Upregulation of PD-L1 by EML4-ALK fusion protein mediates the immune escape in ALK positive NSCLC: Implication for optional anti-PD-1/PD-L1 immune therapy for ALK-TKIs sensitive and resistant NSCLC patients

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ABSTRACT

Driver mutations were reported to upregulate programmed death-ligand 1 (PD-L1) expression. However, how PD-L1 expression and immune function was affected by ALK-TKIs and anti-PD-1/PD-L1 treatment in ALK positive non-small-cell lung cancer (NSCLC) remains poorly understood. In the present study, western-blot, real-time PCR, flow cytometry and immunofluorescence were employed to explore how PD-L1 was regulated by ALK fusion protein. ALK-TKIs and relevant inhibitors were used to identify the downstream signaling pathways involved in PD-L1 regulation. Cell apoptosis, viability and Elisa test were used to study the immune suppression by ALK activation and immune reactivation by ALK-TKIs and/or PD-1 blocking in tumor cells and DC-CIK cells co-culture system. We found that PD-L1 expression was associated with EGFR mutations and ALK fusion genes in NSCLC cell lines. Over-expression of ALK fusion protein increased PD-L1 expression. PD-L1 mediated by ALK fusion protein increased the apoptosis of T cells in tumor cells and DC-CIK cells co-culture system. Inhibiting ALK by sensitive TKIs could enhance the production of IFN-γ. Anti-PD-1 antibody was effective in both crizotinib sensitive and resistant NSCLC cells. Synergistic tumor killing effects were not observed with ALK-TKIs and anti-PD-1 antibody combination in co-culture system. ALK-TKIs not only directly inhibited tumor viability but also indirectly enhanced the antitumor immunity via the downregulation of PD-L1. Anti-PD-1/PD-L1 antibodies could be an optional therapy for crizotinib sensitive, especially crizotinib resistant NSCLC patients with ALK fusion gene. Combination of ALK-TKIs and anti-PD-1/PD-L1 antibodies treatment for ALK positive NSCLC warrants more data before moving into clinical practice.

KEYWORDS

EML4-ALK; immunotherapy; NSCLC; PD-L1; PD-1

Introduction

Lung cancer is a leading cause of cancer mortality worldwide. NSCLC accounts for about 85% of all lung cancer cases. The epidermal growth factor receptor (EGFR) gene is one of the most common driver genes in NSCLC. Up to 47.9% of Asian NSCLC patients harbor EGFR mutation. Fusion of the Echinoderm microtubule-associated protein like-4 (EML4) and anaplastic lymphoma kinase (ALK) represents another distinct mechanism of driver mutation in NSCLC, accounting for about 4–8.1% of NSCLC patients. Although chemotherapy remains the main treatment of advanced NSCLC, small molecular tyrosine kinase inhibitors (TKIs) were recommended for the first line treatment of advanced NSCLC with druggable driver mutations. However, a majority of patients eventually develop acquired resistance and limited strategies are available to handle TKIs resistance. Novel and more effective therapy for NSCLC is urgently warranted.

Currently, immunotherapies have intensively been studied. A large number of immunotherapeutic approaches to cancer treatment have been established. Compared with chemotherapy, anti-PD-1 and anti-PD-L1 antibodies show durable response in a limited subset of NSCLC patients. Exploring effective biomarkers to identify the subset of NSCLC patients that most likely to benefit from this expensive treatment is important. A limited number of studies indicated that PD-L1 expression might be a predictive biomarker for therapeutic response to anti-PD-1 and anti-PD-L1 antibodies. Therefore, it is of clinical importance to explore the genetic background of PD-L1 expression and how the response to this treatment is influenced.

Recently, it was shown that high PD-L1 expression was associated with EGFR mutation and EML4-ALK fusion protein in NSCLC. Another study demonstrated that EGFR mutant tumors display elevated PD-L1 levels and the EGFR mutant mice showed significant response to anti-PD-1...
antibody, indicating EGFR mutation might be a promising biomarker of response to PD-1 blockade. However, how PD-L1 expression and immune function was affected by ALK-TKIs and anti-PD-1/PD-L1 in ALK positive NSCLC were largely unknown.

