Cancer Letters 464 (2019) 5–14

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Cisplatin increases PD-L1 expression and optimizes immune check-point blockade in non-small cell lung cancer

Ludovic Fournel[a,b,c,*], Zherui Wu[b], Nicolas Stadler[b], Diane Damotte[d], Filippo Lococo[e], Geoffroy Boulle[d], Evelyne Ségal-Bendirdjian[b], Antonio Bobbio[b,c], Philippe Icard[c,f], Jean Trédaniel[b,g], Marco Alifano[c], Patricia Forgez[b]

[a] Ecole Doctorale de Cancérologie, Paris Sud-Saclay University, Paris, France
[b] Inserm, UMR 1124, Cellular Homeostasis and Cancer Signaling, Paris-Descartes University, Paris, France
[c] Thoracic Surgery Department, Cochin Hospital, Paris-Center University Hospitals, APHP, Paris-Descartes University, Paris, France
[d] Pathology Department, Cochin Hospital, Paris-Center University Hospitals, APHP, Paris-Descartes University, Paris, France
[e] Thoracic Surgery Department, Azienda Unità Sanitaria Locale/IRCCS of Reggio Emilia, Arcispedale Santa Maria Nuova, Reggio Emilia, Italy
[f] Inserm U1086, BioTICLA Axis, Caen-Normandie University, F-14000, France
[g] Respiratory Medicine and Thoracic-oncology Department, Saint-Joseph Hospital, Paris-Descartes University, Paris, France

ARTICLE INFO

Keywords:
Immune check-point inhibitors
Cisplatin
PD-L1
Lung cancer
Neoadjuvant treatment

ABSTRACT

The number of clinical protocols testing combined therapies including immune check-point inhibitors and platinum salts is currently increasing in lung cancer treatment, however preclinical studies and rationale are often lacking. Here, we evaluated the impact of cisplatin treatment on PD-L1 expression analyzing the clinicopathological characteristics of patients who received cisplatin-based neoadjuvant chemotherapy followed by surgery and showed that cisplatin-based induction treatment significantly increased PD-L1 staining in both tumor and immune cells from the microenvironment. Twenty-two patients exhibited positive PD-L1 staining variation after neoadjuvant chemotherapy; including 9 (23.1%) patients switching from $< 50\%$ to $\geq 50\%$ of stained tumor-cells. We also confirmed the up-regulation of PD-L1 by cisplatin, at both RNA and protein levels, in nude and immunocompetent mice bearing tumors grafted with A549, LNM-R, or LLC1 lung cancer cell lines. The combined administration of anti-PD-L1 antibodies (3 mg/kg) and cisplatin (1 mg/kg) to mice harboring lung carcinoma significantly reduced tumor growth compared to single agent treatments and controls. Overall, these results suggest that cisplatin treatment could synergize with PD-1/PD-L1 blockade to increase the clinical response, in particular through early and sustainable enhancement of PD-L1 expression.

1. Introduction

The survival of patients with metastatic lung cancer has significantly improved with platinum-based treatments and, more recently, with targeted therapies and immunotherapies. Despite therapeutic advances, lung cancer remains the world-leading cause of cancer-related death (approximately 2 million per year), due to innate or acquired tumor resistance to treatments [1]. Identifying theranostic biomarkers to target the right population remains a major objective in the clinical management of this disease. Indeed, programmed cell death 1 (PD-1) and ligand (PD-L1) check-point blockade allows durable disease control and increased survival rates as compared to conventional chemotherapy [2–4]. However, this advantage occurs in only a subset of metastatic non-small cell lung carcinoma (NSCLC) patients, as those exhibiting more than 50% PD-L1-stained tumor cells [2,5]. Considering the widespread use of immune check-points inhibitors (ICIs) in different settings of the NSCLC multimodal treatment, a pronounced interest has been shown for PD-L1 expression and its regulation [6,7]. Several studies evaluated the impact of systemic treatments in tumor microenvironment, most notably with platinum-salts, and the ability of chemotherapy to modulate tumor immunogenicity and more generally to influence protagonists of the adaptive immunity [8,9]. Even though the implicated mechanisms remain undetermined, some authors proposed a transcriptional up-regulation of PD-L1 gene by oncogenic signaling pathways such as mTOR/PI3K/AKT [10,11]. This represented a rationale for clinically testing combination treatments associating chemotherapy to anti-PD-1/PD-L1 [12–15]. In the present study, we evaluated the impact of cisplatin on PD-L1 expression in patients

* Corresponding author. UMR 1124, Cellular homeostasis and cancer signalling, 45 rue des Saints-Pères, Faculté des Saints-Pères, 75006, Paris, France.
E-mail address: ludovic.fournel@aphp.fr (L. Fournel).

https://doi.org/10.1016/j.canlet.2019.08.005
Received 20 June 2019; Received in revised form 5 August 2019; Accepted 7 August 2019

List of abbreviations

AKT	protein kinase B
ALK	anaplastic lymphoma kinase
CDDP	cis-diamminedichloroplatinum, cisplatin
COPD	chronic obstructive pulmonary disease
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
ICI	immune check-point inhibitor
IHC	immunohistochemistry
K-ras	Kirsten rat sarcoma
LLC1	Lewis lung carcinoma 1
mTOR	mammalian target of rapamycin
NAC	neoadjuvant chemotherapy
NSCLC	non-small cell lung carcinoma
PD-1/PD-L1	programmed cell death 1 and ligand
PI3K	Phosphoinositide 3-kinase
PR	partial response
SD	stable disease

receiving cisplatin-based neoadjuvant chemotherapy (NAC) and in preclinical models of lung cancers, along with combined therapies involving ICIs.

2. Materials and methods

2.1. Patients and surgical specimens

We retrospectively reviewed a cohort of 122 patients operated in a single-center of thoracic surgery between 2000 and 2007, for curative-intent resection of locally-advanced lung-carcinoma after NAC which allowed partial response (PR) or stable disease (SD), at computed-tomography evaluation [16]. All cases were preliminarily discussed by a multidisciplinary board. In 39 out of 122 cases, paraffin embedded blocks of metastatic ipsilateral mediastinal lymph-nodes, obtained at initial staging by mediastinoscopy or anterior mediastinotomy, were available for performing matched analysis with post-chemotherapy tumor specimens, subsequently allowing comparative immunochemistry (IHC) assessment of PD-L1 expression. To note, molecular analyses such as EGFR or K-ras mutations and ALK-ROS1 rearrangements testing, were not performed, at that time. Epidemiologic data, clinical and pathological responses to chemotherapy (expressed as percentage of necrosis, fibrosis to viable tumor cells) were collected and analyzed. “Viability” was defined by the presence of tumor cells exhibiting distinct nuclear chromatin, and intact nuclear or cytoplasmic membrane, and the absence of sign of necrosis (i.e. karyorrhexis, karyolysis, and pyknosis).

In addition, we evaluated the PD-L1 status in a second cohort of 20 patients who underwent up-front surgical resection of NSCLC with postoperative pathological mediastinal lymph-node assessment showing minimal pN2 disease. As these patients did not receive any NAC, the comparative evaluation of PD-L1 status between invaded lymph nodes and pulmonary tissues was used as “control” group.

Informed consent was obtained from all patients or relatives (in case of deceased patients). The protocol was approved by the local ethics committee (CPP Ile de France II, n°2008–133 and 2012 06–12) in agreement with French law and declaration of Helsinki.

2.2. Immunohistochemistry staining and evaluation

For each tumor, we performed PD-L1 immunostaining on fresh-cut slides from representative blocks using an anti-PD-L1 antibody (E1L3N, Cell signaling) on Bond automat (Leica) as previously described and validated by the PATTERN French thoracic pathologists group [17]. Staining was double blinded analyzed by at least one expert pathologist. PD-L1 staining evaluation on tumor cells was based on previously published scores [18]. Briefly, tumor cell PD-L1 staining was scored as the percentage of positive cells. The variable ΔPD-L1 was calculated as the difference of the percentage of tumor cells labeled for PD-L1, before and after treatment. Patients who exhibited a switch of PD-L1 labeling from $< 50\%$ to $\geq 50\%$ tumor cells were categorized as $\Delta PD\text{-}L1_{50}$ positive. The immune cells scoring for PD-L1 staining was attributed as follows: score 0: no immune cells positive for PD-L1; score 1: 1–10% of the tumor surface occupied with PD-L1 + immune cells; score 2: more than 10% of the tumor surface occupied with PD-L1 + immune cells. Alveolar macrophages were not counted within the immune cells positive for PD-L1.

