogenicity linked to their mutational burden, thereby attenuating the impact of coexisting COPD. Co-occurring genetic alterations in *KRAS*-mutant lung adenocarcinoma, were associated with different tumor immune patterns, and could be a first argument supporting this hypothesis (33). However, our study showed higher TIL exhaustion in the $COPD^+$ II-III group, an impaired protective impact of immune cells in patients with COPD, and identified CD8 TILs as the most affected population.

The characteristics of the NSCLC immune contexture linked to CD8 TIL exhaustion are not completely defined, and the role of COPD in this phenomenon is not completely elucidated. We identified that CD8 TIL exhaustion was restricted to highly CD8 T cell infiltrated tumors, and these findings were exacerbated in $COPD^+$ patients. Interestingly, PD-1 expression, which is upregulated on T cells after TCR ligation (34), is also upregulated in activated T cells by IL-6 through STAT3-dependent mechanisms (35). Accordingly, high frequency of exhausted TILs observed in $COPD^+$ II-III patients could be driven in part by the increased amounts of IL-6 previously reported in the sputum of $COPD^+$ patients (36). It is also conceivable that when a tumor forms near an emphysematous/inflammatory lesion, the surrounding inflammation, including numerous cytokines (IL-6, TNF-α, IL-1β) and chemokines (CXCL8, CXCL1) (37) modify autocrine and paracrine interactions between malignant cells and infiltrating leucocytes. Interestingly, *ex vivo* infections with influenza virus of lung resections showed an impaired antiviral function of $CD8^+$ T cells in $COPD^+$ patients compared to non-COPD patients, through an up-regulation of PD-1 expression. Moreover, if these PD-1 expressing CD8 T cells, coming from

the non-tumor distal lung tissue, are overrepresented in the tumor immune microenvironment of COPD^{+} patients, they might participate in the deviation of the anti-tumor immune response observed in patients with COPD (38). Nevertheless, orthogonal approaches, including gene expression analysis related to the immune response in cancer, are required to precisely identify the characteristics of the inflammation disrupting the tumor immune contexture of patients with NSCLC and COPD.

In agreement with other studies (17, 27, 39), we observed that PD-L1 expression by NSCLC tumor cells highlights the presence of an active tumor immune microenvironment in lung cancer, independently of COPD. This is probably due to the fact that cancer cells may upregulate PD-L1 expression in response to IFN-γ secretion by TILs (39). Studies of the prognostic value of PD-L1 expression in patients with NSCLC has yielded inconsistent data (40–44). These conflicting results could be due to the fact that the amount of CD8 TILs and patients' COPD status had not been taken into account. In our study, the prognostic value of PD-L1 expression was restricted to COPD^{+} patients belonging to the $CD8_{Tu}^{High}$ group. It probably reflects the effectiveness of mechanisms that cancer cells develop to avoid immune surveillance in a subpopulation of patients characterized by a strong CD8 TIL exhaustion. In $CD8_{Tu}^{Low}$, the lack of PD-L1 prognostic value was probably linked to the lowest impact of the PD-1/PD-L1 pathway on a weakly active anti-tumor immune response. In this situation, PD-L1 expression is probably driven more through oncogenic pathways, including inactivation of STK11/LKB1 (45) or loss of function of the tumor suppressor PTEN (46), and not associated to a strong PD-1 expression by CD8 TILs. Moreover, immune cell prognostic value was completely absent for tumors from COPD^{+} patients expressing PD-L1, probably because the level of TIL exhaustion was higher in this group. Interestingly, in melanoma, preexisting CD8 TILs in tumor microenvironment were required for tumor regression after treatment with pembrolizumab (27), and CD8 TILs

coexpressing PD-1/CTLA-4 had been proposed as a biomarker to predict response to anti-PD-1 (47). Altogether, for NSCLC patients with moderate to severe COPD, our results support an increased predictive potential of PD-L1 expression by tumor cells for the response to checkpoint inhibitors targeting the PD-l/PD-L1 pathway (48).

In accordance with this assumption, our preliminary data obtained in a cohort of 39 nivolumab treated NSCLC patients showed a longer PFS in patients with coexisting COPD (19). Nevertheless, we did not identify any impact of COPD severity on the response to anti-PD-1. It is conceivable that we had not enough patients to observe this kind of effect. Moreover, other confounding factors not explored in our work could explain the lack of association between COPD severity and the response to nivolumab, including among others, CD8 TIL density and PD-L1 expression by tumor cells. Remarkably, at the time of publication, the study from *Mark et al,* also observed that the presence of COPD was associated with longer progression free survival interval in patients treated with anti-PD-1 or anti-PD-L1 (49). However, a smoking-associated mutational signature had previously been suggested to signal a better response to immunotherapy. Since, tobacco and COPD may be confounding factors in the response to nivolumab, we tried to investigate the interplay between these two factors, and if possible their respective impact. Our study suggests that the impact of tobacco on the response to nivolumab would be mainly observed in $COPD^+$ patients. In agreement with this assumption, when anti-PD1/PD-L1 impact was evaluated only in former smokers, *Mark et al* still observed a longer PFS and OS in $COPD^+$ patients (49). We also showed a stronger impact on patients' survival of the immune microenvironment in heavy smokers without COPD. In this situation, tumors are probably more immunogenic and the presence of a strong specific adaptive anti-tumor immune response even more important for patients' survival. Interestingly, in $COPD^+$ patients, the number of pack-years was positively correlated with the level of CD8 TIL exhaustion, and the impact of $CD8_{Tu}$ cell