The purpose of this study is to investigate the detailed regulatory mechanism of PD-L1 by EML4-ALK fusion protein and whether blocking PD-L1/PD-1 could be a novel treatment option in ALK-TKIs sensitive and resistant NSCLC with EML4-ALK fusion gene.

**Materials and methods**

The following studies were conducted in a laboratory that operates under exploratory research principles. These studies were performed using established laboratory protocols. These studies were performed using general research investigative assays. Raw data can be provided per request.

**Cell lines and cell culture**

Human NSCLC cell lines H3122, H2228, H1993, A549, PC-9, HCC827 and H1975 were purchased from the American Type Culture Collection. Immortalized human lung bronchial epithelial cell line (Beas-2B) and DFC1076 were generously provided by Prof. Liang Chen (National Institute of Biological Sciences, Beijing, China). Recombinant Lentivirus expressing either vector (GV230) or GV230 subcloned with EML4-ALK (V1) was constructed by Genechem Corporation (Shanghai, China). The gene sequence of EML4-ALK (V1) was confirmed by PCR-based sequencing. The plasmid DNA was transfected into Beas-2B cell line by using Lipopectamine 

**Western blot analysis**

The detailed protocol was the same as previous reported. The membranes were immunoblotted with primary antibodies against PD-L1 (E1L3N TM, Rabbit mAb) or ALK (D5F3, Rabbit mAb) (Cell Signaling Technology, Danvers, MA); b-actin was normalized to b-actin expression. Different PD-L1 mRNA levels of other cell lines were relative to that of Beas-2B cells.

**Immunochemistry**

Human A549, DFC1076, Beas-2B-EML4-ALK (V1) and Beas-2B-vector cells grown on a chamber slide (BD Biosciences, San Jose, CA) were washed with cold PBS, fixed with 4% paraformaldehyde in PBS for 15 min. After 1h blocking in PBS plus 0.1% Tween-20 and 3% donkey serum, cells were incubated with primary antibodies of PD-L1 (E1L3NTM, Rabbit mAb) or ALK (D5F3, Rabbit mAb) (Cell Signaling Technology, Danvers, MA) at 4°C overnight. After three times of washing, cells were incubated with secondary antibody (Alexa Fluor 488 or 555 donkey anti-rabbit IgG [H+L], Life Technologies, LA) for 1h at room temperature. Then, three times of washing, cells were counterstained with DAPI (P36931, Life technologies, USA) for 5 min. Slides were observed and photographed with fluorescence microscopy after mounting. These experiments were triplicated.

**RNA extraction and PCR**

The extraction of total RNA and synthesis of cDNA were previously described. Quantitative real-time PCR analysis was performed using ABI Prism 7,900-HT Sequence Detection System (96-well, Applied Biosystems, USA). For RT-PCR, the following primers were used for the amplification of PD-L1: forward primer 5#-CCTACTGGCATTTTGTGAAGCTAT-3# and reverse primer 5#-ACCATAGCTGTACTGCAACGGTA-3#; b-actin: 5#-TCTCTGATCCACCACAACT-3# (forward) and 5#-GAAGCATTTGCGGTGGACGAT-3# (reverse). The experiments were triplicated. The relative expression of PD-L1 was normalized to b-actin expression. Different PD-L1 mRNA levels of other cell lines were relative to that of Beas-2B cells.

**ALK siRNA-mediated silencing and inhibitors treatment assay**

For siRNA-mediated silencing of ALK, H3122 cells were transiently transfected with ALK siRNAs by using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The target sequence of ALK siRNA #1 is GAGACATTGCTGCCAGAAA. The target
sequence of ALK siRNA #2 is CGAGGATACATTCTGAAA. The target sequence of ALK siRNA #3 is CGCGTTTGGCGG-TAGAATA. Western blot experiments were conducted after 48 h incubation of transiently transfected cells. To study the signaling pathways involved in PD-L1 mediated by ALK, the following inhibitors were used. H3122 and DFCI076 cells were serum-starved for 2 h then treated with the following kinase inhibitors for 24 h under different doses: ALK inhibitors (TAE684, LDK378, and crizotinib), ERK1/2 inhibitor (SCH772984), AKT1/2 inhibitor (MK-2206 2HCL), JAK3 inhibitor (Tofacitinib), Stat3 inhibitor (Cryptotanshinone), which were all purchased from Selleckchem (Houston, USA). Recombinant humanized anti-PD-1 antibody pembrolizumab (MK-3475, Keytruda) is from Merck Sharp & Dohme Corp (Whitehouse Station, NJ08889, USA).