Staining of CD8 lymphocytes was performed using anti-CD8 antibodies (SP16, Spring Bio). After incubation sections were scanned using Nanozoomer, and count of $CD8^+$ cells performed with Halo® software to calculate the cell density expressed as number of stained cells/mm^2. A secondary analysis was then performed in the tumor zone predefined manually to evaluate the density of intra-tumor T-lymphocytes.

2.3. Cell lines, culture and reagents

The human lung cancer cell lines A549 (CCL-185™), and murine cells from Lewis lung carcinoma model LLC1 (CRL-1642™) were purchased at ATCC®. LNM-R was subcloned from LNM-35 as described previously [19]. All cells were grown in DMEM (Gibco®) supplemented with 10% fetal bovine serum (FBS) (Gibco®) and 2 mM glutamine, at 37 °C, in a humidified atmosphere of 5% CO2 [20]. For *in-vitro* experiments, cells were grown until 80% confluence and then exposed to different treatments in media supplemented with 1% FBS. The AKT-inhibitor MK2206 was purchased from Selleckchem®.

2.4. Quantitative RT-PCR, SDS-Page, Western-blotting and immunocytochemistry assays

Were performed as previously described [21]. See details in supplementary methods.

2.5. Tumor xenografts

Four-week-old male athymic NMRI-Foxn1 nu/nu mice (Janvier™) and C57BL/6j (Janvier™) were used to generate experimental tumors by grafting human lung cancer cell lines (A549, or LNM-R), or mouse lung carcinoma cell line (LLC1), respectively. Mice were injected in the flanks at single or multiple injections, according to the experiment, with 10^6 LNM-R cells, 5×10^6 A549 cells, or 5×10^5 LLC1 cells. LNM-R cells are a very aggressive cell line, highly metastatic, and isolated from LNM-35 [22]. This model is representative of “aggressive” late stage lung carcinoma. When tumors reached a volume of 90–100 mm3 (tumor volumes were calculated using the formula: $(L \times W^2)/2$), animals were randomized in the 5 following treatment groups and received retro-orbital injections of: cisplatin (cis-diamminedichloroplatinum(II): CDDP), vehicle (PBS), anti-PD-L1 (Bio X cell®, 10 mg/kg), murine total-IgGs, or the combination CDDP and anti-PD-L1. The antibodies were injected at the dose of 10 mg/kg at day 1 and 5. The CDDP was injected at the dose of (1 mg/kg) at day 1, 3, 5, 8, and 10. Basing on preliminary experiments, the posology of cisplatin was chosen to remain with a subtoxic dose (LD_{50} for cisplatin-injected i.p in mice of 12 mg/kg). In experiments requiring RT-PCR and Western-Blot comparative analyses for PD-L1 expression, a biopsy was performed under isoflurane-

anesthetized animals, when tumor burdens reached 100 mm3. Animals were then treated with cisplatin (1 mg/kg) or PBS i.v for 24 h, and the whole tumor specimen then was removed. Tumor samples were frozen for further protein or RNA extraction.

All procedures were performed in accordance with the "Guide of the Care and Use of Laboratory Animals". An institutional review board approval was obtained by « Le Comité d'Ethique en l'Expérimentation Animale Charles Darwin #B751201».

2.6. Statistics

Descriptive data were expressed as frequencies for qualitative variables and mean (± SD) or median for continuous variables according to the observed distribution. In columns, curves, or ΔPD-L1 statistics, data were expressed as mean (± SEM). Categorical data were compared using Chi^2, or Mac-Nemar test, as appropriate. Continuous and normally distributed variable resulting from the analysis of matched tissues were performed using paired student t-test. Comparisons between groups were performed using two-way ANOVA. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc. La Jolla, USA).

Table 1
Distribution of NAC free patients according to PD-L1 status and tumor site.

Percentage of PD-L1 stained cells	0%	1–49%	≥50%
Lung primary tumors	n = 13	n = 5	n = 2
Mediastinal lymph nodes	n = 15	n = 3	n = 2

3. Results

3.1. PD-L1 expression is not different in lung primary tumor and associated metastatic mediastinal lymph-nodes

We analyzed PD-L1 expression from both primary lung cancer and associated metastatic lymph nodes in 20 patients with N2 disease who did not receive any induction therapy (minimal N2). As shown in Fig. 1A, no significant difference was observed between the two sites (p = 0.62).

The distribution of PD-L1 status, using a three group classification (50% or higher/1–49%/0%), of primary lung tumors and metastatic mediastinal lymph nodes in the cohort of patients which did not receive neoadjuvant cisplatin based chemotherapy, was nearly identical (Table 1). In most cases (13/20), PD-L1 status was similar between the

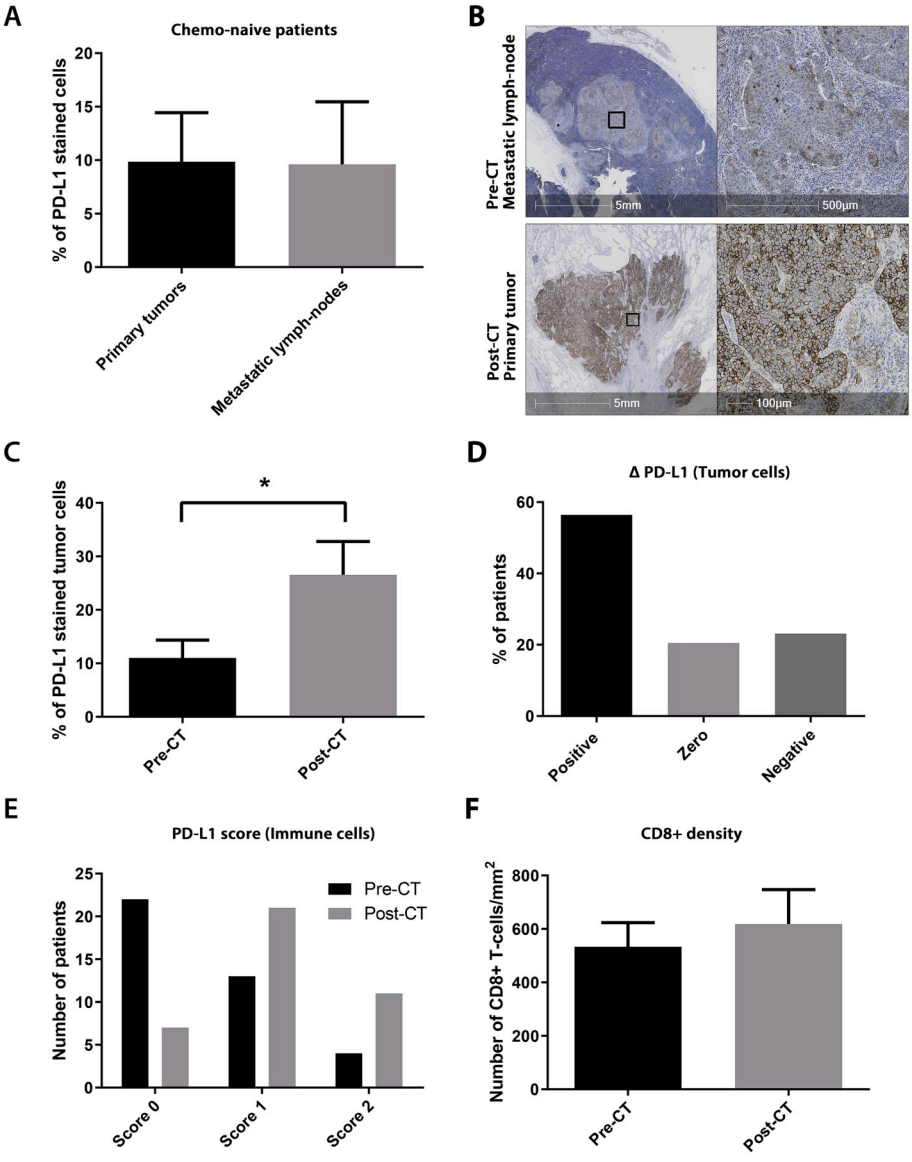


Fig. 1. Influence of neoadjuvant cisplatin-based chemotherapy on PD-L1 expression in operated patients, IHC analyses. (A) PD-L1 staining of tumor cells in patients who underwent up-front surgery for resection of a minimal pN2 NSCLC (n = 20). Quantification was manually performed and expressed as percentage of labeled cells with subsequent matched-pairs comparison between primary tumors and associated metastatic mediastinal lymph-nodes. **(B, C).** IHC analysis from patients who received neoajuvant cisplatin-based chemotherapy (n = 39) for paired-comparison of PD-L1 staining in metastatic mediastinal lymph nodes (pre-CT) and resected primary lung tumors (post-CT), black square indicates the focus area. **(D).** Distribution of ΔPD-L1 (matched post-CT - pre-CT percentage of PD-L1 positive tumor cells) among patients who received induction therapy. **(E)** PD-L1 staining of tumor-infiltrating immune cells in the same cohort of patients, pre- and post-CT samples were classified into 3 different scores (see "methods"). **(F)** Density (number of cells/mm^2) of $CD8^+$ T-lymphocytes in the tumor area and comparison between matched samples. *indicates a P value < 0.05, error-bars: standard error of the mean.

two sites, with 5 tumors harboring a higher percentage of PD-L1 stained cells in the primary than in lymph nodes. Only one patient showed a positive PD-L1 staining ≥50% of tumor cells in the lung primary versus < 50% in lymph nodes. Thus expression of PD-L1 by tumor cells at metastatic nodal locations can be considered as representative of those in the primary tumor, allowing comparative analysis of PD-L1 expression in lymph-nodes cancer cells (baseline) and post-NAC resected lung tumor specimen from the same patient. Respective data were used for calculating ΔPD-L1.

