Expression Analysis and Significance of PD-1, LAG-3, and TIM-3 in Human Non–Small Cell Lung Cancer Using Spatially Resolved and Multiparametric Single-Cell Analysis

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Abstract

**Purpose:** To determine the tumor tissue/cell distribution, functional associations, and clinical significance of PD-1, LAG-3, and TIM-3 protein expression in human non–small cell lung cancer (NSCLC).

**Experimental Design:** Using multiplexed quantitative immunofluorescence, we performed localized measurements of CD3, PD-1, LAG-3, and TIM-3 protein in >800 clinically annotated NSCLCs from three independent cohorts represented in tissue microarrays. Associations between the marker’s expression and major genomic alterations were studied in The Cancer Genome Atlas NSCLC dataset. Using mass cytometry (CyTOF) analysis of leukocytes collected from 20 resected NSCLCs, we determined the levels, coexpression, and functional profile of PD-1, LAG-3, and TIM-3 expressing immune cells. Finally, we measured the markers in baseline samples from 90 patients with advanced NSCLC treated with PD-1 axis blockers and known response to treatment.

**Results:** PD-1, LAG-3, and TIM-3 were detected in tumor-infiltrating lymphocytes (TIL) from 55%, 41.5%, and 25.3% of NSCLC cases, respectively. These markers showed a prominent association with each other and limited association with major clinicopathologic variables and survival in patients not receiving immunotherapy. Expression of the markers was lower in EGFR-mutated adenocarcinomas and displayed limited association with tumor mutational burden. In single-cell CyTOF analysis, PD-1 and LAG-3 were predominantly localized on T-cell subsets/NKT cells, whereas TIM-3 expression was higher in NK cells and macrophages. Coexpression of PD-1, LAG-3, and TIM-3 was associated with prominent T-cell activation (CD69/CD137), effector function (Granzyme-B), and proliferation (Ki-67), but also with elevated levels of proapoptotic markers (FAS/BIM). LAG-3 and TIM-3 were present in TIL subsets lacking PD-1 expression and showed a distinct functional profile. In baseline samples from 90 patients with advanced NSCLC treated with PD-1 axis blockers, elevated LAG-3 was significantly associated with shorter progression-free survival.

**Conclusions:** PD-1, LAG-3, and TIM-3 have distinct tissue/cell distribution, functional implications, and genomic correlates in human NSCLC. Expression of these immune inhibitory receptors in TILs is associated with prominent activation, but also with a proapoptotic T-cell phenotype. Elevated LAG-3 expression is associated with insensitivity to PD-1 axis blockade, suggesting independence of these immune evasion pathways.
Introduction

Immunostimulatory therapies blocking the PD-1 axis pathway have become major antitumor treatment options in diverse malignancies including non–small cell lung cancer (NSCLC; refs. 1–5). To date, single-agent treatment using mAbs targeting PD-1 receptor or its ligand PD-L1 induce lasting clinical responses in approximately 18% of patients with advanced NSCLC. However, primary resistance occurs in the majority of patients and acquired adaptation of tumors to immune pressure in patients initially responding to therapy has also become a clinical challenge (6–9). Therefore, identification of biomarkers for patient selection and characterization of additional nonredundant actionable immunostimulatory targets are needed.

Various immune and tumor genomic metrics are associated with sensitivity to PD-1 axis blockers including tumor PD-L1 expression, measurement of tumor-infiltrating lymphocytes (TIL) or inflammation-related mRNA expression profiles, tumor mutational burden, and microsatellite instability (3, 4, 10–13). To date, however, only detection of PD-L1 protein using IHC and mismatch repair deficiency are approved by the FDA as companion biomarkers for PD-1–blocking antibodies. Additional immune coinhibitory receptors beyond PD-1, such as LAG-3 and TIM-3, are induced after T-cell receptor (TCR) stimulation and mediate T-cell suppression/dysfunction (14–17). The potential role of these receptors in mediating tumor immune evasion in human malignancies and their interaction/independence from PD-1 pathway remain poorly understood.

