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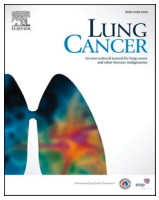

Research Paper

PD-L1 overexpression induces STAT signaling and promotes the secretion of pro-angiogenic cytokines in non-small cell lung cancer (NSCLC)

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ABSTRACT

Background: Monoclonal antibodies (ICI) targeting the immune checkpoint PD-1/PD-L1 alone or in combination with chemotherapy have demonstrated relevant benefits and established new standards of care in first-line treatment for advanced non-oncogene addicted non-small cell lung cancer (NSCLC).
However, a relevant percentage of NSCLC patients, even with high PD-L1 expression, did not respond to ICI, highlighting the presence of intracellular resistance mechanisms that could be dependent on high PD-L1 levels. The intracellular signaling induced by PD-L1 in tumor cells and their correlation with angiogenic signaling pathways are not yet fully elucidated.
Methods: The intrinsic role of PD-L1 was initially checked in two PD-L1 overexpressing NSCLC cells by transcriptome profile and kinase array. The correlation of PD-L1 with VEGF, PECAM-1, and angiogenesis was evaluated in a cohort of advanced NSCLC patients. The secreted cytokines involved in tumor angiogenesis were assessed by Luminex assay and their effect on Huvec migration by a non-contact co-culture system.
Results: PD-L1 overexpressing cells modulated pathways involved in tumor inflammation and JAK-STAT signaling. In NSCLC patients, PD-L1 expression was correlated with high tumor intra-vasculature. When challenged with PBMC, PD-L1 overexpressing cells produced higher levels of pro-angiogenic factors compared to parental cells, as a consequence of STAT signaling activation. This increased production of cytokines involved in tumor angiogenesis largely stimulated Huvec migration. Finally, the addition of the anti-antiangiogenic agent nintedanib significantly reduced the spread of Huvec cells when exposed to high levels of pro-angiogenic factors.
Conclusions: In this study, we reported that high PD-L1 modulates STAT signaling in the presence of PBMC and induces pro-angiogenic factor secretion. This could enforce the role of PD-L1 as a crucial regulator of the tumor microenvironment stimulating tumor progression, both as an inhibitor of T-cell activity and as a promoter of tumor angiogenesis.

1. Introduction

Lung cancer is a leading cause of cancer mortality and can be divided into two broad categories: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). The latter represents 85 % of all lung cancers; it mainly comprises two histological subtypes, adenocarcinoma (ADC, 60 %) and squamous cell carcinoma (SqCC, 35 %), with separate mutational and genomic profiles.

Monoclonal antibodies targeting inhibitory immune checkpoints (e.g., PD-1/PD-L1 and CTLA-4/B7) have demonstrated clinical activity in several malignancies, including NSCLC and other solid tumors. Recently, immunotherapy with anti-PD-1/PD-L1 single agents

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(pembrolizumab, nivolumab, and atezolizumab) has been approved in the first-line treatment of advanced NSCLC with high expression of PD-L1 [1]. Moreover, several trials showed a relevant survival benefit in first-line treatment combining anti-PD-1/PD-L1 with chemotherapy agents [1] or other immune checkpoint inhibitors [2], establishing a new standard of care in first-line treatment of the advanced NSCLC, independently of PD-L1 expression both in squamous and non-squamous histology.

Nevertheless, pembrolizumab is ineffective in around 50 % of NSCLC patients with high PD-L1 levels, and the long-term immunotherapy approach is effective in about one-third of the patients implying the presence of complementary and/or further PD-L1-related resistance mechanisms.

PD-L1 is a membrane protein, that exerts its main function as a ligand for programmed dead receptor-1 (PD-1) on T-cells; recently, emerging roles of PD-L1 lead to pro-survival signals in tumor cells that promote cancer growth, dissemination, and above all resistance to therapies [3], but to date, the intracellular signaling induced by PD-L1 is largely unknown.

In a melanoma murine cancer model, PD-L1 stimulated the tumor progression by counteracting the IFN signaling, via a conserved motif in the intracellular tail of PD-L1 [4,5]. In renal [6], esophageal [7], and breast [8] carcinoma, PD-L1 was reported to induce epithelial to mesenchymal transition (EMT), and in melanoma and ovarian cancers PD-L1 controlled tumor growth and autophagy [9]. Stable knockdown of PD-L1 by CRISPR technology increased sensitivity to chemotherapy in osteosarcoma cells [10].

In contrast, the knockdown of PD-L1 in NSCLC H1299 and Calu-1 cells stimulated tumor cell proliferation, and the addition of antibodies targeting PD-L1 promoted cancer growth, suggesting a tumor suppressor role for PD-L1 [11].

Moreover, although PD-L1 is considered a transmembrane protein, several data demonstrated intracellular PD-L1 localization: in particular, the recycling of PD-L1 from cytoplasmic vesicles to the plasma membrane [12], and the presence of functional PD-L1 in the nucleus [13] have been recently reported, suggesting multiple and distinct functions of PD-L1 in cancer cells.

Among the different intrinsic roles attributed to PD-L1 [14], immunohistochemical data suggested a positive correlation between PD-L1 and VEGFA expression in NSCLC patients with adenocarcinoma histotype [15], in classical Hodgkin lymphoma (cHL) [16], renal cell carcinoma [17] and breast cancer patients [18].

Moreover, some data reported the connection between intracellular PD-L1 role and members of the STAT family, in particular STAT3 [4], STAT1, and STAT2 [19]. It is noteworthy that STAT family members play an active role in several cellular processes including tumor growth, progression, and neo-angiogenesis [20] with different functions attributed to a single STAT transcription factor, in response to different stimuli, suggesting that STAT regulation and function are dependent on the microenvironment surrounding the tumor cells.

Recently, a direct relationship between the intrinsic role of PD-L1 and angiogenesis was reported in uveal melanoma [21] and ovarian cancer [22] patients, enforcing the global role of PD-L1 as a main regulator of processes involved in the tumor progression and resistance to immune cells.

In order to evaluate the intrinsic role of PD-L1 in lung cancer cells and its correlation with angiogenesis, we conducted this *in vitro* study, which allowed us to identify a direct connection between high PD-L1 levels in lung cancer cells and the secretion of pro-angiogenic cytokines. In particular, we observed the induction of STAT signaling in tumor cells with PD-L1 overexpression in the presence of activated PBMC, as responsible for pro-angiogenic factor secretion, thus promoting tumor angiogenesis.

2. Material and methods

2.1. Cell culture and generation of PD-L1 overexpressing cells

Human NSCLC A549 and H460 were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were infected with the lentiviral transfer vector (P-lenti-C-mGFP-P2A-Puro) with or without full-length human PD-L1 cDNA (Origene, Rockville, MD) as previously described [23] and PD-L1 overexpressing cells were selected by puromycin. Human umbilical vein endothelial cells (Huvec) were cultured in EBM-2 basal medium additioned with SingleQuotsTM Supplement Pack, 5 % FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37 °C in a humidified atmosphere containing 37 °C and 5 % CO_2.

2.2. Flow cytometry and confocal analysis

PD-L1 membrane levels were quantified by flow cytometry and confocal analysis as previously reported [24].