density on patients' survival was completely absent in heavy smokers. Altogether, the higher nivolumab efficacy observed in $COPD^+$ patients, probably reflects the effectiveness of PD-1 blockers to unleash anti-tumor CD8 T cell response in a subpopulation of patients characterized by strong CD8 TIL exhaustion.

Finally, we tried to investigated whether the longer PFS observed in nivolumab $COPD^+$ treated patients was linked to a higher TMB induced by a stronger tobacco smoke exposure. Unfortunately, we were able to perform such work only on 22 anti-PD-1 treated patients. However, our preliminary data are not in favor of a higher TMB in $COPD^+$ patients. Moreover, the frequency of *KRAS* and *TP53* mutations, two mutations strongly linked to tobacco smoke exposure (30, 31) that have been suggested as being associated with longer PFS in anti-PD-1 treated patients (32) were not enriched in $COPD^+$ patients. In agreement with these results, our previous work did not detect a higher frequency of *TP53* or *KRAS* mutations in $COPD^+$ patients, again characterized by a stronger smoke exposure, in a cohort of 282 lung adenocarcinomas (31). Beyond the scope of the present work, studies using larger cohorts of patients are mandatory to precisely determine whether the longer PFS after PD-1 blockade observed in $COPD^+$ patients is mostly linked or not to their higher tobacco smoke exposure. Such studies will also allow to determine whether tobacco smoke exposure differentially impacts CD8 TIL exhaustion, TMB and response to PD-1 blockers, in $COPD^+$ patients vs non-COPD patients.

However, there were some limitations to our study. First, our cohort of anti-PD-1 treated patients is restricted to 39 patients, mainly because we needed a follow up of at least one year, combined to fully characterized respiratory functions. Secondly, the use of three cohorts inevitably increased the number of comparisons and the number of false discovery rate, but we tried to reduce this risk using the most appropriate statistical methodology and by applying appropriate adjustments for multiple comparisons. The impact of histological subtypes on our

findings was not fully analyzed, even it does not seem to impact our main results (Data not shown). In fact, we did not address this point to avoid an increase of multiple comparisons. Another limitation, inherent to this kind of work, is related to tissue heterogeneity, slide thickness and surface area covered. However, one strength of our study is that in immunochemistry experiments, we worked on whole tumor sections and not on tumor microarrays (TMA), with senior pathologists, using automates for staining, scanning and counting. All these precautions were intended to increase the accuracy and reproducibility of our data.

By deciphering the immune network of NSCLC, we pinpointed that the use of immunological biomarkers to evaluate patient prognosis (50) and to predict the response to therapy should definitively take into account the coexistence of COPD. This chronic inflammatory disease of the lung is not fully understood and its impact on tumor immune microenvironment functions and on response to immune checkpoint inhibitors will deserve additional experiments. We recommend that clinical trials should investigate whether CD8 TIL density and smoke exposure, together with coexisting COPD may identified the best responders to therapies targeting immune-checkpoints. Overall, our study emphasizes the need to consider the impact of coexisting chronic inflammation on the tumor immune microenvironment in other cancer types. In the era of precision medicine, such studies should extend the clinical success of immunotherapies in cancer.

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758 **Table 1**

Characteristics		COPD⁻ Number	COPD⁻ (%)	COPD⁺ Number	COPD⁺ (%)	P-value
Subjects		238	(55)	197	(45)	
Gender Male/Female		165/73	(69/31)	174/23	(88/12)	**<0.0001***
Age (years) Mean ± SD		61 ± 12		64 ± 9		**0.01**
Smokers		182	(78)	173	(91)	**0.0003***
Smoking history Pack-years ± SD		36 ± 24		49 ± 24		**<0.0001**
Histological Subtypes	**ADC**	173	(73)	136	(69)	0.70*
	SSC	53	(22)	50	(25)	
	Others	12	(5)	11	(6)	
pTNM stages	**I**	102	(43)	94	(48)	0.05*
	II	58	(24)	59	(30)	
	III-IV	78	(33)	44	(22)	
Vascular Emboli	**Yes**	125	(53)	118	(60)	0.21*
	No	98	(41)	65	(33)	
	ND	15	(6)	14	(7)	
Pleural Invasion	**Yes**	124	(52)	110	(56)	0.70#
	No	110	(46)	83	(42)	
	ND	4	(2)	4	(2)	
Lobectomy		210	(88)	171	(87)	0.65*
Pneumonectomy		28	(12)	26	(13)	
% FEV1/FVC Mean ± SD		79 ± 5		60 ± 8		**<0.0001**
FEV1 (% of a predicted value) Mean ± SD		90 ± 17		71 ± 17		**<0.0001**