Beas-2B cells or NSCLC Cells Co-cultured with DC-CIK Cells

Beas-2B-EML4-ALK (V1), Beas-2B-vector, H3122 and DFC1076 cells were respectively seeded into 24-well plates at a density of 3 x 10^4 cells/well overnight. DC-CIK cells were kindly provided by Prof. Jian-chuan Xia (Department of Biotherapy, Sun Yat-sen University Cancer Center, Guangzhou, China) as previously described. Human peripheral blood mononuclear cells (PBMC) were isolated by FicollPaque density centrifugation from 50 mL of heparinized peripheral blood donated by healthy volunteers (details of donors’ information were provided Table S1). Then, PBMC were cultured using complete medium containing 1,000 U/mL IFNγ (Clone-gamma, Shanghai Clone Company, Shanghai, China) and mouse anti-human CD3 monoclonal antibody (R&D Systems, Shanghai, China) and IL-2 (rhIL-2; Beijing Sihuan, Beijing, China) and IL-1α (Life Technologies, Guangzhou, China) at a final concentration of 100 ng/mL, 1,000 U/mL and 100 U/mL, respectively. After 24 h, non-adherent cells were separated from PBMC and cultured into CIK (cytokine-induced Killer) cells. The adherent PBMCs were cultured in AIM-V containing 1,000 U/mL granulocyte macrophage colony-stimulating factor (GM-CSF) (Immunex, Seattle, USA), and 500 U/mL interleukin-4 (IL-4) (StemCell Technologies, Vancouver, Canada) for 6 d. On the sixth day, TNF-α was added into the medium. After 7 days’ culture, monocyte-derived dendritic cells (DC) were harvested from the non-adherent and loosely adherent cells. Then, DC was co-cultured with CIK cells for additional 7 days. Finally, on the 14th day, the acquired DC-CIK cells were co-cultured with Beas-2B-EML4-ALK (V1), Beas-2B-vector, H3122 or DFCI076 cells at the ratio of 2:1. DC-CIK/Beas-2B-EML4-ALK (V1) and DC-CIK/H3122 co-cultured cells were respectively treated with anti-PD-1 antibody (1,000 µg/mL), TAE684 (200 nM) or mock. DC-CIK/DFCI076 co-cultured cells were respectively treated with anti-PD-1 antibody (1,000 µg/mL), crizotinib (200 nM), LDK378 (200 nM) or vehicle. After 48 h treatment, suspended DC-CIK cells were harvested from the co-culture system and stained with anti-human CD3 monoclonal antibody (OKT-3, 11–0037, affymetrix eBioscience) for apoptosis assay. The cell-free supernatant from co-cultured system after 14,000 rpm centrifugation was collected for IFNγ analysis by ELISA.

Annexin V-APC/7-AAD apoptosis assay

CD3 positive T lymphocytes were sorted from DC-CIK cells after staining with CD3 antibody by flow cytometry. The apoptosis of CD3 positive T cells was determined according to the protocol of the Annexin V-APC/7-AAD Apoptosis Detection kit (KG1A023–1026, KeyGEN, Nanjing, China). The Annexin V-APC and 7-AAD fluorescence levels were measured by flow cytometry (BD Biosciences, FACS Calibur). The Annexin V-APC-positive cells (both 7-AAD-negative and -positive) were defined as apoptotic cells.

IFNγ analysis by ELISA

IFNγ level in cell-free supernatant was determined using Human IFNγ ELISA kit (eBioscience, USA) according to the manufacturer’s protocol. These experiments were triplicated.