2.3. Rnaseq

Total RNA was isolated from cellular samples employing the MiRVAna kit (Ambion, Thermo Fisher Scientific). Subsequently, library preparation was conducted using the Illumina TruSeq Stranded Total RNA Library Prep Gold Kit (20020598, Illumina Inc., San Diego, USA) and purified using Agencount AMPure XP beads (Beckman Coulter, Brea, USA). Library concentration was quantified using the Qubit dsDNA BR kit (Thermo Scientific), and the size distribution was assessed with an Agilent Bioanalyzer. The libraries were then subjected to paired-end sequencing (2 × 75 bp) on a NextSeq500 (Illumina). Transcript counts were computed using salmon v1.9.0 (mapping-based mode) and the Ensembl human genome (GRCh38) with genecode v38 annotations [25]. Subsequently, gene expression from 6 samples (3 A549 and 3 A549PD-L1) was filtered to remove genes with a count below 3 in at least 3 samples.

The differential expression (DE) analysis was performed using DESeq2 (R package), and the comparison was set to consider A549wt as the baseline [26]. This means that results with positive Log2FC indicate higher expression in A549-PD-L1 cells compared to A549wt. A negative log2FC value indicates the opposite scenario.

Gene set enrichment analysis (GSEA) was performed on statistically significant DE results (BH adjusted p-value < 0.05) using FGSEA (R package) and probing 3 different collections of gene sets from the Molecular Signature Database (MSigDB): Curated Gene Sets (C2), Ontology gene sets (C5) and the Hallmark gene sets (H). Selected statistically significant results were plotted using ggplot2 (R package) [27].

2.4. Phospho-antibody array analysis

The relative levels of phosphorylation of 45 kinase sites were obtained by Proteome Profiler Human Phospho-kinase Array (Kit ARY003B from R&D System, Minneapolis, MN) according to the manufacturer's guidelines as previously reported [28]. The resulting spots were quantified using Image Studio™ Software, LI-COR Biotechnology (NE, US).

2.5. Bioinformatics analysis of public datasets

Genomics analysis was run on the publicly available The Cancer Genome Atlas (TCGA) lung adenocarcinoma Gene Expression Quantification Dataset (https://portal.gdc.cancer.gov/), retrieved in May 2023. PD-L1, VEGFA, VEGFB, VEGFC, and PECAM1 variables were selected. Gene expression was quantified in Transcripts Per Million (TPM) using the STAR-counts method [29]. Log transformations were used

appropriately to obtain normal distributions suitable for correlation analysis. All variables of interest were studied through correlation analysis after assumption verification. The α-value was set to 0.05. All analyses were run on Python 3.11.4 using NumPy [30], Matplotlib, Seaborn, and R 4.2.0.

2.6. Immunohistochemical analysis

NSCLC patients who underwent lung resections with curative intent at the Unit of Thoracic Surgery, University-Hospital of Parma, were selected from AIRC_Radiomics Cohort (Ethical approval n. 24,934 del 29/06/2020) to investigate the immunohistochemical CD31 expression on tumor tissue. Data related to the IHC expression of PD-L1 was already available, according to clinical practice. The antibody used was PD-L1 (clone SP263, Roche) and its membranous staining pattern was quantified as % of cell surface labeling (TPScore) [31]. The formalin-fixed, paraffin-embedded tissue sections (5 mm thick) were performed on all samples for immunohistochemical staining of CD31 expression. After deparaffinization and rehydration sections were treated with 3 % hydrogen peroxidase for 5 min. For antigen retrieval, sections were treated with pH9 Tris-EDTA buffer for 30 mins in water-bath at 98 °C. Sections were stained with the primary antibody CD31 (clone JC70, Roche), and a polymeric system Ultraview DaB Detection Kit (Ventana-Roche) was used following the manufacturer's specifications. Diaminobenzidine (DAB) was used for staining development and the sections were counterstained with hematoxylin. Negative controls consisted of substituting normal serum for the primary antibody. The section was scanned by a pathologist and the CD31 positivity was observed in the membrane and cytoplasm of endothelial cells. The intratumoral microvessel density (number of vessels/mm^2) was assessed by light microscopic analysis (20x) as the most representative tumor area that contained the largest numbers of capillaries and small venules (microvessels). The technique used to measure microvessel density in tumor tissue sections stained with CD31 (endothelial cell marker) is the hotspot method (Weidner's method) [32].

2.7. PBMC isolation

PBMC isolation was described elsewhere [24]. PBMC were activated with soluble anti-CD3 (1 μg/mL) and anti-CD28 (2 μg/mL) (BioLegend, San Diego, CA, USA).

2.8. Co-culture system

A non-contact co-culture system of cancer cells with PBMC was established using a transwell suspension culture chamber with a polyethylene terephthalate film (PET), with 0.4 μm pore size (Corning, NY, USA). Briefly, $80x10^3$ cancer cells were seeded in a 24-well plate. After 24 h, $6x10^5$ freshly isolated PBMC, activated with anti-CD3/CD28, were added to the transwell; at the end, PBMC were removed, and the culture medium was recovered and tested for cytokine production [24].

2.9. Luminex assay

The media from fresh isolated PBMC, A549, A549 PD-L1, H460, and H460 PD-L1 cells were collected and assayed for Human Th1/Th2 Luminex Performance Assay Fixed Panel or Human pre-mixed Multi-analyte kit (Angiogenin, Angiopoietin 1–2, FGF-a, FGF-b, PDGF-aa, PDGF-bb, VEGF, VEGF-C) from Biotechne (Minneapolis, MN) using Luminex technology with custom-made plate and read on Magpix machine (BioRad) according to the manufacturer's instructions. The total protein content of tumor cells was quantified with Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA, US). The concentration of each analyte in the media was normalized to the total cellular protein of tumor cells per well and the data are presented as pg of analyte/μg of the protein or pg of analyite/ml.

2.10. Western blotting

The procedures for protein extraction, solubilization, and protein analysis by western blotting were performed as described previously [33]. Antibodies against p-STAT2, STAT2, p-STAT5, STAT5, p-STAT6, and STAT6, were from Cell Signaling Technology, Incorporated (Danvers, MA). Anti-b-actin (clone B11V08) was from BioVision (Milpitas, CA). Horseradish peroxidase-conjugated secondary antibodies and the chemiluminescence system were from Millipore (Millipore, MA). Reagents for electrophoresis and blotting analysis were from BIO-RAD Laboratories (Hercules, CA, US).

2.11. Huvec migration assay

After 48 h of cancer cells/PBMC non-contact co-culture, the transwell with immune cells was substituted with a new transwell chamber with 6.5-mm diameter polycarbonate filters (8 μm pore size, BD Biosciences, Erembodegem, Belgium) with 5×10^4 Huvec cells. After 16 h, cell migration was quantified as previously reported [34].

2.12. Reagents

Nintedanib (Boehringer Ingelheim, Germany) and Ruxolitinib (Selleckchem Houston, TX) were dissolved in DMSO. DMSO concentration never exceeded 0.1 % (v/v); equal amounts of the solvent were added to control cells.