759

760 **Table 1. Baseline characteristics of 435 patients with NSCLC (retrospective cohort).**

761 All variables were evaluated among the 435 patients with NSCLC. Quantitative data were
762 compared between COPD⁻ and COPD⁺ patients by Student's t test. Categorical data were
763 compared by chi-square test (*) or Fisher exact test (#), as appropriate. *P* values <0.05 were
764 considered statistically significant and appear in bold. Abbreviations: ADC, adenocarcinoma;
765 SSC, squamous cell carcinoma; ND, Not determined.

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Figures and figure legends:

Figure 1

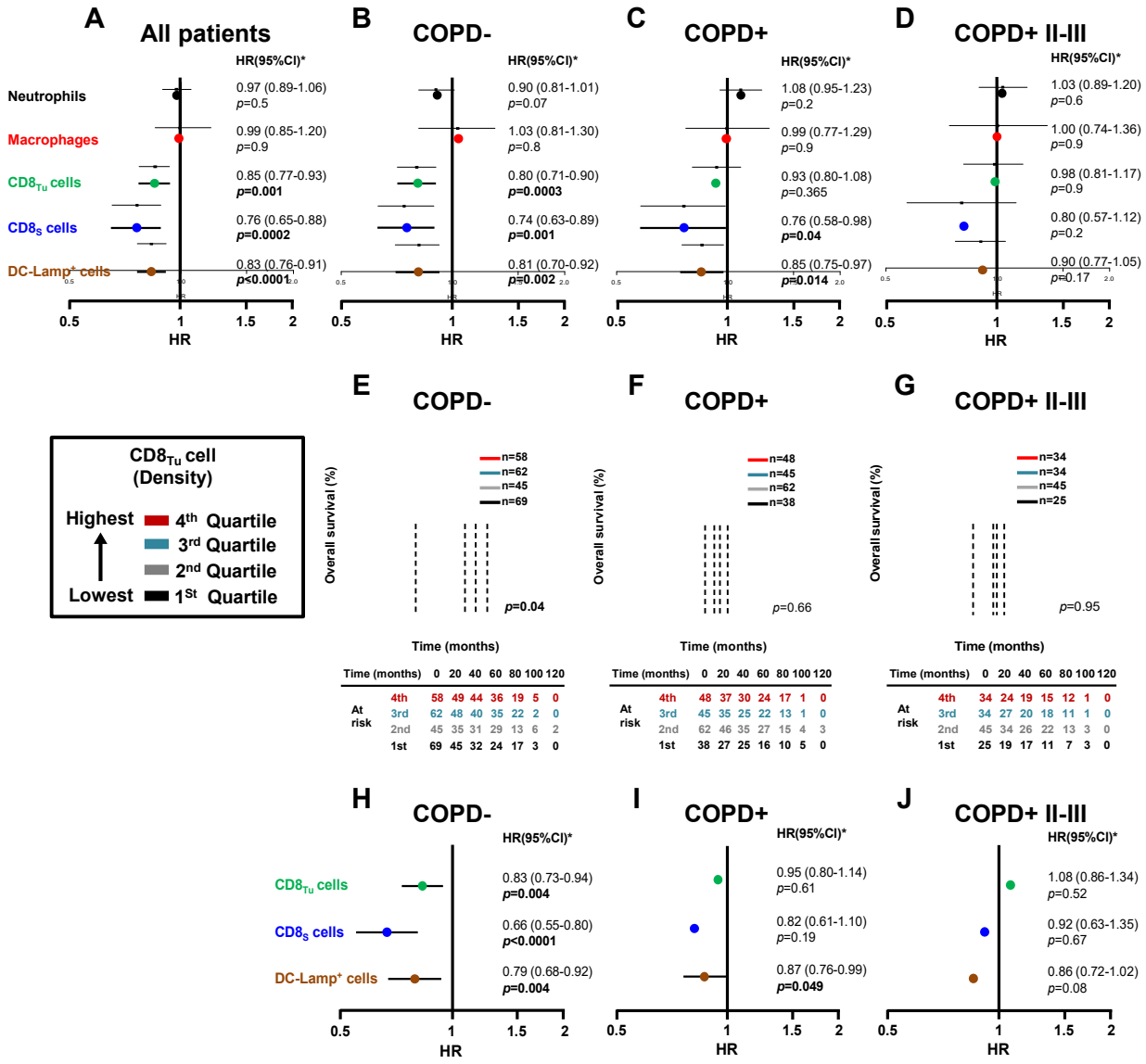

Figure 1. Prognostic value of immune cell densities in NSCLC according to COPD status (retrospective cohort). (**A-D**) Forest plots of univariate Cox-regression analysis showing the impact of neutrophil (n=435), macrophage (n=435), $CD8_{Tu}$ (n=427), CD8s (n=435) and DC-Lamp$^+$ cell (n=435) density on overall survival (OS) in the whole cohort (all patients) (**A**), COPD$^-$ (**B**), COPD$^+$ (**C**) and COPD$^+$ II-III patients (**D**). (**E-G**) The quartile method was used to stratify patients into four groups by density of $CD8_{Tu}$ cells, from the lowest (1st quartile, black curves) to the highest density of $CD8_{Tu}$ cells (4th quartile, red curves). Kaplan-Meier curves of OS according to $CD8_{Tu}$ cell density in COPD$^-$ (**E**), COPD$^+$

(**F**) and COPD^{+} II-III patients (**G**). The horizontal dashed lines represent the median survivals. (**H-J**) Forest plots of multivariate Cox-regression analysis showing the impact of $CD8_{Tu}$, CD8s and DC-Lamp^{+} cell density on OS adjusted for age, gender, vascular emboli, smoking history and stratified on the stage of the tumor, in COPD^{-} (**H**), COPD^{+} (**I**) and COPD^{+} II-III patients (**J**). *P* values <0.05 were considered statistically significant and appear in bold.