PD-L1 staining of immune cells in the microenvironment was also evaluated and compared between metastatic mediastinal lymph nodes and lung primary. The distribution of patients exhibiting a PD-L1 immune-score > 1 was not significantly different between nodal and pulmonary sites (15 ± 8.2% versus 35 ± 10.9%, p = 0.27.

3.2. Cisplatin-based NAC induces PD-L1 expression by tumor and immune cells in resected specimens of human NSCLC

Thirty-nine patients with available pre- and post-chemotherapy matched tumor tissues were identified. There were 29 (74.4%) men and 10 (25.6%) women, with mean age 56.5 (± 8.1) years. Most of patients were smokers (76.9%) with a mean tobacco consumption of 37.1 (± 6.2) pack-years. Chronic obstructive pulmonary disease (COPD) patients represented 51.3% of the whole cohort. Induction chemotherapy consisted in administrations of a doublet including cisplatin which allowed PR or SD in 15 (38.5%) and 24 (61.5%) patients, respectively.

The pathological analysis of resected lung specimens revealed that tumors were predominantly adenocarcinomas) and that mean rates of histologic changes were distributed as follows: necrosis 16.1 ± 20.3%, fibrosis 48.2 ± 27.7%, and viable tumor cells 35.7 ± 23.0% (Table 2).

PD-L1 expression was evaluated on tissue of the resected primary lung tumor, in this cohort of 39 patients. The IHC results of PD-L1 staining showed a higher percentage of labeled tumor cells after NAC than baseline (26% versus 11%, respectively, p = 0.017), suggesting an inducing effect of cisplatin on PD-L1 expression (Fig. 1B and C). Interestingly, ΔPD-L1 was positive, zero or negative in 22 (56.4%), 8 (20.5%) and 9 (23.1%) of the cases, respectively (Fig. 1D). Among the 22 ΔPD-L1 positive patients, 9 (23.1% overall) exhibited a switch of PD-L1 labeling from < 50% to ≥ 50% and were categorized as $\Delta PD\text{-}L1_{50}$ positive. Analyzing the possible associations between PD-L1 expression and pathological changes after NAC, we noted that the subgroup exhibiting < 5% of PD-L1 positive tumor cells in lymph-nodes (pre-treatment) showed significantly higher rate of fibrosis (p = 0.034) and a trend towards lower rate of viable tumor cells (p = 0.101) after NAC. Inversely, more than 50% of PD-L1 positive tumor cells in the lung primary (post-treatment) was significantly associated to a lower rate of fibrosis (p = 0.030), but a higher rate of necrosis (p = 0.011) after NAC. Furthermore, we observed that the rate of specimens with more than 50% viable tumor cells was significantly higher in the subgroup of $\Delta PD\text{-}L1_{50}$ positive cases versus $\Delta PD\text{-}L1_{50}$ negative ones (p = 0.019) (Table 3). In exploring potential interrelationships between clinical characteristics and ΔPD-L1, we did not find significant association with clinical variables such as, age (p = 0.60), gender (p = 0.78), smoking (p = 0.84), COPD (0.23), histological type of the tumor (p = 0.39), or number of NAC cures (p = 0.40).

We also performed PD-L1 staining on resected metastatic mediastinal lymph-nodes after NAC. Amongst 39 patients, 15 were downstaged during definitive pathological analysis, as harvested mediastinal lymph nodes were tumor free (ypN2 negative). We were able to perform PD-L1 staining on 22 patients, with available metastatic nodal tissue, and found a trend (p = 0.17) towards higher PD-L1 expression after NAC, with a mean percentage of labeled tumor cells in pre- and post-NAC of 5.7 ± 2.1% and 13.3 ± 5.4%, respectively. This suggested that NAC could increase PDL-1 expression of tumor cells in nodal secondary sites.

PD-L1 staining of immune cells in the microenvironment was also evaluated and compared between matched pre- and post-chemotherapy tissues. The percentage of tumors exhibiting a PD-L1 immune-score > 1 was significantly higher in the post-treatment group (25.6% versus 10.3%, p = 0.001), suggesting an up-regulation of PD-L1 by cisplatin in immune cells (Fig. 1E). The rate of viable tumor cells shows a pronounced trend towards an increase in the group of patients with positive immune-score variation (43.6% versus 31.8% mean rate of viable tumor cells in positive ΔPD-L1 immune-score versus others, p = 0.11).

Concerning $CD8^+$ staining in matched samples, the mean number of $CD8^+$ lymphocytes in the tumor area did not significantly change after induction therapy (p = 0.59, Fig. 1F). In addition, there was no significant association between variations of PD-L1 in tumor cells and corresponding number of $CD8^+$ lymphocytes (p = 0.92).

Table 2
Characteristics of patients included in the NAC cohort.

Variable	Number of patients (%)
Gender	
male	29 (74.4%)
female	10 (25.6%)
Age	
≤60 years	25 (64.1%)
> 60 years	14 (35.9%)
Smoking	
Never	9 (23.1%)
Current/former	30 (76.9%)
COPD	
Yes	20 (51.3%)
No	19 (48.7%)
cStage	
Ia,b-IIa,b	0 (0.0%)
IIIa	38 (97.4%)
IIIb	1 (2.6%)
IV	0 (0.0%)
Type of NAC	
Cis + gemci	18 (46.2%)
Cis + vino	13 (33.3%)
Cis + other	8 (20.5%)
Number of Cures of NAC	
2	16 (41.0%)
3	16 (41.0%)
4	7 (18.0%)
Resection	
Lobectomy	20 (51.3%)
Bilobectomy	4 (10.3%)
Pneumonectom	15 (38.5%)
pStage	
Ia,b	7 (17.9%)
Ia,b	8 (20.5%)
IIIa	22 (56.4%)
IIIb	2 (5.1%)
IV	0 (0.0%)
Histology	
ADK	21 (53.8%)
SCC	11 (28.2%)
NSCLC	6 (15.4%)
LCNEC	1 (2.6%)
yp Viable tumor	
≤50%	10 (25.6%)
> 50%	29 (74.4%)
yp Fibrosis	
≤30%	13 (33.3%)
> 30%	26 (66.7%)
yp Necrosis	
≤30%	30 (76.9%)
> 30%	9 (23.1%)

Cis: cisplatin; gemci: gemcitabine; vino: vinorelbine; ADK: adenocarcinoma; SCC: squamous-cell carcinoma; LCNEC: large-cell neuroendocrine carcinoma; yp: post-NAC pathological assessment.

Table 3
Interrelationship between PD-L1 staining and clinicopathological features of NAC patients.

Variable	Mean (± SEM) ΔPD-L1	P-value	Proportion of $\Delta PD\text{-}L1_{50}$ positive patients in the category (%)	P-value
Gender				
Male	13.7 (6.8)	0.78	20.7	0.66
Female	17.6 (12.3)		30.0	
Age > 60 years				
Yes	11.7 (8.4)	0.60	14.3	0.28
No	17.8 (8.5)		28.0	
Smoker				
Current/former	14.7 (6.9)	0.84	23.3	0.66
Never	17.7 (14.5)		22.2	
COPD				
Yes	22.6 (8.2)	0.23	30.0	0.25
No	7.8 (9.2)		15.8	
Type of NAC				
Cis + gemci	16.7 (5.8)	0.67	22.2	0.80
Cis + vino	8.6 (4.2)		20.0	
Cis + other	23.2 (8.3)		33.3	
Number of NAC cures				
< 4	17.3 (6.2)	0.40	20.0	0.67
≥ 4	1.8 (25.6)		23.5	
yp Viable tumor > 50%				
Yes	30.8 (13.2)	0.09	50.0	0.02
No	8.7 (6.3)		13.8	
yp Necrosis > 30%				
Yes	6.6 (15.4)	0.47	22.2	0.66
No	16.9 (6.3)		23.3	
yp Fibrosis > 30%				
Yes	17.2 (6.9)	0.55	19.2	0.33
No	9.6 (11.3)		30.8	

COPD: chronic obstructive pulmonary disease; Cis: cisplatin; gemci: gemcitabine; vino: vinorelbine; yp: post-NAC pathological assessment.