Cells survival analysis

To suppress interference of DC-CIK cells, the proliferation rate of H3122 or DFCI076 cells was dynamically monitored in real time using the xCELLigence system (E-plate, Roche). To establish a background reading for the RTCA system, firstly, 50 µL of growth complete media was added to each well of the 96-well E-plates and tested. H3122 and DFCI076 cells were respectively seeded into 96-well plates at a density of 1.0 x 10^4 cells/well. Then, they were respectively co-cultured with 50 µL/well DC-CIK cells acquired previously at the ratio of 1:1. Next, an additional 50 µL/well of the growth medium containing different drugs such as 1,000 µg/mL of anti-PD-1 combined with 200 nM of TAE684 or either agent alone or vehicle was respectively added to the different wells of E-plate in DC-CIK/H3122 co-cultured system. Likely, 200 nM of crizotinib, 200 nM of LDK378, 1,000 µg/mL of anti-PD-1 or combined with 200 nM of LDK378 or vehicle was respectively added to the different wells of E-plate in DC-CIK/DFCI076 co-cultured system for a total volume of 200 µL/well before plating the plates at 37°C incubators with 5% CO2. Cell growth status from each E-plate were monitored every 15 min and cell index values were expressed accordingly based on observing dynamic cell growth curves.

Statistical analysis

Representative results from three independent experiments were shown in the present study. Numerical data were presented as the mean ± standard deviation of the mean (SD). The p-values between two experimental groups were tested by two-tailed Student’s t-test and p-values less than 0.05 were considered significant.

Results

PD-L1 expression was associated with EML4-ALK fusion and EGFR mutational status in human NSCLC cell lines

The protein level of PD-L1 in EML4-ALK fusion NSCLC cell lines (H3122, H2228 and DFCI076) and EGFR mutant NSCLC cell lines (PC9, HCC827 and H1975) was significantly higher than that in
double-wild type cell lines (A549, H1993) or that in an immortalized human lung Beas-2B cell (Fig. 1A), which was consistent with the results of PD-L1 mRNA level (Fig. 1B). Moreover, we employed immunofluorescence to locate PD-L1 in DFCI076 cell line (relatively higher PD-L1 expression) and A549 cell line (very weak PD-L1 expression). Both cell membrane and cytoplasm in DFCI076

Figure 1. PD-L1 expression was associated with EML4-ALK fusion and EGFR mutational status in human NSCLC cell lines. (A) The protein expression level of PD-L1 (detected by western blot) in several common NSCLC cell lines and an immortalized human lung Beas-2B. GAPDH was used to verify equal loading. (B) The relative expression level of PD-L1 mRNA (detected by real time PCR) in different NSCLC cell lines and Beas-2B as described above. The relative expression level of PD-L1 mRNA was normalized to that in Beas-2B cell line. (C) The localization of PD-L1 (green signal) in DFCI076 and A549 cell lines was shown by immunofluorescence counterstained with DAPI (blue signal). (D) Flow cytometry analysis of cell-surface PD-L1 expression in DFCI076 and A549 cell lines (PD-L1, red line; isotype controls, gray zone). All the experiments were repeated three times. Representative data are shown.
cells showed strong PD-L1 signal (green fluorescence) (Fig. 1C). The different levels of PD-L1 expression in A549 and DFCI076 were further confirmed by flow cytometry (Fig. 1D).

**Overexpression exogenous EML4-ALK induced PD-L1 expression**

EML4-ALK (V1) over-expression plasmid was constructed in an immortalized human lung Beas-2B cell and HEK 293T cells. We found that the expression of PD-L1 was induced by exogenous EML4-ALK (V1) and the induction of PD-L1 by EML4-ALK (V1) fusion protein was antagonized by a selective ALK inhibitor TAE684 (Fig. 2A, B). The induction of PD-L1 by exogenous overexpression of EML4-ALK (V1) was further confirmed by flow cytometry (Fig. 2C, D). Finally, we employed immunofluorescence to show the association between EML4-ALK (V1) and PD-L1
expression. The higher expression of EML4-ALK (V1) fusion protein and PD-L1 were both presented on the membrane of Beas-2B with EML4-ALK (V1) over-expression (Fig. 2E). Taken together, these results indicate that overexpression of exogenous EML4-ALK induced PD-L1 expression.