2.13. Statistical analysis

Western blot images are representative of two independent experiments. Statistical analyses were carried out using the GraphPad Prism 6.00 software. Comparisons were performed by the two-tailed Student's *t*-test, and the ANOVA test and p-values are indicated where appropriate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3. Results

3.1. PD-L1 overexpression induces changes in inflammatory and Jak/STAT pathway

With the aim to elucidate the biological role associated with PD-L1 overexpression, A549, and H460 NSCLC cell lines were infected with lentiparticles with/without the full-length cDNA for the human PD-L1 gene. After selection, we evaluated PD-L1 membrane level (mPD-L1) by both flow cytometry and confocal microscopy in A549 and H460 cells infected with PD-L1 vector (A549PD-L1 and H460PD-L1) compared to cells infected with empty vector (hereafter reported as parental cells). As reported (Fig. 1A), a marked increase of mPD-L1 was documented for both PD-L1 overexpressing cells compared to the corresponding parental cell lines. Data were confirmed by confocal analysis for the A549PD-L1 cells (Fig. 1B).

To elucidate the gene expression modification caused by PD-L1 overexpression, we analyzed the difference in transcriptomes between A549 and A549PD-L1 overexpressing cells by RNA-seq. In total, we found 4482 genes that were differentially expressed in A549PD-L1 cells compared to control ones. To have a better view of the pathway involved, we cataloged them in different pathways in a Gene Set Enrichment Analysis (GSEA) (Fig. 2A). Among the analyzed pathways, we observed a significant alteration of pro-inflammatory pathways and IFN-γ-related effectors. Moreover, the Jak-STAT axis was one of the most perturbated signaling, pointing to a possible role of PD-L1 in the control of this protein family.

To further extend our analysis, we explored the signaling pathways affected by PD-L1 overexpression: 45 specific Ser/Thr or Tyr phosphorylation sites of 35 different proteins were analyzed by a human phospho-antibody array. As reported in Fig. 2B, three phosphorylation

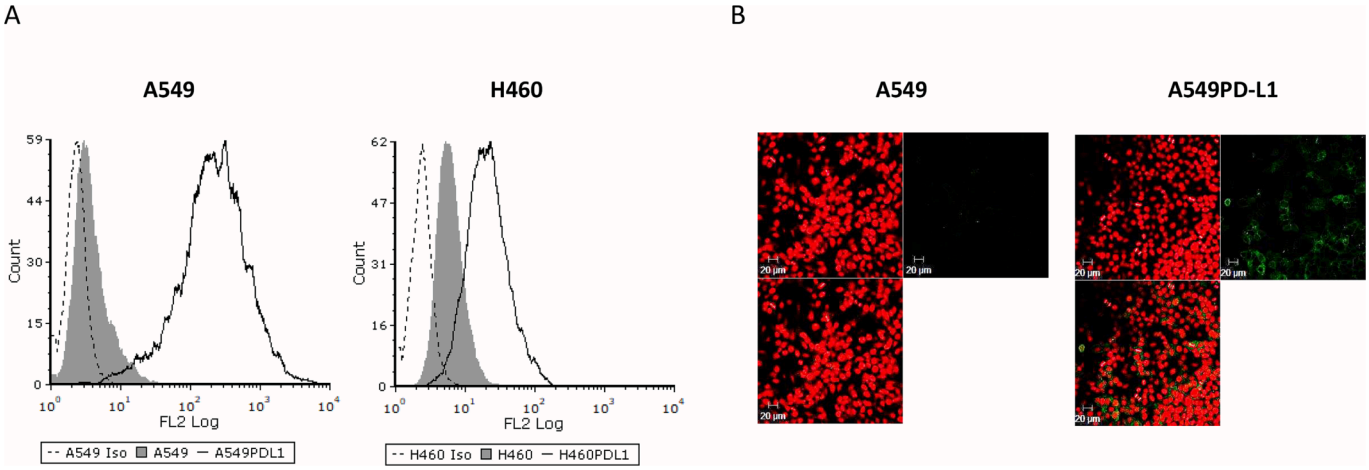


Fig. 1. Evaluation of PD-L1 overexpression in NSCLC cell lines (A) PD-L1 levels were analyzed by flow cytometry in A549, and H460 NSCLC cell lines infected with empty vector and in the corresponding PD-L1 overexpressing cells. (B) Confocal immunofluorescence analysis of PD-L1 expression in A549 and A549PD-L1 cells; green fluorescence indicates the positivity to PD-L1. The nuclei were stained with Draq5 (red fluorescence). Scale bar: 20 µm. Results are representative of two independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

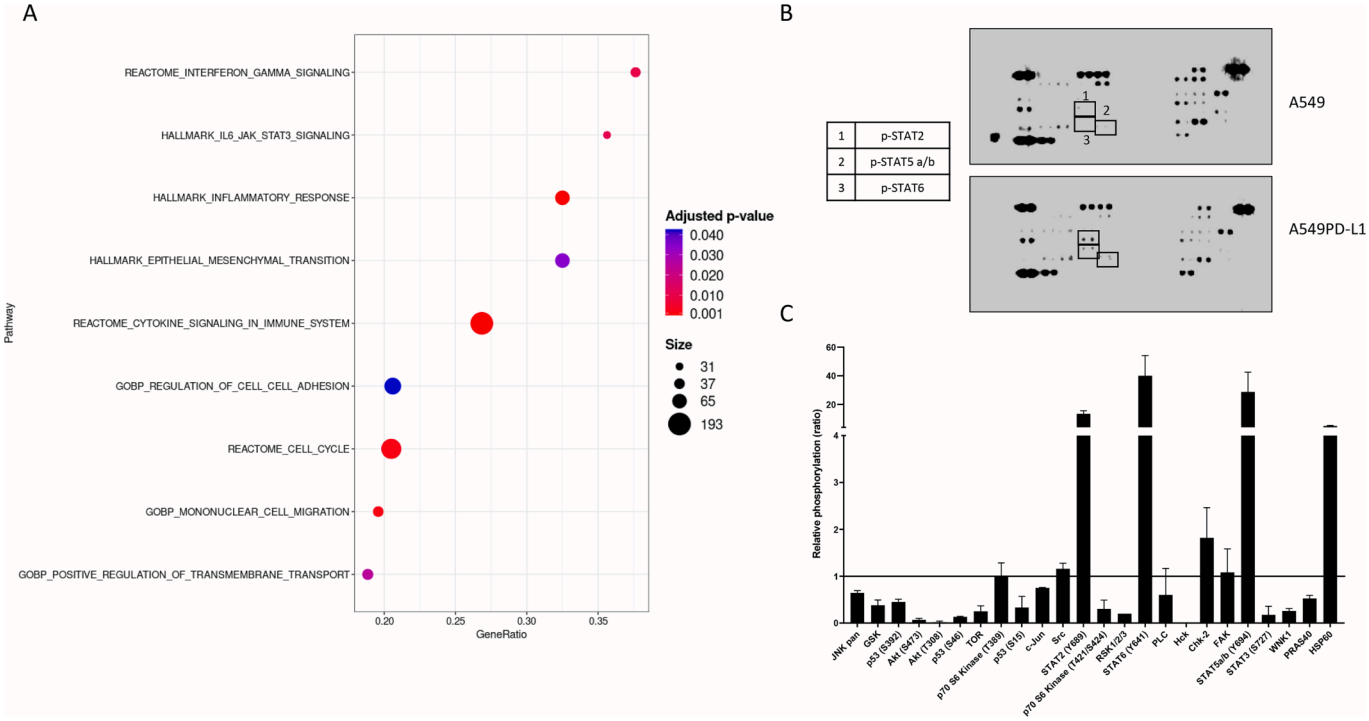


Fig. 2. Signaling pathways altered by PD-L1 overexpression (A) Cells were incubated for 48 h in culture medium, then the RNA was extracted. The graph was generated through the R Studio software and relative programming language. In particular, the code package ggplot2 was used. (B) A549 and A549PD-L1 cells were cultured for 24 h and protein lysates were subjected to a phospho-RTK array according to kit instructions. Each membrane contains specific kinase and positive control antibodies spotted in duplicate. (C) Highly expressed kinases are evidenced by bars in densitometric analysis as a ratio over corresponding spots on A549 parental cells.

sites presented a significant increase in A549PD-L1 cells, compared to the parental one. In particular, the phosphorylation of tyrosine 689 in STAT2 (Y689), 694 in STAT5 (Y694), and 641 in STAT6 (Y641) was strongly increased in A549PD-L1 cells, as reported in Fig. 2C.