3.3. Cisplatin increases PD-L1 expression in experimental tumors

To confirm our clinical results and better investigate the influence of cisplatin on PD-L1 regulation, we established experimental tumors using the syngeneic model of Lewis lung carcinoma. First, we confirmed that LLC1 cells express PD-L1, as a strong band for PD-L1 transcript was observed by RT-PCR (Fig. 2A). We then analyzed the level of PD-L1 protein content in the LLC1 tumors before or after cisplatin treatment for 24 h. An example of a Western blot is shown in Fig. 2B. The semi-quantitative estimation demonstrated a significant increase of PD-L1 protein in the tumor after treatment ($p = 0.02$, Fig. 2C).

To eliminate the influence of the immune component in this regulation, especially T-lymphocytes, we evaluated PD-L1 expression in a model of athymic nude mice bearing tumors from human lung adenocarcinoma cell line (A549). Comparative analysis of tumor samples showed a significant increase of PD-L1 mRNA level after treatment with 24-h cisplatin ($p = 0.03$, Fig. 2D). In control groups receiving injection of PBS alone, PD-L1 transcript levels were not modified ($p = 0.53$).

3.4. Cisplatin rapidly increases PD-L1 expression in a dose-dependent manner, via AKT activation

To further explore the modality of actions of cisplatin on tumor cells, we incubated different human and murine lung carcinoma cell-lines, expressing PD-L1 at baseline, with increasing concentrations of cisplatin. After treatment, the rates of PD-L1 mRNA were increased in both human and murine cell lines, by 1.5–7.5 folds as compared to control groups (Fig. 3A–C). In human LNM-R cells, in which we found an IC_{50}% of 12 ± 3.1 μM cisplatin, the enhancement of PD-L1 expression was observed at a very low dose, then dose escalation of cisplatin was positively correlated to PD-L1 mRNA levels and reached a plateau around 8 μM (Fig. 3A). This increase of PD-L1 transcript was also observed in others cell lines (Fig. 3B and C). Concomitantly, the PD-L1 protein levels were also increased in the three cell lines, as compared to respective controls. This PDL-1 overexpression was associated to AKT activation by cisplatin (Fig. 3D). As PD-L1 is a *trans*-membranous protein, we subsequently performed an immunocytochemistry analysis showing cisplatin-induced enhanced labeling of the cytoplasmic membrane as compared to control (Fig. 3E).

Furthermore, we evaluated the role of the PI3K/AKT pathway in PD-L1 up-regulation by cisplatin. LLC1 cells were treated for 24 h with

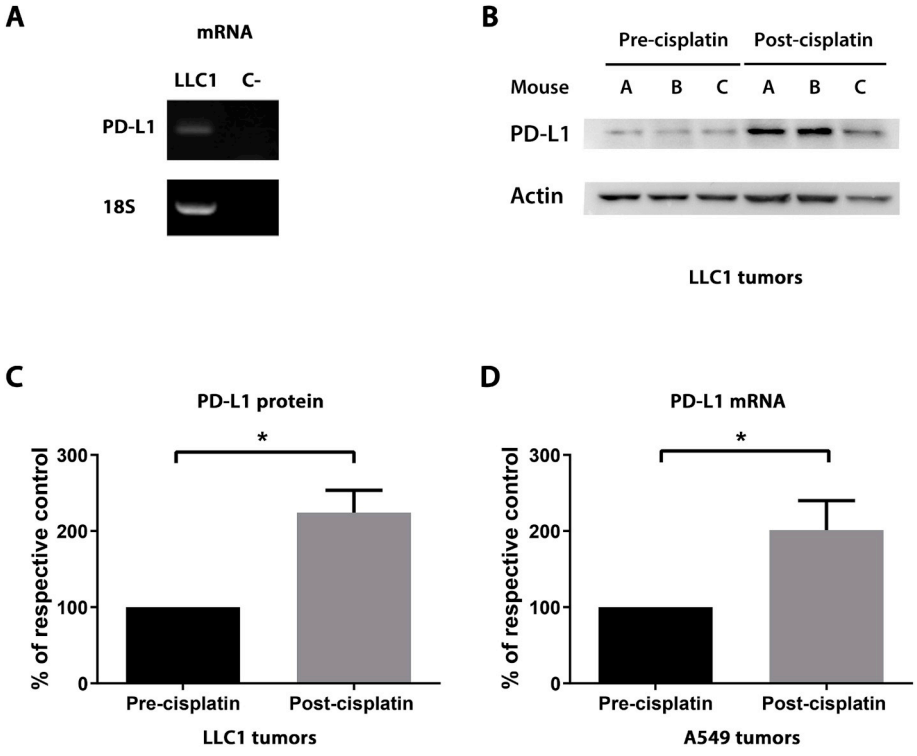


Fig. 2. Cisplatin increases PD-L1 expression in experimental tumor grafts. **(A)** Qualitative PCR showing PD-L1 and 18S mRNAs extracted from LLC1 cells and negative control. **(B, C)** Western blotting of PD-L1 protein in pre- and post-cisplatin treatment of LLC1 tumors (n = 6), each animal being its own control. Semi-quantification of PD-L1 protein content was performed and normalized with actin, and as calculated the percentage of each respective control. **(D)** Q-PCR quantification of PD-L1 mRNA extracted from A549 tumor-bearing nude mice (n = 10). A paired analysis was performed on matched samples to compare pre- and post-cisplatin treatment for 24 h. Results are expressed as the percentage of respective control. * means a P value < 0.05, error-bars: standard error of the mean.