Figure 2

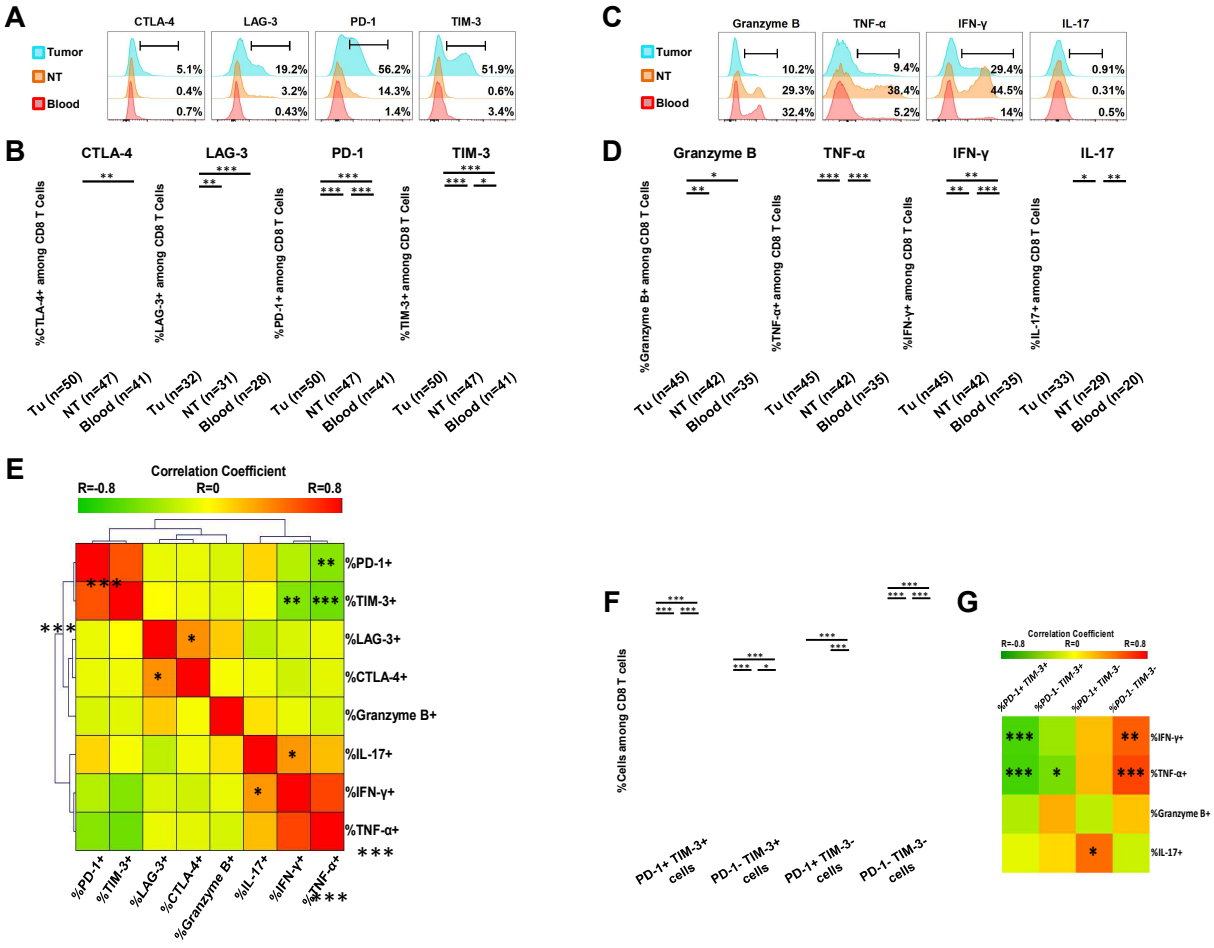

Figure 2. Characterization of CD8 tumor-infiltrating T lymphocytes (CD8 TILs) in NSCLC (prospective cohort).

(**A**) Histograms showing, for one representative patient, the frequency of CTLA-4$^+$, LAG-3$^+$, PD-1$^+$ and TIM-3$^+$ cells among CD8 T cells in Tumor, NT and blood. (**B**) Frequency of CTLA-4$^+$, LAG-3$^+$, PD-1$^+$ and TIM-3$^+$ cells among CD8 T cells in Tumor, NT and blood. (**C**) Histograms showing, for one representative patient, the frequency of Granzyme B$^+$, TNF-α$^+$, IFN-γ$^+$ and IL-17$^+$ cells among CD8 T cells in Tumor, NT and blood. (**D**) Frequency of Granzyme B$^+$, TNF-α$^+$, IFN-γ$^+$ and IL-17$^+$ cells among CD8 T cells in Tumor, NT and blood. (**E**) Spearman-correlation matrix of parameters studied by flow cytometry. Each colored square illustrates the correlation between two parameters. Red color illustrates a strong

positive correlation, and green color illustrates a strong negative correlation. (**F**) Frequencies of PD-1^{+} TIM-3^{+}, PD-1^{+} TIM-3^{-}, PD-1^{-} TIM-3^{+}, PD-1^{-} TIM-3^{-} cells among CD8 T cells in Tumor (black circle, n=50), NT (grey circle, n=47) and blood (white circle, n=41). (**G**) Correlation between the frequencies of PD-1^{+} TIM-3^{+}, PD-1^{-} TIM-3^{+}, PD-1^{+} TIM-3^{-} and PD-1^{-} TIM-3^{-} cells among CD8 TILs and frequencies of Granzyme B^{+}, IL-17^{+}, TNF-α^{+} and IFN-γ^{+} cells among CD8 TILs. In (**B**), (**D**) and (**F**) data are expressed as mean and an ANOVA or a Kruskal–Wallis test followed by an appropriate correction was applied based on Shapiro normality test. In (**E**) and (**G**) a spearman test was applied. *p<0.05, **p< 0.01 and ***p<0.001.