**Inhibiting EML4-ALK could reduce PD-L1 expression**

Since PD-L1 was induced by exogenous EML4-ALK (V1) protein, it should be feasible that PD-L1 expression in ALK fusion NSCLC cells be suppressed by blocking ALK. Firstly, we employed three different targeted ALK-siRNAs to knock down ALK expression. As shown in Fig. 3A, all the ALK-siRNAs significantly knocked down ALK expression, followed by the downregulation of PD-L1 in H3122 with endogenous EML4-ALK expression. The downregulation of PD-L1 by ALK knockdown was confirmed at mRNA level (Fig. 3B). Then, we use specific ALK inhibitor TAE684 to inhibit ALK activity. As expected, PD-L1 was reduced after ALK activity was inhibited (Fig. 3C). DFCI076 cells, which harbor EML4-ALK fusion gene but are resistant to crizotinib, were treated with crizotinib and LDK378, respectively. The PD-L1 expression of DFCI076 cell line was not changed due to unaltered ALK kinase activity after crizotinib treatment. However, LDK378 significantly inhibited the activation of ALK and downregulated PD-L1 protein level (Fig. 3D). Taken together, our data showed that inhibiting EML4-ALK could reduce PD-L1 expression in NSCLC with EML4-ALK fusion protein.

**EML4-ALK regulated PD-L1 through p-ERK1/2 and p-AKT but not p-JAK3 signaling**

Here we tried to identify the signaling pathways involved in ALK-induced PD-L1 expression in NSCLC. P-ERK1/2, p-AKT and p-Stat3 were activated by EML4-ALK fusion protein and inhibited by TAE684 (Fig. 4A, B). Furthermore, the inhibitors of ERK1/2, AKT1/2/3, JAK3 and Stat3 were used to block the downstream pathways of EML4-ALK. We found ERK1/2 inhibitor (SCH772984) could effectively inhibit p-ERK1/2, resulting in the decrease of PD-L1 expression (Fig. 4C). In addition, AKT1/2/3 inhibitor (MK-2206 2HCL) could effectively suppress p-AKT1/2/3 in a dose-dependent manner and the expression of PD-L1 was also downregulated (Fig. 4D). However, the expression of PD-L1 was not altered after p-JAK3 was inhibited by JAK3 inhibitor or after p-Stat3 was inhibited by STAT3 inhibitor (Fig. 4E, F). These results show that EML4-ALK regulated PD-L1 through p-ERK1/2 and p-AKT signaling but not p-JAK3/Stat3 signaling in NSCLC cells with EML4-ALK fusion protein.
Figure 4. EML4-ALK regulated PD-L1 through p-ERK1/2 and p-AKT but not p-JAK3 signaling. (A) The protein expression level of ALK, ERK1/2, p-ERK1/2, AKT, p-AKT, JAK3, p-JAK3, STAT3, p-STAT3 Tyr705, GAPDH in Beas-2B-vector, Beas-2B-EML4-ALK (V1) cell lines. (B) The protein expression level of ALK, ERK1/2, p-ERK1/2, AKT, p-AKT, JAK3, p-JAK3, STAT3, p-STAT3 Tyr705, GAPDH in H3122 cells which were treated with 0, 0.1, 0.2, 0.4 μM ALK inhibitor (TAE684). (C) The protein expression level of ERK1/2, p-ERK1/2, PD-L1 and GAPDH in H3122 cells which were treated with 0, 0.25, 0.5, 1.0 μM ERK1/2 inhibitor (SCH772984). (D) The protein expression level of p-AKT, AKT, PD-L1 and GAPDH in H3122 cell which were treated with 0, 0.5, 1.0, 2.0 μM AKT1/2/3 inhibitor (MK-2206 2HCL). (E) The protein expression level of JAK3, p-JAK3 and PD-L1 in H3122 cells which were treated with 0, 1.0, 2.0, 4.0 μM p-JAK3 inhibitor (Tofacitinib). (F) The protein expression level of Stat3, p-Stat3 (Tyr 705) and PD-L1 in H3122 cells which were treated with 0, 3.0, 7.0, 10.0 μM Stat3 inhibitor (Cryptotanshinone). Representative results from three independent experiments are shown.
**Upregulation of PD-L1 mediated by EML4-ALK fusion protein could induce the apoptosis of T Cells through PD-L1/PD-1 Axis**