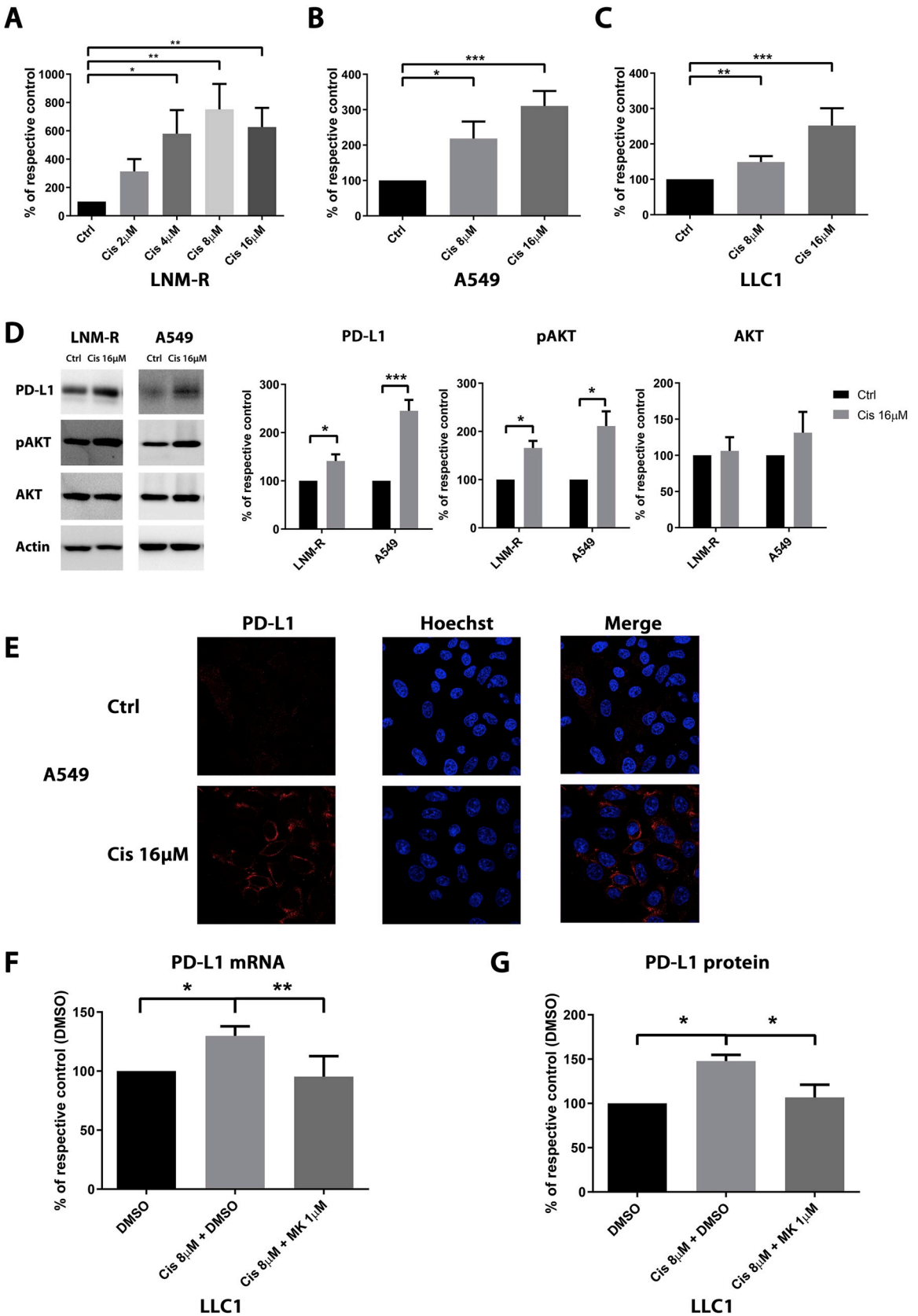
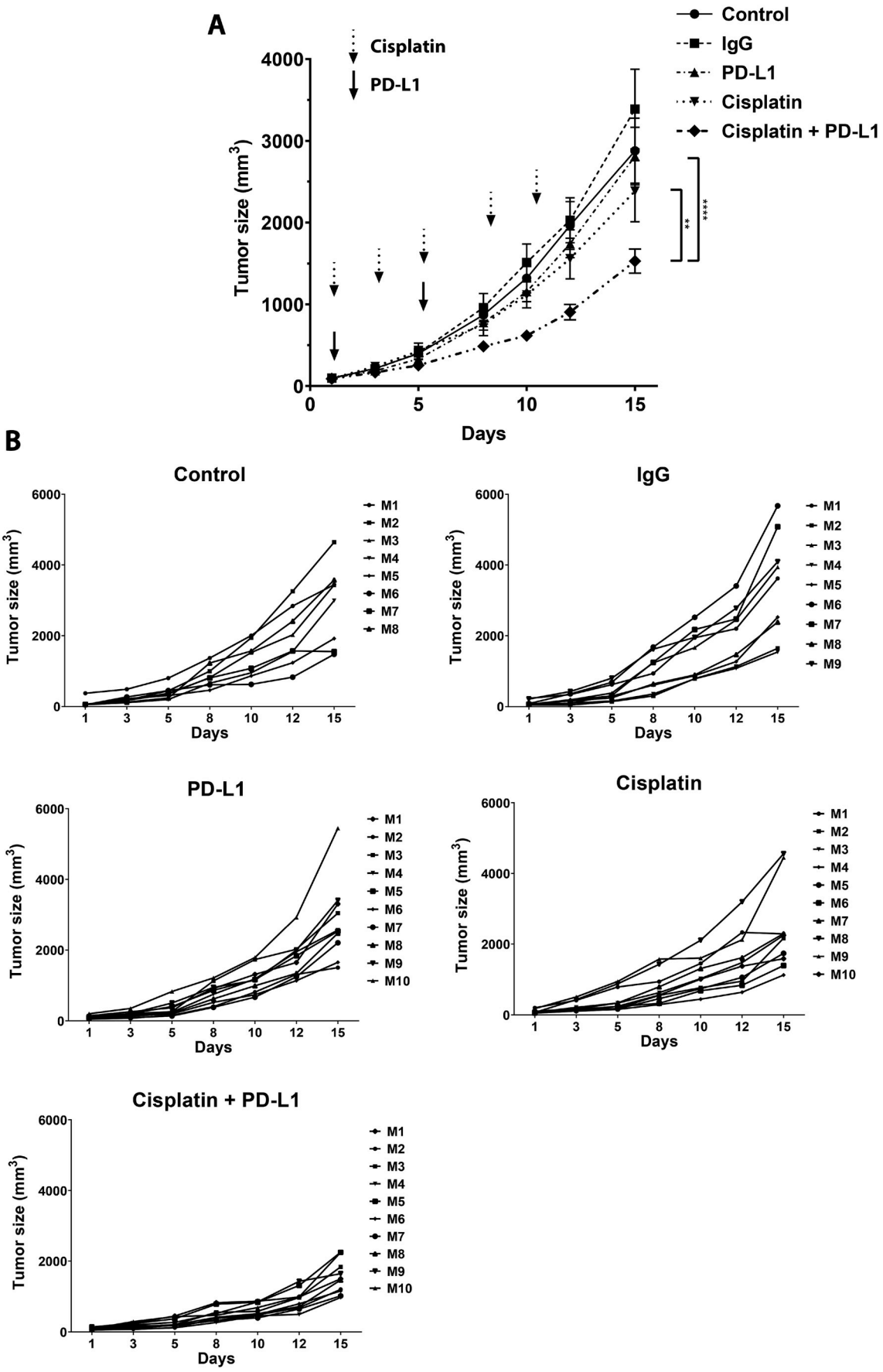


Fig. 3. Cisplatin-induced PD-L1 expression in NSCLC cell-lines. Q-PCR quantification of PD-L1 mRNA after 24-h incubation with cisplatin or equivalent concentrations of PBS (control) in **(A)** LNM-R (n = 6), **(B)** A549 (n = 8), and **(C)** LLC1 cells (n = 8). **(D)** Western-blot analyses of PD-L1, pAKT and AKT proteins after extraction from LNM-R (n = 4), and A549 (n = 8) cells, incubated during 24 h with PBS or 16 μM cisplatin, quantification is expressed as percentage of respective control. **(E)** Immunocytochemistry of A549 cells incubated during 24 h with PBS or 16 μM cisplatin, PD-L1 protein (red labeling), nuclei (Hoechst blue labeling). Quantification of PD-L1 expression at the mRNA **(F)** and protein **(G)** levels after 24-h incubation of LLC1 cells (n = 4) with DMSO (control), cisplatin (8 μM), or cisplatin and AKT-inhibitor (MK2206, 1 μM). * indicates a P value < 0.05, ** < 0.01, *** < 0.001, error-bars: standard error of the mean.



(caption on next page)

Fig. 4. Cisplatin and anti-PD-L1 combined treatment reduced tumor growth, in the Lewis model. (A) Tumor growth curves of LLC1 grafted on C57BL/6JRj j mice. When tumors reached 90–100 mm3, mice were treated (i.v.) with PBS (n = 8), total IgG1 control (BioXcell®) (n = 9), anti PD-L1 (n = 10), cisplatin (n = 10), or the combination cisplatin and anti PD-L1 (n = 10). The arrow indicates the day of injection. **(B)** Individual follow up of LLC1 tumor size treated as described above. **indicates a P value < 0.01, $^{****} < 0.0001$, error-bars: standard error of the mean.

8 μM of cisplatin plus or without MK2206, an AKT blocker, or DMSO. The RNA quantification revealed that cisplatin-induced PD-L1 up-regulation was significantly counteracted by MK2206 (Fig. 3F). This effect was confirmed at the protein level with western-blot analyses showing a significantly lower PD-L1 expression in the group of cells incubated with combined cisplatin and MK2206 as compared to cisplatin and control (Fig. 3G). Concomitantly, pAKT was increased by 3 fold when LLC1 cells were treated with 8 μM of cisplatin and return to half of the basal level in the presence of MK 2206 (data not shown).Overall, these results suggest that PD-L1 up-regulation by cisplatin could involve the PI3K/AKT signaling pathway.

3.5. The combined treatment associating cisplatin and anti-PD-L1 improves therapeutic response compared to single agent regimens

As PD-L1 expression was increased after cisplatin exposure, we addressed the question of a potential improvement of tumor response with the combined treatment associating cisplatin and anti-PD-L1 antibodies, as compared to single agent regimen. As previously described, we performed syngeneic grafts of LLC1 tumors to C57BL/6j mice. The treatment began when the tumor burden reached 90–100 mm^3, two weeks after cell injection. The tumor growth was rapid as the control group reached 3000 mm3 after 15 days. Consequently, in accordance with ethical guidelines, we terminated the experiment. The tumor growth rate was not significantly different between the control, the isotope IgG, the PD-L1, and the cisplatin groups. In contrast, the tumor volume was reduced by 47% in mice treated with the combined treatment as compared to control (1530 ± 147 *vs* 2879 ± 400 mm^3) (Fig. 4A). Individual responses, shown in Fig. 4B, revealed that in control, and IgG groups, the tumor growth rate was dispersed among animals, and more homogenous in the single agent regimen groups except some mice exhibiting high tumor progression. Tumor response in the group receiving combined cisplatin and anti-PD-L1 was also homogeneous, all 10 tumor-bearing mice showed almost similar tumor growth delay (Fig. 4B).