Lymphocyte-activation Gene-3 (LAG-3 or CD223) is a 498 amino acid type I transmembrane protein with high structural homology with CD4 protein and capacity to bind MHC class II molecules (18, 19). Alternative ligands have been proposed to explain some of the suppressive effects of LAG-3 in CD8+ cytotoxic cells in the absence of MHC class II, such as Galectin-3, LSECtin, and α-synuclein fibers (20–22). Recent work from our group identified FGL-1 as a novel high-affinity and cell-free ligand for LAG-3 involved in tumor immune evasion (23). Despite being studied in diverse anticancer clinical trials (19), the tissue distribution and functional role of LAG-3 in human malignancies has not been clearly defined.

T-cell immunoglobulin and mucin domain-3 (TIM-3) is a 281 amino acid long type 1 transmembrane protein containing a variable N-terminal Ig domain and a mucin stalk domain (24). An immunosuppressive effect of TIM-3 signaling on T cells was reported to be related with the binding of Galectin-9 and CEA-CAM1 (25, 26). Additional candidate ligands have also been shown to be able to modulate TIM-3 functions including phosphatidyl serine (PtdSer) and high-mobility group protein B1 (HMGB1; ref. 14). Clinical studies assessing the safety and antitumor effect of TIM-3 blockers alone or in combination with other therapies are currently ongoing: NCT02817633, NCT03099109, NCT03066648. The expression, tissue distribution, and association of TIM-3 with other immune-inhibitory receptors in human lung cancer are not well understood.

Here, we used multiplexed tissue imaging of large tumor collections and single-cell phenotypic analysis of primary cancers to evaluate the distribution and significance of PD-1, LAG-3, and TIM-3 expression in NSCLC. Our results reveal complex functional associations and support independent functions of these receptors to suppress T-cell function.

Materials and Methods
Patient, cohorts, and tissue microarrays

Formalin-fixed, paraffin-embedded (FFPE) samples from previously reported retrospective collections of NSCLC not treated with immune checkpoint blockers and represented in tissue microarrays (TMA) were analyzed (27, 28). The first collection includes samples from 426 patients with NSCLC seen at Yale Pathology between 1988 and 2012 (cohort #1). The second cohort includes samples from 304 patients with NSCLC collected at Sotiria General Hospital and Patras University General Hospital (Greece) between 1991 and 2001 (cohort #2). All cases in the cohorts were reviewed by a pathologist using hematoxylin and eosin–stained preparations and tumor histology variant was confirmed by morphology analysis. Tumor cores for TMA construction were obtained from case areas selected by a pathologist to represent the disease. Tumor core selection was not based on specific tumor segments or location. Clinicopathologic information from patients in both cohorts was collected from clinical records and pathology reports. Analysis of mRNA expression and nonsynonymous mutations was performed using the lung cancer dataset from TCGA (cohort #3, n = 370). Another TMA-based cohort from Yale (cohort #4), including retrospective samples from 108 lung adenocarcinomas clinically tested for EGFR and KRAS mutations was also studied. Fresh immune cell/leukocyte extracts from 20 primary resected NSCLCs were also included for mass cytometry analysis (cohort #5). To assess the value of the markers in patients treated with PD-1 axis blockers, we analyzed a combined retrospective cohort from Yale, Cleveland Clinic, and Navarra University including 90 baseline/pretreatment cases treated with PD-1–blocking antibodies (nivolumab or pembrolizumab) or a PD-L1 blocking Ab (atezolizumab; cohort #6). A summary description of all 6 cohorts/datasets included in the study is provided in Supplementary Table S1. All the studies were conducted in accordance with recognized ethical guidelines (e.g., Declaration of Helsinki, CIOMS, Belmont Report, U.S. Common Rule) and tissue and clinical information were used after approval of the Yale Human Investigation Committee protocols 9505008219, 1412015109, 1608018220, and 1603017333 or local institutional protocols, which approved the patient consent forms or waiver of consent.
Multiplexed quantitative immunofluorescence