Figure 3

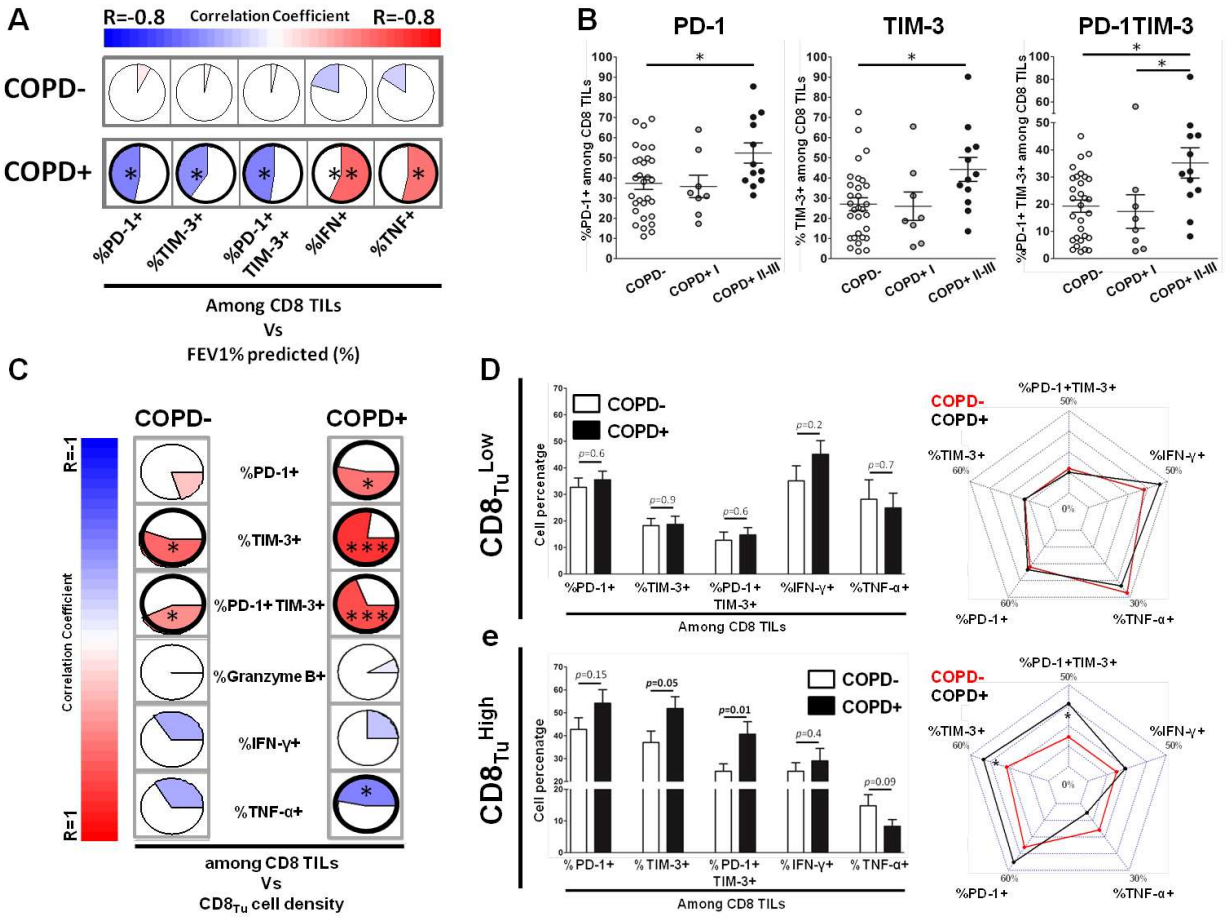

Figure 3. CD8 TIL exhaustion according to the COPD status of the patients (prospective cohort).

(**A-E**) CD8 TIL characterization in COPD$^-$ (n=30), COPD$^+$ I (n=8) and COPD$^+$ II-III (n=12) patients. (**A**) Graphical representation of Spearman correlations between the FEV1% predicted and the frequencies of PD-1$^+$, TIM-3$^+$, PD-1$^+$ TIM-3$^+$, Granzyme B$^+$, IFN-γ$^+$ and TNF-α$^+$ cells among CD8 TILs, in COPD$^-$ patients and COPD$^+$ patients. Red and blue colors indicate positive and negative correlations, respectively; the lighter is the color, the less significant is the corresponding correlation. The filled fraction of the circle in each of the pie charts corresponds to the absolute value of the associated Spearman correlation coefficient. (**B**) Frequencies of PD-1$^+$, TIM-3$^+$ and PD-1$^+$ TIM-3$^+$ cells among CD8 TILs. (**C**) Graphical representation of Spearman correlations in COPD$^-$ and COPD$^+$ patients, between $CD8_{Tu}$ cell