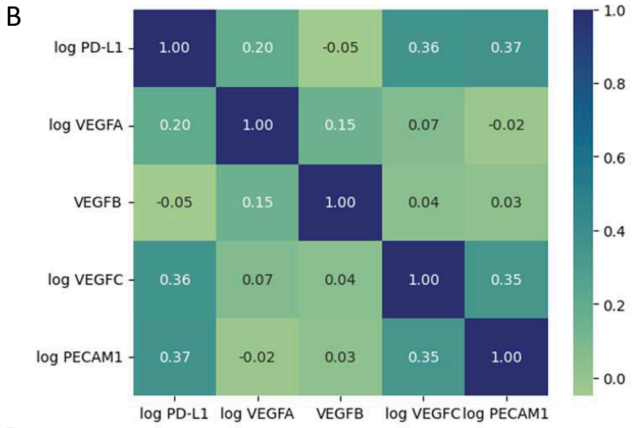
3.2. High PD-L1 expression is correlated with tumor angiogenesis in NSCLC patients

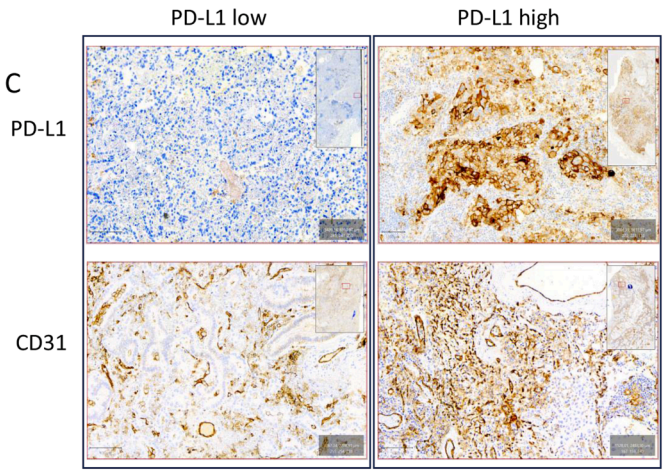
Since STAT family proteins are involved in tumor angiogenesis regulation, we checked a correlation between high PD-L1 and tumor vascularization. The TCGA ADC Dataset, retrieved in May 2023 from the Genomic Data Commons (GCD), contained 592 observations. Gene expression relationships were investigated to assess any correlation between log PD-L1, and other target genes involved in tumor angiogenesis. Statistically significant correlations were found between log PD-L1 and log VEGFA, log VEGFC, and log PECAM1 but not with log VEGFB. Correlation analysis results are reported in Fig. 3A. The correlation matrix is reported in Fig. 3B.

To further confirm these observations, we selected 22 NSCLC patients (17 ADC and 5 SqCC, stage I/III) (Supplementary Table 1) who

A

	log PD-L1
TCGA ADC	
log VEGFA	**0.20 (0.12, 0.27)***
VEGFB	-0.05 (-0.12, 0.03)
log VEGFC	**0.36 (0.28, 0.42)***
log PECAM1	**0.37 (0.29, 0.43)***





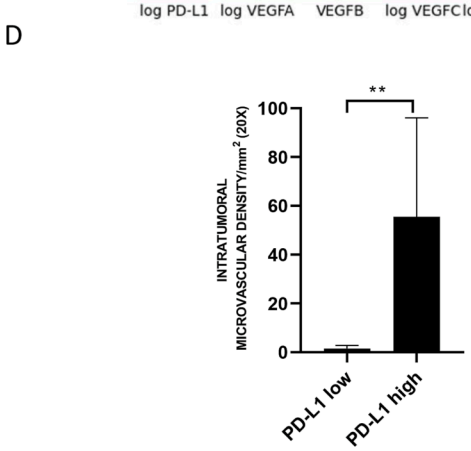


Fig. 3. PD-L1 levels correlate with VEGF and CD31 angiogenic markers and tumor intravasculature (A) Correlation analysis of gene expression levels. Results are reported as Pearson r correlation coefficients with 95 % confidence intervals. Statistically significant correlations are highlighted in bold with asterisks. (B) TCGA ADC dataset correlation matrix. Pearson correlation analysis on a panel of gene expression values. Confidence intervals of log-transformed PD-L1 correlations are reported in (A). (C) Immunoperoxidase-stained sections for PD-L1 and CD31 from a surgically resected NSCLC, scanned with Motic EasyScan One. The black rectangle shows the whole slide section, while the small red rectangle identifies the area shown at higher magnification. Scale bars: 100 μm. (D) Intratumoral microvascular density in NSCLC patients with high vs. low PD-L1 expression. The density of microvessels was assessed by light microscopic analysis (20x) on the most representative tumor areas. *P < 0.05; **P < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

underwent lung resections with curative intent at the Unit of Thoracic Surgery, University-Hospital of Parma. The tumor stage was scored using the staging system from the 8th American Joint Committee on Cancer [35]. The cohort consisted of 14 females and 8 males with a mean age of 76 years. Most of these were ex-smokers (14), 6 smokers and 2 no-smokers. Fifteen patients presented high PD-L1 expression (>50 %) while 7 were negative (<1%).

By immunohistochemistry areas of highest neovascularization were found by light microscopic analysis of the tumor sections at low power and those areas of the tumor with the greatest density of microvessels were selected (Fig. 3C). Interestingly, we observed a significant intratumoral increase in the number of capillaries and small venules in tumors with high PD-L1 expression compared to tumors with low PD-L1 levels (Fig. 3D).

These data globally suggested a correlation between the expression of PD-L1 and tumor angiogenesis.

3.3. Cytokines involved in tumor angiogenesis are secreted by cells with high PD-L1 expression

It has been reported that STAT family proteins transduce signals regulating the expression of a multitude of genes at the transcriptional level. These genes can control cell proliferation and migration, tumor progression, chemotherapy resistance, and neo-angiogenesis. Since we observed STAT signaling activation in PD-L1 overexpressing cells (Fig. 2) and a correlation between PD-L1 and CD31, VEGF A-C, and tumor intra-vasculature (Fig. 3), and considering data reported in the literature [36,37], we evaluated if altered STAT signaling could improve the expression/secretion of VEGF and other pro-angiogenic cytokines and finally enhance the angiogenesis.