A 5-color quantitative immunofluorescence (QIF) protocol for FFPE tissue specimens was developed for simultaneous detection of DAPI, CD3, PD-1, LAG-3, and TIM-3 using isotype-specific antibodies and different fluorescence conjugates as described previously by our group (28). To reliably measure the markers, we first validated individual assays using control preparations from cell line transfectants and human tissues. As shown in Supplementary Fig. S1, PD-1, LAG-3, and TIM-3 were detected exclusively in FFPE cell preparations from HEK293 cells transfected with each respective target, but not in parental cells lacking endogenous expression. The markers were then integrated into a multiplexed panel together with 4',6-diamidino-2-phenylindole (DAPI) to highlight every cell in the sample and CD3 to map T cells (Fig. 1). For the multiplexed staining, sections were deparaffinized and subjected to antigen retrieval using EDTA buffer (Sigma-Aldrich) pH 8.0 and boiled for 20 minutes at 97°C in a pressure-boiling container (PT module; Lab Vision). Slides were then incubated with dual endogenous peroxidase block (#S2003; Dako) for 10 minutes at room temperature and subsequently with a blocking solution containing 0.3% BSA in 0.05% Tween solution for 30 minutes. Primary antibodies included CD3 (rabbit polyclonal; Dako), PD-1 (clone EH33), LAG-3 (clone 17B4), and TIM-3 (clone D5D5R). Secondary antibodies and fluorescent reagents used were anti-rabbit Envision (K4003; Dako) with fluorescein-tyramide (PerkinElmer), antimouse IgG2a antibody (Abcam) with Cy3 plus (PerkinElmer), goat anti-rabbit (Abcam) with biotinylated tyramide/Streptavidine–Alexa Fluor 750 conjugate (PerkinElmer), and anti-mouse Envision (K40001) with Cy5-tyramide (PerkinElmer). Residual horseradish peroxidase activity between incubations with secondary antibodies was eliminated by exposing the slides twice for 10 minutes to a solution containing benzoic hydradize (0.136 mg) and hydrogen peroxide (50 μL). To determine the reproducibility of the QIF assay, we measured serial sections from an index TMA containing positive and negative controls (YTM3A45S) at different time points. The linear regression coefficients of scores obtained between independent runs were high (R > 0.9, P < 0.001; Supplementary Fig. S2), supporting the consistency of the measurements.

Tissue fluorescence measurement and scoring

Quantitative measurement of the fluorescent signal was performed using the AQUA method that enables objective and sensitive measurement of targets within user-defined tissue compartments (28). Briefly, the QIF score of each target in CD3+ T-cell compartment from the whole TMA spot was calculated by dividing the target pixel intensities by the area of CD3 positivity. Scores were normalized to the exposure time and bit depth at which the images were captured, allowing scores collected at different exposure times to be comparable. Markers were also measured in the total tissue compartment by collecting the signal score in the area defined by DAPI staining (e.g., all cells in the sample). For graphical representation of the retrospective TMA collections, the scores of LAG-3 and TIM-3 were mean-normalized relative to PD-1 scores to display them in a comparable scale.

Cell preparation and cytometry by time-of-flight analysis

As described previously (29), primary resected NSCLC tissues were finely minced and mechanically dissociated with the GentleMACS Dissociator (Miltenyi Biotec) in the presence of RPMI1640 with 0.5% BSA and 5 mmol/L EDTA. The resulting cell suspension was filtered using a 70-μm cell strainer (BD Falcon). Cells were centrifuged at 600 × g for 7 minutes at 4°C and resuspended in PBS with 0.5% BSA and 0.02% NaN3. A total of 2 × 106 cells from each tumor were incubated with antibodies against CD16/32 at 50 μg/mL in a total volume of 50 μL for 10 minutes at room temperature to block Fc receptors. Surface marker metal-conjugated antibodies’ cocktail were then added, yielding 100-μL final reaction volume and stained for 30 minutes at 4°C. After staining, cells were washed two times with PBS with 0.5% BSA and 0.02% NaN3. Then, cells were resuspended with RPMI1640 and 10 μmol/L cisplatin (Fluidigm) in a total volume of 400 μL for 60 seconds before quenching 1:1 with pure FBS to determine viability. Cells were centrifuged at 600 × g for 7 minutes at 4°C and washed once with PBS with 0.5% BSA and 0.02% NaN3. Cells were then fixed using fixation/permeabilization buffer (Tubiscience) for 30 minutes at 4°C. After two washes with permeabilization buffer (Tubiscience), cells were incubated with intracellular metal-conjugated antibodies cocktail in 100 μL for 30 minutes at 4°C. A summary of the antibodies/clones used in the mass cytometry analyses is presented in the Supplementary Table S2. Antibodies were either purchased preconjugated from Fluidigm or purchased purified and conjugated in-house using mass cytometry antibody conjugation kits according to the manufacturer’s instructions. Cells were washed twice in PBS with 0.5% BSA and 0.02% NaN3, and then stained with 1 mL of 14,000 191/193r DNA Intercalator (Fluidigm) diluted in PBS with 1.6% FFA overnight. Cells were then washed once with PBS with 0.5% BSA and 0.02% NaN3 and then two times with double-deionized ddH2O. All mass cytometry files were normalized together using the mass cytometry data normalization algorithm (30). For analysis, FCS files were manually pre gated on Ir193 DNA+CD45+ events, excluding cisplatin+ dead cells, doublets, and DNA-negative debris by Cytobank. The gated CD45+ population was then clustered on the basis of all labeled phenotypic markers using spanning-tree progression analysis of density-normalized events (SPADE; ref. 31). Putative cell populations were visualized by SPADE trees and manually annotated on the basis of the expression of key markers as shown in Supplementary Fig. S3.