densities and flow cytometry data, including the frequencies of PD-1$^+$, of TIM-3$^+$, of PD-1$^+$ TIM-3$^+$, Granzyme B$^+$, IFN-γ$^+$ and TNF-α$^+$ cells among CD8 TILs. Graphical representation is the same than in Figure 3A. (**D**-**E**) The median $CD8_{Tu}$ cell density was used to stratify patients into $CD8_{Tu}^{Low}$ (**D**) or $CD8_{Tu}^{High}$ (**E**) groups. (**D-E**) Histograms and radar plots showing the frequencies of PD-1$^+$, TIM-3$^+$, PD-1$^+$ TIM-3$^+$, IFN- γ$^+$ and TNF-α$^+$ cells among CD8 TILs according to COPD status in $CD8_{Tu}^{Low}$ group and $CD8_{Tu}^{High}$ group. In (**A**) and (**C**) a Spearman Test was performed. In **(B)** data are expressed as mean ± SEM and a parametric test (ANOVA with post-hoc Bonferroni correction) or a nonparametric test (Kruskal–Wallis test followed by a post-hoc Dunn's test) was applied based on Shapiro normality test. In (**D**-**E**) data are expressed as mean, and a parametric test (Student't test) or a nonparametric test (Mann Whitney test) was applied based on Shapiro normality test. *p<0.05, **p< 0.01 and ***p<0.001.