As reported in Supplementary Fig. 1, the secretion of pro-angiogenic factors by A549PD-L1 cells over parental ones was not detected in our experimental conditions by employing the Luminex-based assay, suggesting that it could be necessary to further stimulate STAT family members. It is known that cytokines from immune cells promote STAT activation in tumor cells [39]. As expected, activated PBMC released different cytokines (Fig. 4A), in particular, GMC-SF, IFN-γ, IL-10, IL-2, and TNF-α (IL1-b, IL-4, IL-5, and IL-6 were barely increased). For this reason, we enhanced the activation of STAT family members by employing a non-contact co-culture system with activated PBMC (Fig. 4B). We observed an increase of STAT2, STAT5, and STAT6 phosphorylation, in both A549 and H460 PD-L1 overexpressing cells (Fig. 4C) over parental ones.

Therefore, we explored whether STAT activation could be associated with the pro-angiogenic factors secretion; results from multiplex Luminex-based assay (Fig. 4D, E) evidenced that a significant increase in angiogenic factor release in culture medium was observed in both PD-L1 overexpressing cells co-cultured with activated PBMC. In particular, in A549PD-L1 cells we observed increased levels of Angiopoietin-1, PDGF-bb, FGF-a and VEGF-c; likewise, in H460PD-L1 cells, we detected an increase of Angiopoietin-1, PDGF-aa, PDGF-bb and VEGF. Globally, we observed an enhanced production of pro-angiogenic cytokines in PD-L1

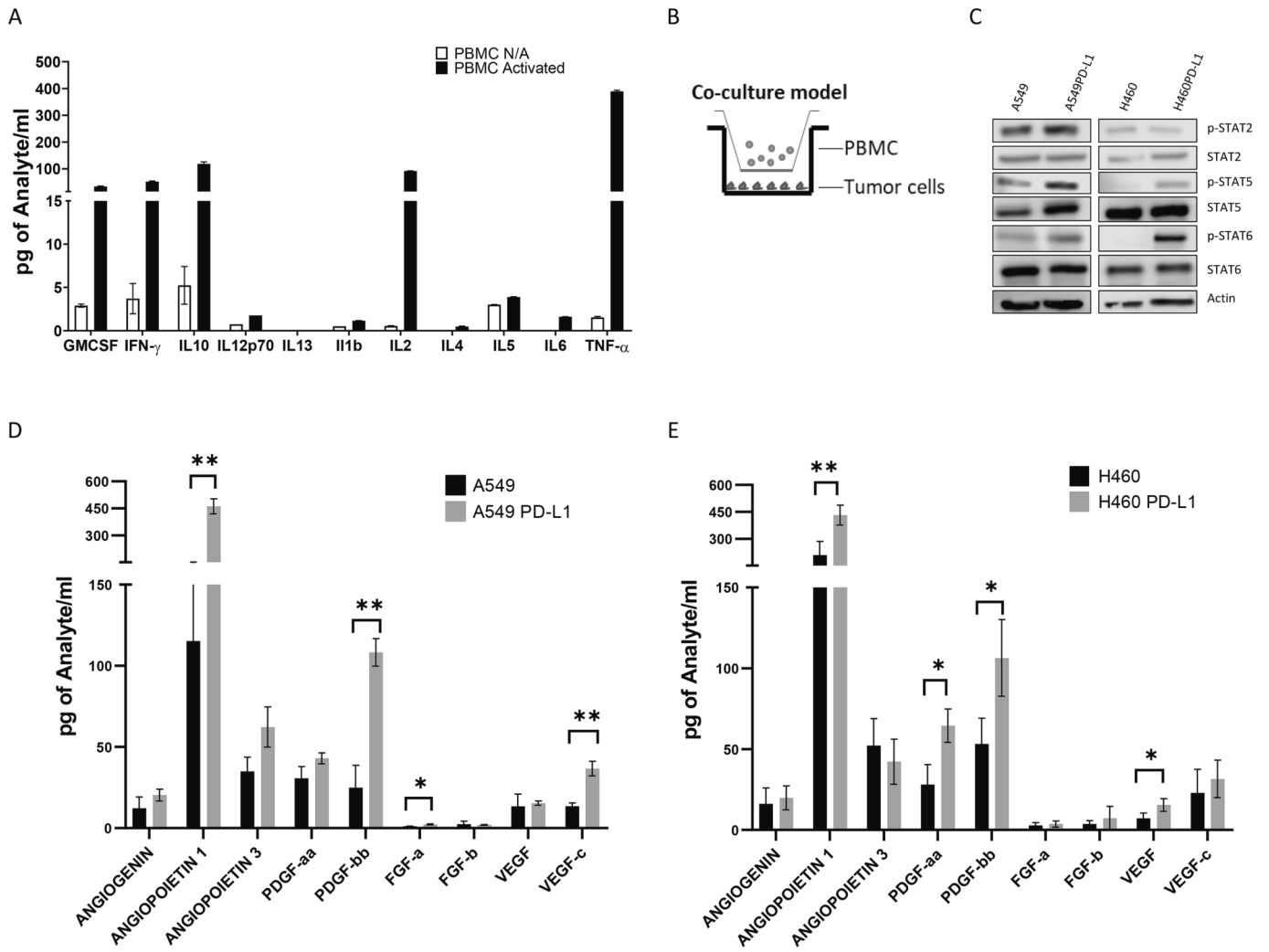


Fig. 4. PD-L1 high cells secrete more pro-angiogenic cytokines when challenged with immune cells (A) Fresh isolated PBMC were cultured for 2 days in medium with/without anti-CD3 and anti-CD28: the media were then collected and submitted to Luminex-based assay for the indicated secreted analytes. The data points represented double determinations and are presented as pg of analyte/ml of medium. (B) Schematic representation of the non-contact co-culture transwell assay. (C) A549, H460, and corresponding PD-L1 overexpressing cells were co-cultured for 2 days in a non-contact condition with activated PBMC; at the end of the experiment, tumor cells were lysed and the phosphorylation of STAT2, STAT5, and STAT6 was evaluated by western blotting. Results are representative of two independent experiments. (D-E) A549, H460, and the corresponding PD-L1 overexpressing cells were co-cultured with activated PBMC in a non-contact cell culture assay for 2 days: at the end the media were submitted to Luminex-based assay for the indicated secreted analytes. The data points represented double determinations, normalized to the total protein content of tumor cells, and are presented as pg analyte/ml of medium. Results are mean ± SD of two independent experiments. *P < 0.05; **P < 0.01.

overexpressing cells.

To ensure that these angiogenic factors were exclusively secreted by tumor cells, we performed a Luminex-based assay on activated PBMC without cancer cells, as reported in Supplementary Fig. 2. The analysis showed that PBMC were not able to secrete relevant amounts of these cytokines, indicating that they were exclusively produced by tumor cells.

3.4. Ruxolitinib markedly reduces pro-angiogenic cytokine secretion

As shown in Fig. 2B and 4C, PD-L1 overexpression increased STAT signaling mainly in the presence of activated PBMC, with an enhanced secretion of angiogenic factors (Fig. 4D, E). Based on these data, we hypothesize that this production may be a consequence of STAT activation. With the aim to demonstrate this assumption, we pharmacologically inhibited STAT signaling.

Ruxolitinib is reported to target Janus kinase 1 and 2 (JAK1 and 2), two intracellular enzymes that positively control the STAT signaling. When 10 μM of ruxolitinib, known to completely inhibit STAT6 phosphorylation [38], was added to our co-culture system, it significantly reduced the PBMC cytokine secretion (Fig. 5A) and the phosphorylation of STAT2, STAT5, and STAT6 in both A549 and H460 PD-L1 overexpressing cells (Fig. 5B). As a consequence, we observed a significant shutdown of the cytokines amount involved in angiogenesis in A549PD-L1 and H460PD-L1 cells (Fig. 5C), demonstrating that the high levels of angiogenic factors released by these cells is a consequence of the STAT activation. Only FGF-b secretion increased after ruxolitinib treatment, underlining the complex and heterogenic regulation of biosynthesis of these pro-angiogenic factors (Fig. 5C).