4. Discussion

Our unique experimental approach consisting of the study of PD-L1 expression in the same series of patients before and after NAC, confirmed the previous findings at the level of human tumor: chemotherapy increases the level of PDL-1. This result underscores and strengthens the existing data found in the literature, and thereby opens the debate for the positioning of PD-1/PD-L1 blockade within the sequence of systemic treatments for lung cancer.

In addition, the clinical and pathological results observed in the NAC cohort, our preclinical experiments revealed an early induction of PD-L1 by cisplatin associated with significant biological effect. The paired analysis of human tumor samples revealed an increase of PD-L1 expression several weeks following cisplatin administration, which suggests prolonged up-regulation in tumor cells. These results is in contrast to previous observations in head and neck squamous-cell carcinoma, suggesting that cisplatin-induced PD-L1 up-regulation might not be sustainable over time [23]. An up-regulation of PD-L1 by cisplatin, being both early and sustainable, would have a major impact in the design of combined systemic therapies protocols including cisplatin and ICIs. Similarly, some authors previously demonstrated that platinum-based NAC can induce PD-L1 expression in various cancers which could favor the results of consecutive ICIs treatment [23–26]. Even though true benefit of such therapeutic association probably needs to be overbalanced with the theoretical negative impact of NAC on immune cells, our team previously demonstrated that platinum based chemotherapy does not affect the immune contexture of post-induction NSCLC [27]. In this study, we chose to mimic the clinical context by using a subtoxic dose of cisplatin, and obtained an improved tumor response combining cisplatin to anti-PD-L1 antibodies compared to single agent regimen. Thus we can emphasize that administration of cisplatin can up-regulate PD-L1 while not significantly impairing anticancer immunity.

The independent role of cisplatin on PD-L1 expression in NAC patients could be challenged as cisplatin was combined to other chemotherapeutic agents. However, we did not find any statistical association between ΔPD-L1 and type of doublet. In addition, several retrospective studies analyzing the effect of cisplatin-based NAC in other neoplasms than NSCLC found similar results concerning its influence on PD-L1 expression whereas regimen protocols generally involve different associations than in lung cancer [24,25]. Thus it can be concluded that cisplatin could play a significant role in PD-L1 expression by tumor cells.

The methodology consisting in analyzing ΔPD-L1 after NAC by comparing IHC results in metastatic and primary tissues seems relevant and advantageous. Indeed, our results on chemo-naive minimal-N2 patients, showed no significant difference between PD-L1 staining in lung tumor and invaded mediastinal lymph nodes. Although relying on the analysis of “only” 20 patients, because of the rarity of minimal N2 condition, this result suggests that PD-L1 labeling in lymph-nodes is representative of those from primary lung tumors. Despite the potential for slightly different clinical and biological entities, other authors have corroborated findings and reported similar or higher PD-L1 expression in tumor cells of metastatic sites as compared to primary sites [28,29].

Our results outline a strong association between changes in PD-L1 expression by tumor cells and pathological features of tumors after NAC, in line with similar findings [9]. It has been reported that high PD-L1 expression in tumors was associated with resistance to cisplatin, and also that ΔPD-L1 after induction therapy might reflect chemosensitivity, with low or no ΔPD-L1 positivity in those patients responding to cisplatin [9,30]. Out results concur with this hypothesis based on the finding that $\Delta PD\text{-}L1_{50}$ positive patients exhibit worse pathologic criteria of response to NAC. To explain this correlation, we may hypothesize that neoadjuvant treatment “selected” the subset of tumor cells resistant to cisplatin and, thus, still viable after induction therapy, these resistant clones exhibiting higher PD-L1 expression. Moreover, this latter observation possibly links to some mechanisms involved in PD-L1 up-regulation which are currently under investigation. Indeed, the regulation of this protein is very complex and involves many transcriptional, post-transcriptional and post-translational actions, the clarification of which would be best suited for individual studies [10]. Our preliminary mechanistic exploration focused on a relatively ubiquitous oncogenic signaling pathway but specifically reported as up-regulating PD-L1, and known to sustain survival and resistance to cisplatin-induced apoptosis in cancer cells: PI3K/AKT [11,31,32]. The present study supports these observations showing that inhibition of PI3K/AKT pathway resulted in a significant reduction of PD-L1 expression in tumor cells exposed to cisplatin.

Increased PD-L1 expression in immune cells of the microenvironment after NAC represents another important result. Indeed, sustained stimulation of the PD-1/PD-L1 axis may alter anti-tumor activity as it has been shown that high proportions of PD-L1+ regulatory and PD-1+CD8$^+$ T-lymphocytes in the stroma of NSCLCs were significantly associated with good response to ICIs [33]. In further studies, it would

be interesting to explore the impact of treatments combining cisplatin-based chemotherapy and ICIs, in a concomitant or sequential manner, on anti-tumor immunity. Interestingly, the influence of number and function of immune cells of the microenvironment on the outcome of combined ICIs and NAC should be further investigated.

4.1. Limitations

All PD-L1 IHC evaluations relied on a retrospective cohort including a limited number of operated patients harboring heterogeneous clinical and pathological characteristics. Molecular analyses were not routinely performed at time of initial management and could not be performed for the current study. Thus, we could not analyze the impact of tumor mutational burden on ΔPD-L1 and its microenvironment. In addition, the reliability of pre- and post-cisplatin matched tumor biopsies analyses could be challenged by the likely heterogeneity of PD-L1 expression within the whole tumor area, encouraging a cautious interpretation of observed differences [34,35]. This methodological limitation also occurs in our mice experiments which, moreover, harbor drawbacks for mimicking “normal” tumor stroma and microenvironment due to the heterotopic location and the rapid growth of injected pulmonary tumors [36].

5. Conclusion

In conclusion, cisplatin enhanced PD-L1 expression in both preclinical models and cohorts of patients with NSCLC. The results suggested reproducible, persistent, and a significant inducing impact on tumor cells. This study will hopefully provide a solid and reliable basis for further explorations, as well as reinforcing the rationale for several ongoing clinical trials testing combination therapies including PD-L1 and cytotoxic drugs like cisplatin. At the time of protocols combining chemo and/or radiotherapy with ICIs, it also raises the question of the relevance of performing sequential reevaluation of PD-L1 status to select the ideal subset of patients.

Competing interests

The authors declare that they have no competing interests.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Ludovic Fournel: Conceptualization, Data curation, Formal analysis, Writing - original draft. **Zherui Wu:** Data curation, Writing - review & editing. **Nicolas Stadler:** Data curation, Investigation. **Diane Damotte:** Methodology, Validation. **Filippo Lococo:** Supervision, Investigation. **Geoffroy Boulle:** Software, Investigation. **Evelyne Ségal-Bendirdjian:** Methodology, Visualization. **Antonio Bobbio:** Visualization, Resources. **Philippe Icard:** Validation. **Jean Trédaniel:** Conceptualization, Writing - review & editing. **Marco Alifano:** Conceptualization, Writing - review & editing. **Patricia Forgez:** Conceptualization, Methodology, Project administration, Writing - review & editing.

Acknowledgements

We thank Dr Neil Insdorf for his kind help in editing the manuscript. We thank Tatiana Ledent (animal facility, Saint-Antoine Hospital, Paris, France) and staff of the microscopy platform SCM (Service Commun de Microscopie, Faculté des Sciences Fondamentales et Biomédicales, Paris, France)". We also thank Dr Cecilia Mengoli for her help in immunochemistry analyses (pathology department of Santa-Maria-Nuova Hospital, Reggio Emilia, Italy).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.08.005.