PD-L1/PD-1 signal axis could lead to the apoptosis or suppression of T cells, which could be reversed by anti-PD-1 antibody. To investigate whether PD-L1 upregulation mediated by EML4-ALK fusion protein could also affect T cell function, we co-cultured DC-CIK with Beas-2B-EML4-ALK (V1) or Beas-2B-vector cells. As Fig. 2A shows, Beas-2B-EML4-ALK (V1) cells expressed higher level of PD-L1 than Beas-2B-vector cells did. The apoptosis rate of CD3+ T cells co-cultured with Beas-2B-EML4-ALK (V1) cells is higher than that with Beas-vector cells (24.9 ± 3.4% vs. 9.8 ± 1.0%, p < 0.05). Blocking PD-1 with anti-PD-1 antibody in Beas-2B-EML4-ALK (V1)/DC-CIK co-culture system reduced the apoptosis rate of T cells to 12.2 ± 1.7% (p < 0.05). Next, we used TAE684 to reduce the expression of PD-L1 mediated by EML4-ALK. We found the apoptosis rate of T cells decreased to 17.1 ± 1.3% (p < 0.05). Our results show that PD-L1 upregulation mediated by EML4-ALK could induce the apoptosis of T cells through PD-L1/PD-1 axis.

**Downregulation PD-L1 by ALK-TKIs in NSCLC with EML4-ALK fusion could relieve the suppression of T cells**

To determine whether inhibiting EML4-ALK by ALK inhibitors could reverse the apoptosis of T cells, we co-cultured H3122 cells with DC-CIK cells and found the apoptosis rate of T cells decreased from 28.8 ± 1.4% to 19.3 ± 1.5% (p < 0.05) when treated with TAE684 in H3122/DC-CIK co-culture system (Fig. 6A, B). In DFCI076/DC-CIK co-culture system, LDK378 decreased the apoptosis rate of T cells from 35.5 ± 2.3% to 23.7 ± 3.4% (p < 0.05). However, crizotinib did not affect the apoptosis rate of T cells (p = 0.7). Blockade of PD-1 could reverse the apoptosis of T cells when DC-CIK cells were co-cultured with either H3122 or DFCI076 (13.4 ± 2.1% and 16.4 ± 1.9%, p < 0.05). (Fig. 6A, C)

To understand whether ALK-TKIs could affect the immune function of T cells when T cells were co-cultured with EML4-ALK positive NSCLC, the co-cultured supernatant was collected for IFNγ analysis. We found IFNγ was markedly increased in the supernatant of H3122/DC-CIK cells co-culture system after TAE648 treatment. As expected, crizotinib did not affect the IFNγ level while LDK378 remarkably enhanced the production of IFNγ in the supernatant of DFCI076/DC-CIK cells co-culture system (Fig. 6E, F). These results indicate that inhibiting ALK activity by sensitive ALK inhibitors could...
reverse the apoptosis of T cells and enhance the production of IFNγ in vitro.

Survival inhibition of NSCLC with EML4-ALK by anti-PD-1 or ALK blocking

The above data showed that blockade of PD-1 or inhibiting EML4-ALK by sensitive ALK inhibitors could relieve the suppression of T cells. In order to determine the effect of PD-1 blocking or ALK inhibitors on tumors cells in DC-CIK and tumor cells co-culture system, we tested the real-time survival signal of attached tumors cells. The cell index represents the survival rate of attached tumors cells. Tumor cells co-cultured with DC-CIK cells were treated with anti-PD-1 antibody and/or plus ALK inhibitors. We found blockade of PD-1 decreased the survival of H3122 cells and crizotinib resistant DFCI076 cells. The survival rates of H3122 cells but not DFCI076 cells decreased after TAE684 administration. However, LDK378 significantly decreased the survival rate of DFCI076 cells. Then, we tried to explore whether ALK inhibitor plus anti-PD-1 antibody could have additional inhibition of tumor cells in the co-culture system. The result shows that no synergistic tumor killing effect of ALK inhibitor and anti-PD-1 antibody was observed (Fig. 7A, B). These results demonstrated that anti-PD-1 antibody or sensitive ALK inhibitor could activate the antitumor immunity of T cells and decrease the survival rates of crizotinib sensitive and resistant NSCLC cells in tumors cells and DC-CIK cells co-culture system.