3.5. Huvec migration is increased by high levels of angiogenic cytokines

Angiogenic cytokines derived from tumor cells exert their function on stroma cells and, in particular, they stimulate the formation of new endothelial vessels that provide the nutrients necessary for tumor expansion. The new vessels are generated by the migration of

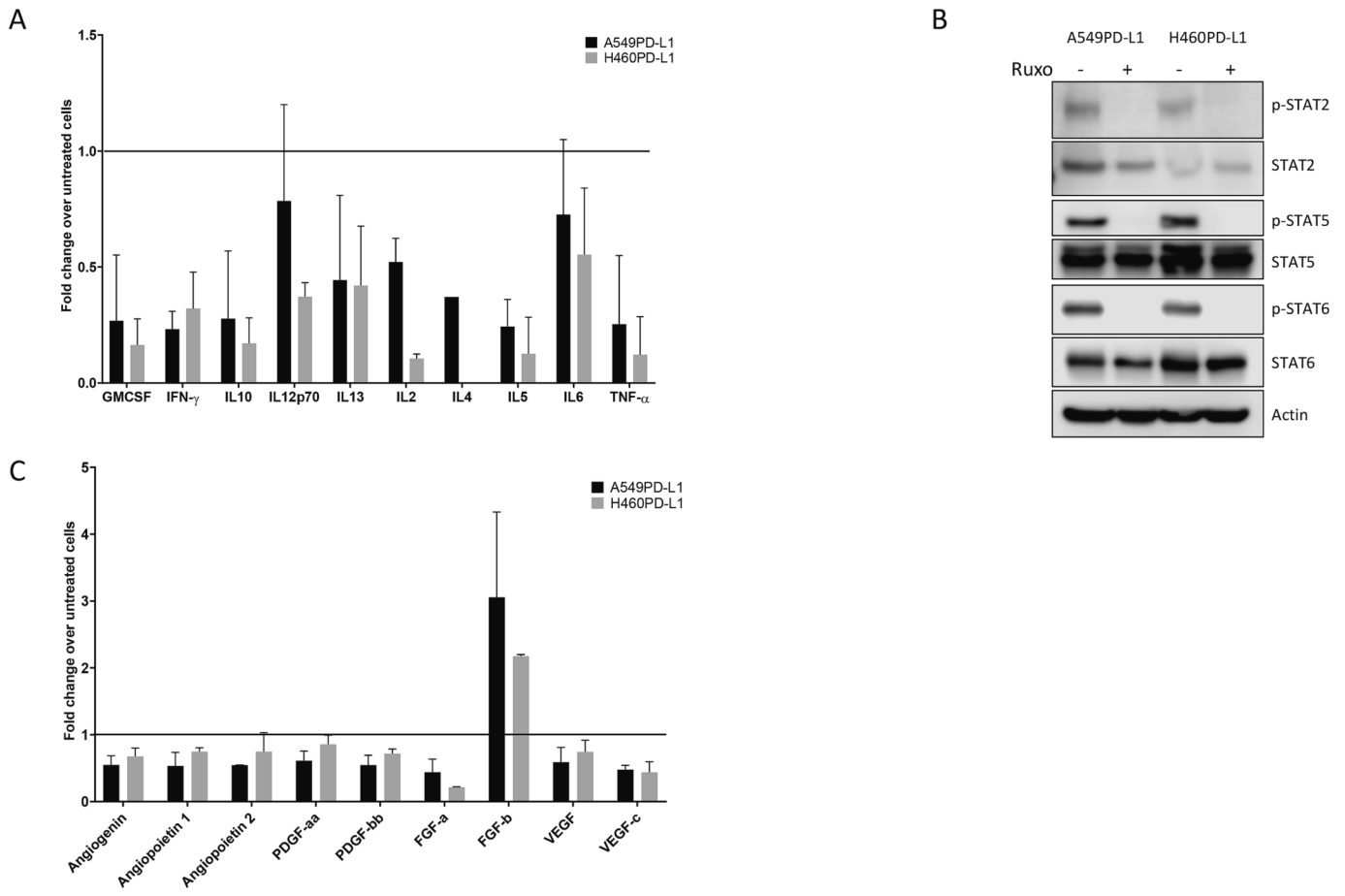


Fig. 5. Effect of ruxolitinib treatment on cytokine and pro-angiogenic factor production (A) A549 and H460 PD-L1 overexpressing cells were incubated with activated PBMC in a non-contact cell culture assay for 2 days in the absence or presence of ruxolitinib 10 μM: at the end, the culture media were submitted to Luminex-based analysis for the indicated secreted cytokines. (B) Western blot analysis of the STAT2, STAT5, and STAT6 phosphorylation in A549 and H460 PD-L1 overexpressing cells, co-cultured for 48 h in a non-contact condition with fresh isolated PBMC in the absence or presence of Ruxolitinib 10 μM. Results are representative of two independent experiments. (C) A549 and H460 PD-L1 overexpressing cells were incubated with activated PBMC in a non-contact cell culture assay for 2 days in the absence or presence of ruxolitinib 10 μM: at the end, the culture media were submitted to Luminex-based analysis for the indicated pro-angiogenic factors. The data points in A and C represent double determinations, normalized to the total protein content, and presented as fold change over untreated PD-L1 overexpressing cells (dot line). The value below the dotted line indicated a reduction of indicated cytokines or pro-angiogenic factors in the culture media.

endothelial cells through basal membranes, a critical phase in the development of new blood vasculature. To test if the secreted pro-angiogenic factors can stimulate endothelial cell migration, Human Umbilical Vein Endothelial Cells (Huvec), were employed in a co-culture assay, as described in Fig. 6A. After 48 h of non-contact co-culture of activated PBMC with tumor cells, the insert with PBMC was removed and a new transwell with Huvec cells was added to the co-culture medium (CM), in order to directly place Huvec cells in contact with medium enriched with angiogenic cytokines.

The analysis reported in Fig. 6B, C demonstrated that the secreted cytokines stimulated Huvec migration and in particular a higher migration index was reported for Huvec cells cultured in the presence of co-culture medium (CM) from both PD-L1 overexpressing cells (CM A549PD-L1 vs CM A549 and CM H460PD-L1 vs CM H460).

These findings may suggest the rationale for the use of targeting agents that selectively suppress the vasculature in high PD-L1-expressing cells. Therefore, we evaluated if an antiangiogenic compound could be more effective at reducing Huvec migration in the presence of high angiogenic factor levels released by PD-L1 overexpressing cells.

The previously reported pro-angiogenic factors exert their function by binding with transmembrane receptors involved in endothelial cell spread, such as VEGFR, PDGFR, and FGFR family members, which represent the main target for the pan-kinase inhibitor nintedanib. As shown in Fig. 6D, the addiction of nintedanib almost completely suppressed the migratory properties of Huvec cells in PD-L1 overexpressing cells (CM A549PD-L1 and CM H460PD-L1) over parental ones (CM A549 and CM H460), suggesting that the antiangiogenic properties of nintedanib are more pronounced in the presence of PD-L1 overexpressing cells.