References

[1] L.A. Torre, R.L. Siegel, A. Jemal, Lung cancer statistics, Adv. Exp. Med. Biol. 893 (2016) 1–19, https://doi.org/10.1007/978-3-319-24223-1_1.
[2] J. von Pawel, R. Bordoni, M. Satouchi, L. Fehrenbacher, M. Cobo, J.Y. Han, T. Hida, D. Moro-Sibilot, P. Conkling, D.R. Gandara, A. Rittmeyer, M. Gandhi, W. Yu, C. Matheny, H. Patel, A. Sandler, M. Ballinger, M. Kowanetz, K. Park, Long-term survival in patients with advanced non-small-cell lung cancer treated with atezolizumab versus docetaxel: results from the randomised phase III OAK study, Eur. J. Canc. Oxf. Engl. 107 (2019) (1990) 124–132, https://doi.org/10.1016/j.ejca.2018.11.020.
[3] R.S. Herbst, P. Baas, D.-W. Kim, E. Felip, J.L. Pérez-Gracia, J.-Y. Han, J. Molina, J.-H. Kim, C.D. Arvis, M.-J. Ahn, M. Majem, M.J. Fidler, G. de Castro, M. Garrido, G.M. Lubiniecki, Y. Shentu, E. Im, M. Dolled-Filhart, E.B. Garon, Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial, Lancet Lond. Engl. 387 (2016) 1540–1550, https://doi.org/10.1016/S0140-6736(15)01281-7.
[4] M. Reck, D. Rodríguez-Abreu, A.G. Robinson, R. Hui, T. Csőszi, A. Fülöp, M. Gottfried, N. Peled, A. Tafreshi, S. Cuffe, M. O'Brien, S. Rao, K. Hotta, M.A. Leiby, G.M. Lubiniecki, Y. Shentu, R. Rangwala, J.R. Brahmer, KEYNOTE-024 investigators, pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer, N. Engl. J. Med. 375 (2016) 1823–1833, https://doi.org/10.1056/NEJMoa1606774.
[5] M. Reck, D. Rodríguez-Abreu, A.G. Robinson, R. Hui, T. Csőszi, A. Fülöp, M. Gottfried, N. Peled, A. Tafreshi, S. Cuffe, M. O'Brien, S. Rao, K. Hotta, K. Vandormael, A. Riccio, J. Yang, M.C. Pietanza, J.R. Brahmer, Updated analysis of KEYNOTE-024: pembrolizumab versus platinum-based chemotherapy for advanced non-small-cell lung cancer with PD-L1 tumor proportion score of 50% or greater, J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 37 (2019) 537–546, https://doi.org/10.1200/JCO.18.00149.
[6] S. Maleki Vareki, C. Garrigós, I. Duran, Biomarkers of response to PD-1/PD-L1 inhibition, Crit. Rev. Oncol. Hematol. 116 (2017) 116–124, https://doi.org/10.1016/j.critrevonc.2017.06.001.
[7] Z.-Y. Dong, S.-P. Wu, R.-Q. Liao, S.-M. Huang, Y.-L. Wu, Potential biomarker for checkpoint blockade immunotherapy and treatment strategy, Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med. 37 (2016) 4251–4261, https://doi.org/10.1007/s13277-016-4812-9.
[8] J. Shin, J.-H. Chung, S.H. Kim, K.S. Lee, K.J. Suh, J.Y. Lee, J.-W. Kim, J.-O. Lee, J.-W. Kim, Y.-J. Kim, K.-W. Lee, J.H. Kim, S.-M. Bang, J.-S. Lee, Effect of platinum-based chemotherapy on PD-L1 expression on tumor cells in non-small cell lung cancer, Canc. Res. Treat. Off. J. Korean Canc. Assoc. (2018), https://doi.org/10.4143/crt.2018.537.
[9] P. Zhang, Y. Ma, C. Lv, M. Huang, M. Li, B. Dong, X. Liu, G. An, W. Zhang, J. Zhang, L. Zhang, S. Zhang, Y. Yang, Upregulation of programmed cell death ligand 1 promotes resistance response in non-small-cell lung cancer patients treated with neo-adjuvant chemotherapy, Cancer Sci. 107 (2016) 1563–1571, https://doi.org/10.1111/cas.13072.
[10] I. Zerdes, A. Matikas, J. Bergh, G.Z. Rassidakis, T. Foukakis, Genetic, transcriptional and post-translational regulation of the programmed death protein ligand 1 in cancer: biology and clinical correlations, Oncogene 37 (2018) 4639–4661, https://doi.org/10.1038/s41388-018-0303-3.
[11] K.J. Lastwika, W. Wilson, Q.K. Li, J. Norris, H. Xu, S.R. Ghazarian, H. Kitagawa, S. Kawabata, J.M. Taube, S. Yao, L.N. Liu, J.J. Gills, P.A. Dennis, Control of PD-L1 expression by oncogenic activation of the AKT-mTOR pathway in non-small cell lung cancer, Cancer Res. 76 (2016) 227–238, https://doi.org/10.1158/0008-5472.CAN-14-3362.
[12] L. Paz-Ares, A. Luft, D. Vicente, A. Tafreshi, M. Gümüş, J. Mazières, B. Hermes, F. Çay Şenler, T. Csőszi, A. Fülöp, J. Rodríguez-Cid, J. Wilson, S. Sugawara, T. Kato, K.H. Lee, Y. Cheng, S. Novello, B. Halmos, X. Li, G.M. Lubiniecki, B. Piperdi, D.M. Kowalski, KEYNOTE-407 investigators, pembrolizumab plus chemotherapy for squamous non-small-cell lung cancer, N. Engl. J. Med. 379 (2018) 2040–2051, https://doi.org/10.1056/NEJMoa1810865.
[13] L. Gandhi, D. Rodríguez-Abreu, S. Gadgeel, E. Esteban, E. Felip, F. De Angelis, M. Domine, P. Clingan, M.J. Hochmair, S.F. Powell, S.Y.-S. Cheng, H.G. Bischoff, N. Peled, F. Grossi, R.R. Jennens, M. Reck, R. Hui, E.B. Garon, M. Boyer, B. Rubio-Viqueira, S. Novello, T. Kurata, J.E. Gray, J. Vida, Z. Wei, J. Yang, H. Raftopoulos, M.C. Pietanza, M.C. Garassino, KEYNOTE-189 investigators, pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer, N. Engl. J. Med. 378 (2018) 2078–2092, https://doi.org/10.1056/NEJMoa1801005.
[14] S.V. Liu, D.R. Camidge, S.N. Gettinger, G. Giaccone, R.S. Heist, F.S. Hodi, N.E. Ready, W. Zhang, J. Wallin, R. Funke, D. Waterkamp, P. Foster, K. Iizuka, J. Powderly, Long-term survival follow-up of atezolizumab in combination with platinum-based doublet chemotherapy in patients with advanced non-small-cell lung cancer, Eur. J. Canc. Oxf. Engl. 101 (2018) (1990) 114–122, https://doi.org/10.1016/j.ejca.2018.06.033.

[15] S.J. Antonia, A. Villegas, D. Daniel, D. Vicente, S. Murakami, R. Hui, T. Yokoi, A. Chiappori, K.H. Lee, M. de Wit, B.C. Cho, M. Bourhaba, X. Quantin, T. Tokito, T. Mekhail, D. Planchard, Y.-C. Kim, C.S. Karapetis, S. Hiret, G. Ostoros, K. Kubota, J.E. Gray, L. Paz-Ares, J. de Castro Carpeño, C. Wadsworth, G. Melillo, H. Jiang, Y. Huang, P.A. Dennis, M. Özgüroğlu, PACIFIC investigators, durvalumab after chemoradiotherapy in stage III non-small-cell lung cancer, N. Engl. J. Med. 377 (2017) 1919–1929, https://doi.org/10.1056/NEJMoa1709937.

[16] E.A. Eisenhauer, P. Therasse, J. Bogaerts, L.H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, L. Rubinstein, L. Shankar, L. Dodd, R. Kaplan, D. Lacombe, J. Verweij, New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1), Eur. J. Canc. Oxf. Engl. 45 (1990) 228–247, https://doi.org/10.1016/j.ejca.2008.10.026 2009.

[17] J. Adam, N. Le Stang, I. Rouquette, A. Cazes, C. Badoual, H. Pinot-Roussel, L. Tixier, C. Danel, F. Damiola, D. Damotte, F. Penault-Llorca, S. Lantuéjoul, Multicenter harmonization study for PD-L1 IHC testing in non-small-cell lung cancer, Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 29 (2018) 953–958, https://doi.org/10.1093/annonc/mdy014.

[18] R.S. Herbst, J.-C. Soria, M. Kowanetz, G.D. Fine, O. Hamid, M.S. Gordon, J.A. Sosman, D.F. McDermott, J.D. Powderly, S.N. Gettinger, H.E.K. Kohrt, L. Horn, D.P. Lawrence, S. Rost, M. Leabman, Y. Xiao, A. Mokatrin, H. Koeppen, P.S. Hegde, I. Mellman, D.S. Chen, F.S. Hodi, Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients, Nature 515 (2014) 563–567, https://doi.org/10.1038/nature14011.

[19] M. Alifano, F. Souazé, S. Dupouy, S. Camilleri-Broët, M. Younes, S.-M. Ahmed-Zaïd, T. Takahashi, A. Cancellieri, S. Damiani, M. Boaron, P. Broët, L.D. Miller, C. Gespach, J.F. Regnard, P. Forgez, Neurotensin receptor 1 determines the outcome of non-small cell lung cancer, Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 16 (2010) 4401–4410, https://doi.org/10.1158/1078-0432.CCR-10-0659.