Figure 4

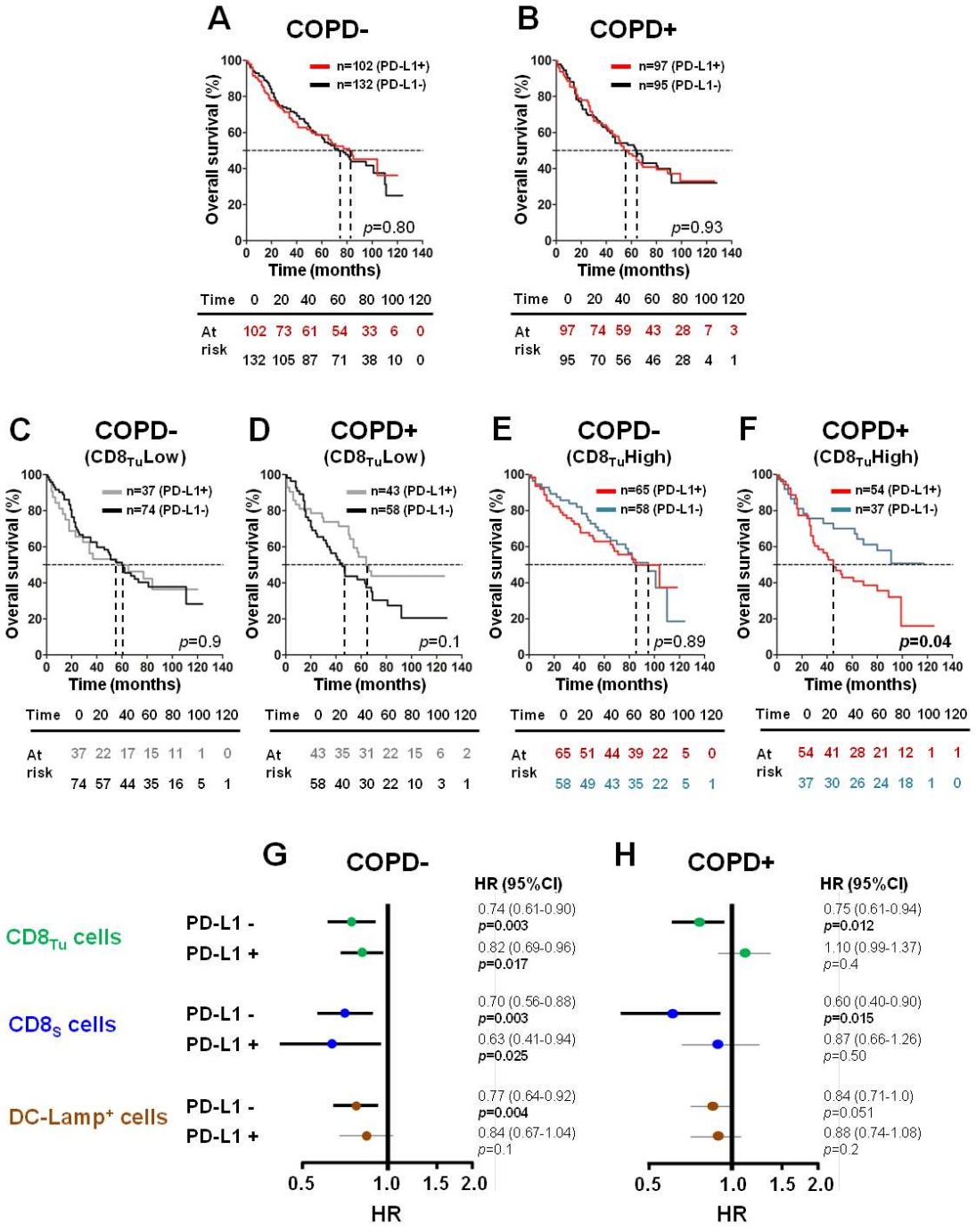

Figure 4. Immune cell prognostic value according to COPD status and PD-L1 expression by tumor cells (retrospective cohort).

Kaplan-Meier curves of OS in NSCLC patients according to PD-L1 expression by tumor cells, in $COPD^-$ patients (n=234) (**A**) and in $COPD^+$ patients (n=192) (**B**). (**C**) and (**D**) Kaplan-Meier curves of OS in the $CD8_{Tu}^{Low}$ group according to PD-L1 expression by tumor

cells in $COPD^-$ (n=111) (**C**) and $COPD^+$ patients (n=101) (**D**). (**E**) and (**F**) Kaplan-Meier curves of OS in the $CD8_{Tu}^{High}$ group according to PD-L1 expression by tumor cells in $COPD^-$ (n=123) (**E**) and $COPD^+$ patients (n=91) (**F**). (**G**) and (**H**) Forest plots of univariate Cox-regression analyses showing the impact of $CD8_{Tu}$ cell, CD8s cell and DC-$Lamp^+$ cell density on the OS according to PD-L1 expression by tumor cells, in $COPD^-$ patients (**G**) and in $COPD^+$ patients (**H**). *P* values <0.05 were considered statistically significant and appear in bold.