Finally, consistent with the data reported in Fig. 5B, where Ruxolitinib significantly downregulated the production of cytokines involved in the angiogenesis, we tested the effect of this JAK 1–2 inhibitor on Huvec migration, employing the experimental system reported in Fig. 6A. Ruxolitinib, causing STAT pathway inhibition and depletion of angiogenic factors, significantly inhibited Huvec migration (Fig. 6E).

4. Discussion

In this *in vitro* study, we reported for the first time that PD-L1 can stimulate the production of pro-angiogenic factors, via the STAT signaling pathway, suggesting a novel understanding of the crosstalk between angiogenesis and PD-L1 expression.

It is well known that PD-L1, by binding PD-1 on $CD8^+$ lymphocytes, leads to inhibition of T-cell activation and induction of anergy [39].

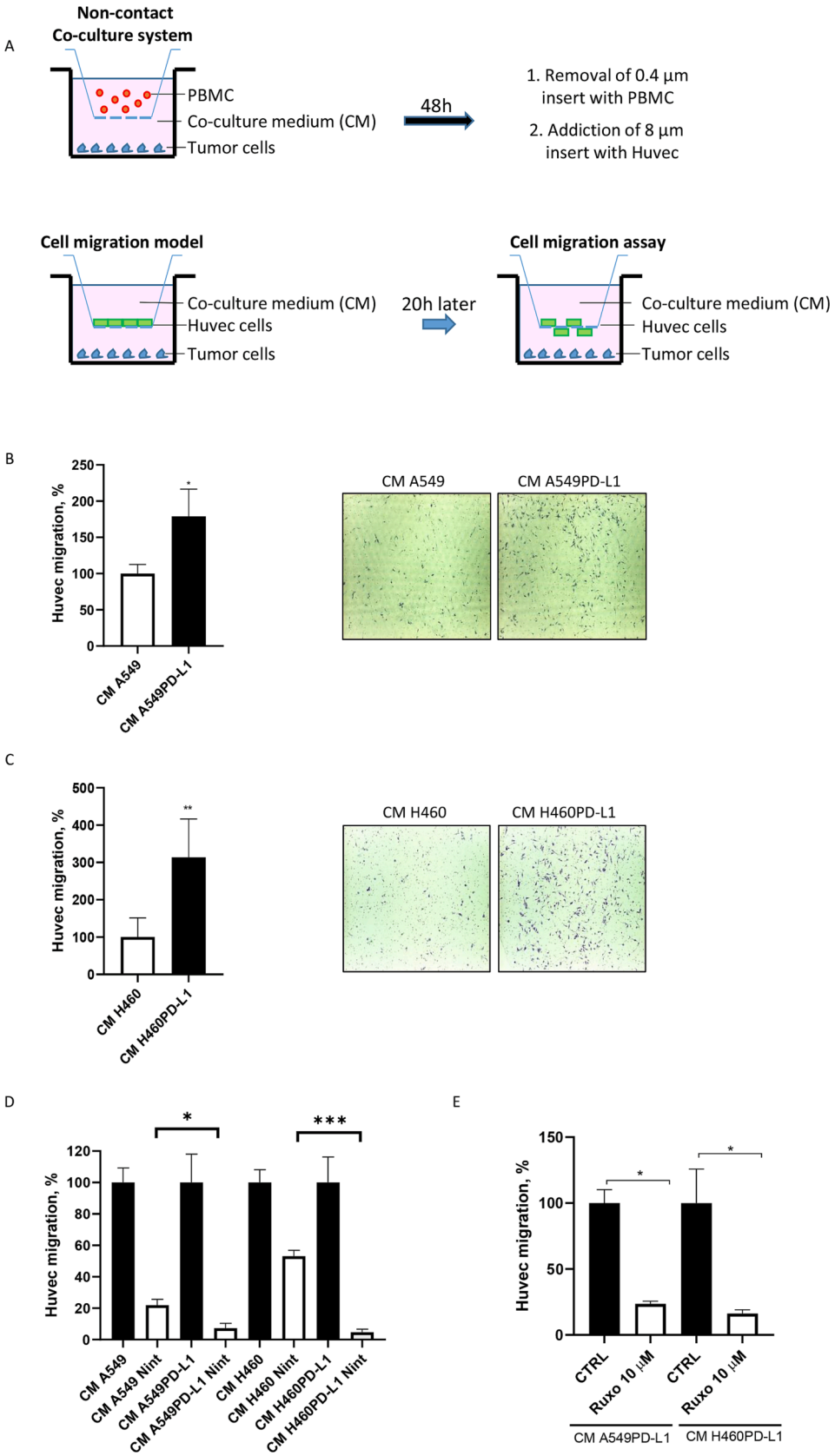


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Fig. 6. Huvec migration in the presence of high angiogenic cytokines levels (A) Schematic representation of Huvec migration in a non-contact co-culture assay. After 2 days of cancer cells and PBMC co-culture, the transwell (pore 0.4 μm diameters) with PBMC was removed and a new transwell (pore 8 μm diameters) with Huvec cells was added. After 16 h, cell migration was evaluated. (B-C) Huvec cells were placed in the co-culture medium from A549 (CM A549), H460 (CM H460), and corresponding PD-L1 overexpressing cells with PBMC, and maintained for 16 h. Representative images of migrated cells are shown. (D) Huvec cells were placed in the co-culture medium from A549 (CM A549), A549PD-L1 (CM A549PD-L1), H460 (CM H460), and H460 PD-L1 (CM H460PD-L1) and maintained in the absence or presence of nintedanib 2 μM for 16 h. (E) Huvec cells were placed in the co-culture medium from A549PD-L1 (CM A549PD-L1) and H460PD-L1 (CM H460PD-L1) with PBMC previously additioned with ruxolitinib 10 μM as for Fig. 5, and maintained for 16 h. Columns, means of 10 fields counted: bars, SD. Results are mean ± SD of two independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Additionally, the use of ICIs, by blocking the PD-1/PD-L1 interaction, causes a marked increase in T-cell activity [40]. Despite some patients experienced durable response after ICI treatment, in a significant number of cases this therapy failed [41], suggesting the existence of undiscovered PD-L1-related mechanisms of tumor progression and immune evasion. Recently, several studies have confirmed an intrinsic role of PD-L1 in cancer cells not related to PD-1 interaction, suggesting further properties of tumor PD-L1 involved in tumor progression [14].

In our *in vitro* models, PD-L1 overexpression in NSCLC cell lines caused a relevant perturbation of signaling pathways involved in tumor inflammation, associated with activation of members of the STAT family, in particular, STAT2, STAT5, and STAT6. The role of PD-L1 as a regulator of STAT family members is quite ambiguous; in a mouse melanoma model, PD-L1 negatively regulates STAT3 activation [4], but this has not been confirmed in preclinical models of NSCLC [19], suggesting that the connection between PD-L1 and STAT family is still unclear.

The observation that PD-L1 modulates pathways related to inflammation and immune cell response suggests that PD-L1 on tumor cells can exert an important intrinsic role in the presence of cells from the immune setting. In our experimental co-culture system, the cytokines produced by immune cells increased the phosphorylation of STAT proteins in PD-L1 overexpressing cells. Moreover, we observed higher production of secreted factors involved in neo-angiogenesis by cells with high PD-L1 levels and this result is strictly dependent on STAT activation. Many studies reported that constitutive activation of STAT family members is directly related to tumor angiogenesis along with other processes of cancer progression [42,43]. In particular, STAT3 has been strictly connected with the induction of pro-angiogenic factors, in response to hypoxia [44] and to pro-inflammatory cytokines [20], and STAT5 induces the expression and secretion of factors that promote angiogenesis [45–47].