[20] M. Younes, Z. Wu, S. Dupouy, A.M. Lupo, N. Mourra, T. Takahashi, J.F. Fléjou, J. Trédaniel, J.F. Régnard, D. Damotte, M. Alifano, P. Forgez, Neurotensin (NTS) and its receptor (NTSR1) causes EGFR, HER2 and HER3 over-expression and their autocrine/paracrine activation in lung tumors, confirming responsiveness to erlotinib, Oncotarget 5 (2014) 8252–8269, https://doi.org/10.18632/oncotarget.1633.

[21] Z. Wu, L. Fournel, N. Stadler, J. Liu, A. Boullier, N. Hoyeau, J.F. Fléjou, V. Duchatelle, N. Djebrani-Oussedik, M. Agopiantz, E. Ségal-Bendirdjian, A. Gompel, M. Alifano, O. Melander, J. Trédaniel, P. Forgez, Modulation of lung cancer cell plasticity and heterogeneity with the restoration of cisplatin sensitivity by neurotensin antibody, Cancer Lett. 444 (2019) 147–161, https://doi.org/10.1016/j.canlet.2018.12.007.

[22] K. Kozaki, O. Miyaishi, T. Tsukamoto, Y. Tatematsu, T. Hida, T. Takahashi, T. Takahashi, Establishment and characterization of a human lung cancer cell line NCI-H460-LNM35 with consistent lymphogenous metastasis via both subcutaneous and orthotopic propagation, Cancer Res. 60 (2000) 2535–2540.

[23] L. Tran, C.T. Allen, R. Xiao, E. Moore, R. Davis, S.-J. Park, K. Spielbauer, C. Van Waes, N.C. Schmitt, Cisplatin alters antitumor immunity and synergizes with PD-1/PD-L1 inhibition in head and neck squamous cell carcinoma, Cancer Immunol. Res. 5 (2017) 1141–1151, https://doi.org/10.1158/2326-6066.CIR-17-0235.

[24] S. Grabosch, M. Bulatovic, F. Zeng, T. Ma, L. Zhang, M. Ross, J. Brozick, Y. Fang, G. Tseng, E. Kim, A. Gambotto, E. Elishaev, R.P. Edwards, A.M. Vlad, Cisplatin-induced immune modulation in ovarian cancer mouse models with distinct inflammation profiles, Oncogene (2018), https://doi.org/10.1038/s41388-018-0581-9.

[25] H.Y. Ng, J. Li, L. Tao, A.K.-Y. Lam, K.W. Chan, J.M.Y. Ko, V.Z. Yu, M. Wong, B. Li, M.L. Lung, Chemotherapeutic treatments increase PD-L1 expression in esophageal squamous cell carcinoma through EGFR/ERK activation, Transl. Oncol. Times 11 (2018) 1323–1333, https://doi.org/10.1016/j.tranon.2018.08.005.

[26] S.J.L. Mesnage, A. Auguste, C. Genestie, A. Dunant, E. Pain, F. Drusch, S. Gouy, P. Morice, E. Bentivegna, C. Lhomme, P. Pautier, J. Michels, A. Le Formal, B. Cheaib, J. Adam, A.F. Leary, Neoadjuvant chemotherapy (NACT) increases immune infiltration and programmed death-ligand 1 (PD-L1) expression in epithelial ovarian cancer (EOC), Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 28 (2017) 651–657, https://doi.org/10.1093/annonc/mdw625.

[27] R. Remark, A. Lupo, M. Alifano, J. Biton, H. Ouakrim, A. Stefani, I. Cremer, J. Goc, J.-F. Régnard, M.-C. Dieu-Nosjean, D. Damotte, Immune contexture and histological response after neoadjuvant chemotherapy predict clinical outcome of lung cancer patients, OncoImmunology 5 (2016) e1255394, , https://doi.org/10.1080/2162402X.2016.1255394.

[28] Y. Liu, Z. Dong, T. Jiang, L. Hou, F. Wu, G. Gao, Y. He, J. Zhao, X. Li, C. Zhao, W. Zhang, Q. Tian, Y. Pan, Y. Wang, S. Yang, C. Wu, S. Ren, C. Zhou, J. Zhang, F.R. Hirsch, Heterogeneity of PD-L1 expression among the different histological components and metastatic lymph nodes in patients with resected lung adenosquamous carcinoma, Clin. Lung Cancer 19 (2018) e421–e430, https://doi.org/10.1016/j.cllc.2018.02.008.

[29] H. Uruga, E. Bozkurtlar, T.G. Huynh, A. Muzikansky, Y. Goto, M. Gomez-Caraballo, A.N. Hata, J.F. Gainor, E.J. Mark, J.A. Engelman, M.D. Lanuti, M. Mino-Kenudson, Programmed cell death ligand (PD-L1) expression in stage II and III lung adenocarcinomas and nodal metastases, J. Thorac. Oncol. Off. Publ. Int. Assoc. Stud. Lung Cancer 12 (2017) 458–466, https://doi.org/10.1016/j.jtho.2016.10.015.

[30] S.Y. Lee, D.K. Jung, J.E. Choi, C.C. Jin, M.J. Hong, S.K. Do, H.-G. Kang, W.K. Lee, Y. Seok, E.B. Lee, J.Y. Jeong, K.M. Shin, S.S. Yoo, J. Lee, S.I. Cha, C.H. Kim, J.Y. Park, PD-L1 polymorphism can predict clinical outcomes of non-small cell lung cancer patients treated with first-line paclitaxel-cisplatin chemotherapy, Sci. Rep. 6 (2016) 25952, https://doi.org/10.1038/srep25952.

[31] E.A. Mittendorf, A.V. Philips, F. Meric-Bernstam, N. Qiao, Y. Wu, S. Harrington, X. Su, Y. Wang, A.M. Gonzalez-Angulo, A. Akcakanat, A. Chawla, M. Curran, P. Hwu, P. Sharma, J.K. Litton, J.J. Molldrem, G. Alatrash, PD-L1 expression in triple-negative breast cancer, Cancer Immunol. Res. 2 (2014) 361–370, https://doi.org/10.1158/2326-6066.CIR-13-0127.

[32] E.G. Sarris, M.W. Saif, K.N. Syrigos, The biological role of PI3K pathway in lung cancer, Pharm. Basel Switz. 5 (2012) 1236–1264, https://doi.org/10.3390/ph5111236.

[33] S.-P. Wu, R.-Q. Liao, H.-Y. Tu, W.-J. Wang, Z.-Y. Dong, S.-M. Huang, W.-B. Guo, L.-Y. Gou, H.-W. Sun, Q. Zhang, Z. Xie, L.-X. Yan, J. Su, J.-J. Yang, W.-Z. Zhong, X.-C. Zhang, Y.-L. Wu, Stromal PD-L1-positive regulatory T cells and PD-1-positive CD8-positive T cells define the response of different subsets of non-small cell lung cancer to PD-1/PD-L1 blockade immunotherapy, J. Thorac. Oncol. Off. Publ. Int. Assoc. Stud. Lung Cancer 13 (2018) 521–532, https://doi.org/10.1016/j.jtho.2017.11.132.

[34] M. Ilie, E. Long-Mira, C. Bence, C. Butori, S. Lassalle, L. Bouhlel, L. Fazzalari, K. Zahaf, S. Lalvée, K. Washetine, J. Mouroux, N. Vénissac, M. Poudenx, J. Otto, J.C. Sabourin, C.H. Marquette, V. Hofman, P. Hofman, Comparative study of the PD-L1 status between surgically resected specimens and matched biopsies of NSCLC patients reveal major discordances: a potential issue for anti-PD-L1 therapeutic strategies, Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 27 (2016) 147–153, https://doi.org/10.1093/annonc/mdv489.

[35] E. Munari, G. Zamboni, G. Lunardi, L. Marchionni, M. Marconi, M. Sommaggio, M. Brunelli, G. Martignoni, G.J. Netto, M.O. Hoque, F. Moretta, M.C. Mingari, M.C. Pegoraro, A. Inno, S. Paiano, A. Terzi, A. Cavazza, G. Rossi, F.R. Mariotti, P. Vacca, L. Moretta, G. Bogina, PD-L1 expression heterogeneity in non-small cell lung cancer: defining criteria for harmonization between biopsy specimens and whole sections, J. Thorac. Oncol. Off. Publ. Int. Assoc. Stud. Lung Cancer 13 (2018) 1113–1120, https://doi.org/10.1016/j.jtho.2018.04.017.

[36] L. Zitvogel, J.M. Pitt, R. Daillère, M.J. Smyth, G. Kroemer, Mouse models in oncoimmunology, Nat. Rev. Cancer 16 (2016) 759–773, https://doi.org/10.1038/nrc.2016.91.