Discussion

Recent studies demonstrated that PD-L1 was over-expressed in 19.63%–65.38% of NSCLC. However, the genetic determinants of PD-L1 in NSCLC cancer patients remain unclear. Our and several other groups’ studies found that PD-L1 expression was associated with driver mutations such as EGFR mutation and EML4-ALK fusion gene in NSCLC, which could potentially be manipulated by immunotarget therapy.

In the present study, we demonstrated the expression of PD-L1 in NSCLC cell lines with EML4-ALK fusion gene or EGFR mutation was higher than that in cell lines with double wild-type. We further found over-expression of exogenous EML4-ALK could induce PD-L1 expression, which could be
reversed by ALK-siRNAs or sensitive ALK-inhibitors. Further studies found the downstream pathways p-ERK1/2 and p-AKT but not p-JAK3/p-STAT3 were involved in the regulation of PD-L1 by EML4-ALK fusion protein. These results suggested that EML4-ALK fusion protein represents an intrinsic regulation of PD-L1 in NSCLC with EML4-ALK fusion gene. Similarly, constitutive activation of NPM/ALK was reported to drive PD-L1 expression in lymphoma.29 Loss of phosphatase and tensin homolog (PTEN) could also induce PD-L1 expression in glioma.30 LMP1, a well-recognized oncogene of EBV-related carcinomas also participated in PD-L1 upregulation in NPC.20 These data imply that constitutive oncogene pathway activation could upregulate PD-L1 expression.

Figure 7. Survival inhibition of NSCLC with EML4-ALK by anti-PD-1 or ALK blocking (A) The real time survival curve of H3122 cells co-cultured with DC-CIK cells for 0~100h were measured in the presence of mock, anti-PD-1 antibody (1,000 μg/mL), TAE684 (0.2 μM) or both. (B) The real time survival curve of DFCI076 cells were measured after co-cultured with DC-CIK cells for 0~100h in the presence of mock, crizotinib (0.2 μM) LDK378 (0.2 μM) and/or plus anti-PD-1 antibody (1,000 μg/mL). Representative results from three independent experiments are shown.

Figure 8. Proposed treatment paradigms for EML4-ALK-driven NSCLC.
Previous study shows that that upregulation of PD-L1 by EGFR activation could mediate the immune escape in EGFR-driven NSCLC.38 EGFR-TKIs could not only have direct tumor killing effect but also have immune enhancement function.18 However, how the immune function of T cells was affected by ALK fusion gene and ALK-TKIs in ALK positive NSCLC were largely unknown.

Here we found upregulation of PD-L1 by EML4-ALK fusion protein could induce the apoptosis of T cells through PD-L1/ PD-1 axis. Inhibiting ALK by sensitive TKIs could reduce PD-L1 expression and reverse the suppression of T cells. IFNγ, an indicator of T cell function was increased in the co-culture medium of EML4-ALK positive NSCLC cells and T cells after sensitive ALK-TKI treatment. Our study suggested that upregulation of PD-L1 by EML4-ALK fusion protein mediates the immune escape in EML4-ALK positive NSCLC. EML4-ALK fusion protein induced PD-L1 upregulation represents an innate immune resistance mechanism in EML4-ALK positive NSCLC.

Recent studies found that PD-L1 expression was associated with the response to anti-PD1/PD-L1 treatment in NSCLC. We further analyzed the tumor inhibition by ALK-TKIs, PD-1 blocking or both. As expected, TAE684 had strong tumor inhibition in H3122 cells. LDK378 but not crizotinib could significantly inhibit the viability of DFCI076 cells. Interestingly, the viability of both crizotinib sensitive and resistant cells was inhibited by the blockade of PD-1. This implies that anti-PD-1 or anti-PD-L1 therapy maybe a promising optional treatment of EML4-ALK positive NSCLC. EML4-ALK fusion protein induced PD-L1 upregulation represents an innate immune resistance mechanism in EML4-ALK positive NSCLC.

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Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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