Although the role of STAT6 has not been fully dissected as for STAT3 or STAT5, emerging results confirmed its involvement in the promotion of transcription factors involved in tumor angiogenesis [48].

The enhanced pro-angiogenic secretion promoted endothelial cell migration, suggesting that, in our NSCLC models, PD-L1 overexpression could exert its function even by promoting tumor angiogenesis. The existence of this correlation has been supported by both TCGA analysis and the analysis of samples from NSCLC patients with low/high tumor PD-L1 levels, where a significant intra-tumoral vasculature was observed only in high PD-L1-expressing NSCLC patients.

Using *in vitro* co-culture assay, we observed an increase of Huvec migration in the presence of high pro-angiogenic factors secretion, by PD-L1 overexpressing cells, and pharmacological treatment with the anti-angiogenic drug nintedanib significantly blocked Huvec spread.

Moreover, the anti-inflammatory agent ruxolitinib [49], by inhibiting the STAT family in both PBMC and tumor cells, abrogated pro-angiogenic factor secretion and finally Huvec dissemination.

Based on our results defining a consistent increase in pro-angiogenic factor secretion in PD-L1 overexpressing cells, we can trace a new intrinsic role for PD-L1, in the context of the tumor microenvironment. In the presence of high PD-L1 levels as a consequence of the release of pro-inflammatory cytokines as IFN-γ [50] or as a direct effect of oncogenic signals in tumor cells [51], PD-L1 exerts its main function by inhibiting $CD8^+$ T-cell killing properties, but parallelly it can promote tumor angiogenesis by stimulating the secretion of pro-angiogenic cytokines.

Some members of this pro-angiogenic family, and in particular VEGF, can influence immune cells to some extent. In certain conditions, high levels of VEGF by driving aberrant angiogenesis in tumors, can have immunosuppressive effects, inhibiting some aspects of the immune response [52]. Compared to normal blood vessels, tumor vasculature is characterized by abnormal capillary circulation and heterogeneous aspect of vascular endothelial cells, with loss of connections between pericytes and basement membrane. These abnormalities of structure alter tumor blood perfusion and tumor microenvironment, with increased interstitial fluid pressure and hypoxia [53]. In this context, immune effector cells have a reduced capacity to invade solid tumors and often exhibit altered functions.

Moreover, one of the ways VEGF can directly modulate immune cells is by promoting the recruitment and activity of regulatory T-cells (Tregs) [54]. Additionally, VEGF and other angiogenic factors, such as Angiogenin-2 [55] have been shown to affect the function of other immune cells, such as dendritic cells [56] and macrophages [57]. VEGF can impair their antigen-presenting capabilities and alter their cytokine production, which can influence the immune response.

VEGF can also prevent T-cell spread and infiltration by stopping adhesion between T cells and vascular endothelial cells and reducing CXCL10 and CXCL11 levels [58].

Globally, these angiogenic factors can modify the tumor microenvironment, leading to the recruitment of immunosuppressive Tregs and myeloid-derived suppressor cells (MDSCs) that can suppress the activity of immune cells and promote resistance to immune checkpoint inhibitors.

The overall findings of this study support the role of PD-L1 as a critical regulator of the TME: by generating an immune suppressive microenvironment through the production of pro-angiogenic factors, PD-L1 could make ineffective the ICI based treatment in NSCLC patients.

To overcome the resistance associated with pro-angiogenic factors, combination therapies are being explored [55]. For example, the combination of pembrolizumab with anti-VEGF/VEGFR agents, such as bevacizumab or ramucirumab, could be a promising strategy in certain cancers by targeting both the immune checkpoint and the angiogenesis pathways simultaneously. Additionally, other strategies like the dual blockade of immune checkpoints and VEGF signaling pathways are being investigated to enhance treatment responses [59].

Ongoing clinical trials have been assessing anti-angiogenic agents in combination with ICI, both in neoadjuvant setting (NCT04040361; NCT04875585), adjuvant setting (NCT00324805), first-line (NCT03829319; NCT03836066; NCT05859217; NCT04925986) and in later lines after immunotherapy failure (NCT03976375; NCT05633602; NCT03689855; NCT04340882; SAFFRON-301; NCT04921358).

Considering that different pro-angiogenic factors are secreted by PD-L1 overexpressing cells, the choice of the correct anti-angiogenic compound is critical. The combination of ICI with bevacizumab is certainly relevant but may present some problems: for example, it has been recently reported that in KRAS mutated tumor epithelial cells, high levels of Angiopoietin-2 are responsible for resistance to the anti-angiogenic compound bevacizumab, as it stimulates tumor angiogenesis in a VEGF-independent manner [60]. While VEGF is mainly involved in vasculature development by promoting endothelial tips, Angiopoietin-2 is responsible for the repeal of pericytes from vessels to allow sprouting [61]. Based on these data, the combination encompassing an ICI with a

multikinase inhibitor, such as nintedanib used in our study, or with a combination of specific angiopoietin targeting agents should be particularly beneficial for the subset of PD-L1 overexpressing NSCLC patients.

5. Conclusions

Our results reported for the first time that, independently from the binding with PD-1 on T-lymphocytes, PD-L1 overexpressing cells produced high levels of pro-angiogenic factors via STAT signaling in the presence of immune cells. This sheds light on a previously unrecognized function of PD-L1 in the tumor microenvironment, where it emerges as a critical regulator of tumor vasculature. This role has significant implications for the effectiveness of ICIs, potentially providing the rationale for including antiangiogenic agents together with ICIs for NSCLC patients with high PD-L1 expression levels in the first-line setting, or at the tumor progression as a second-line approach.

Conflict of interest

No potential conflicts of interest that might be relevant to the contents of this manuscript were disclosed. MT received speakers' and consultants' fees from AstraZeneca, Pfizer, Eli-Lilly, BMS, Novartis, Roche, MSD, Boehringer Ingelheim, Otsuka, Takeda, and Pierre Fabre. TM received institutional research grants from AstraZeneca and Boehringer Ingelheim. AR received institutional research grants from AstraZeneca.

LA received speakers' fees for AstraZeneca and MSD. LA has been on advisory boards for BeiGene, Sanofi, and Novartis.

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CRediT authorship contribution statement

A. Cavazzoni: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. **G. Digiacomo:** Conceptualization, Methodology. **F. Volta:** Conceptualization, Investigation. **R. Alfieri:** Formal analysis, Visualization, Writing – review & editing. **E. Giovannetti:** Methodology. **L. Gnetti:** Data curation. **L. Bellini:** Methodology. **M. Galetti:** Methodology. **C. Fumarola:** Formal analysis. **G. Xu:** Methodology. **M. Bonelli:** Data curation. **S. La Monica:** Data curation. **M. Verzè:** Validation. **A. Leonetti:** Validation. **K. Eltayeb:** Methodology. **S. D'Agnelli:** Data curation. **L. Moron Dalla Tor:** Software. **R. Minari:** Validation. **P.G. Petronini:** Writing – review & editing, Supervision. **M. Tiseo:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lungcan.2023.107438.

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