Figure 5

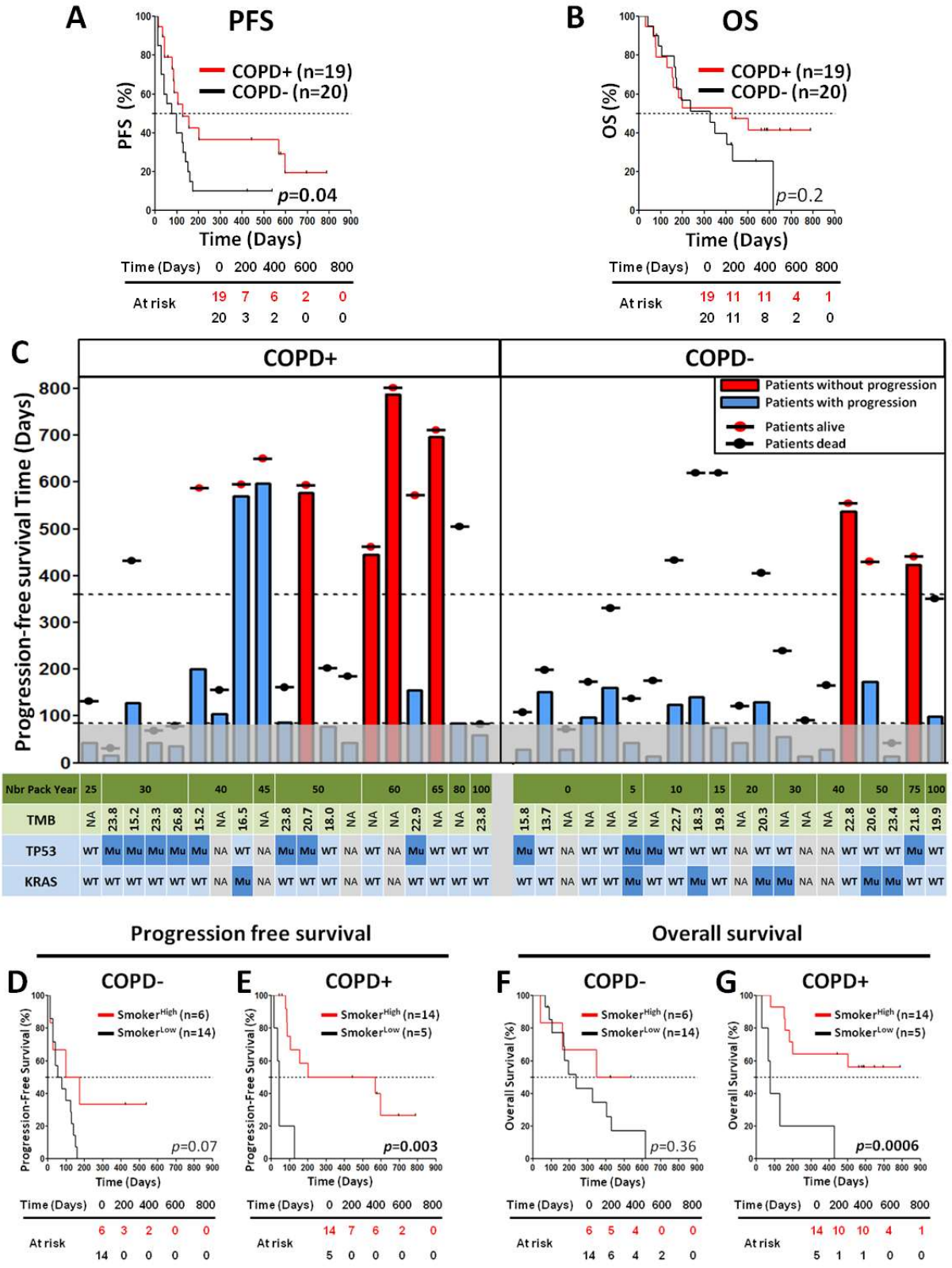

Figure 5. Nivolumab efficacy in advanced-stage NSCLC patients according to a coexisting COPD and to smoke exposure. (**A-B**) Kaplan-Meier curves of progression-free

survival (PFS) and overall survival (OS) in $COPD^-$ (n=20) and $COPD^+$ patients (n=19). Progression-free survival was defined as the time from the start date of nivolumab treatment to the date of the first documented event of tumor progression. (**C**) Characteristics of the response to nivolumab treatment according to patients' COPD status and to smoking exposure. TMB, Tumor mutation burden expressed as number of nonsynonymous mutations per megabase and determined using whole exome sequencing experiments; Mu, Mutated-tumor; WT, Wild type-Tumor, NA; Not available. (**D-G**) Kaplan-Meier curves of progression-free survival (**D-E**) and of overall survival (**F-G**) in $COPD^-$ (n=20) (**D**,**F**) and $COPD^+$ (n=19) (**E,G**) patients according to smoking exposure. Patients were stratified into two groups, $Smoker^{High}$ (>30 pack-years) and $Smoker^{Low}$ (≤30 pack-years). Tick marks indicate censoring events. *P* values <0.05 were considered statistically significant and appear in bold.