Cell culture and transfections

For assay validation experiments, HEK293 parental cells were transiently transfected with 1 μg of full-length cDNA coding each target using Lipofectamine 3000 (Thermo Fisher Scientific) for 24 hours. Cells were used fresh for protein extraction and immunoblotting; or fixed in 10% neutral buffered formalin for 8 to 12 hours and embedded in paraffin for quantification by QIF. Cell lines used in this study were purchased from the ATCC and authentication was performed every 3 to 6 months using the GenePrint 10 System in the Yale University DNA Analysis Facility.

TCGA data analysis for mRNA expression and genomic alterations

We analyzed the NSCLC samples from The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/). Briefly, we downloaded the RNA-sequencing (RNA-seq) and DNA whole-exome sequencing data from 370 NSCLC cases including 250 adenocarcinomas and 120 squamous cell carcinomas. Using data
Figure 1. Distribution and frequency of T-cell PD-1, TIM-3, and LAG-3 expression in NSCLC. A, Representative fluorescence microphotographs showing the simultaneous detection of PD-1 (red), LAG-3 (white), TIM-3 (green), and CD3 (yellow) positive cells in NSCLC. B, Level of the markers measured using QIF in 2 retrospective NSCLC cohorts—cohort #1 (n=426) (A) and #2 (n=304) (B). The markers were measured in CD3+ T cells and showed a continuous distribution and strong association with CD3 (insets). The frequency of expression of each marker is indicated with white-colored text within the charts. R, Spearman correlation coefficient.
processed through the cbioPortal interface (http://www.cbioportal.org/). DNA segments and RNA transcripts were aligned and DNA variant calling was performed using default TCGA pipelines. We conducted single scatterplot analysis between the mRNA scores of PDCD1 (PD-1), LAG-3, and HAVCR2 (TIM-3) genes. The total number of nonsynonymous mutations detected in the whole-exome sequencing data relative to germline DNA was used as the tumor mutational burden.

**Statistical analysis**

QIF signals between compartments were analyzed using linear regression, correlation functions, and expressed as regression/correlation coefficients. For experiments including numerous fields of view (FOV) per case slide, we analyzed the top 10% marker scores in each preparation. Patient characteristics were compared using the Student t test for continuous variables and χ² test for categorical variables. Survival functions were compared using Kaplan–Meier estimates and statistical significance was determined using the log-rank test. Correlation studies were performed calculating linear regression coefficients and/or Spearman rho-rank functions. Associations between the markers and statistical significance were determined using JMP Pro. v11 and GraphPad Prism v7.0a software.

**Results**

**Tumor tissue distribution of PD-1, LAG-3, and TIM-3 in human NSCLCs**

We standardized a QIF panel for simultaneous measurement of the markers DAPI, CD3, PD-1, LAG-3, and TIM-3 and studied 730 retrospectively collected NSCLC samples from two independent populations represented in TMA format [cohort #1 from Yale (N = 426) and cohort #2 from Greece (N = 304)]. All the markers showed a predominant membranous staining pattern and were detected in CD3⁺ TILs (Fig. 1A). However, PD-1 and LAG-3 were predominantly localized in CD3⁺ T cells while TIM-3 was recognized frequently in CD3⁻ populations. Using the visual detection threshold, we detected T-cell PD-1, LAG-3, and TIM-3 expression in 65%, 33%, and 24% of NSCLCs in the first cohort; and in 45%, 49%, and 26% of cases in the second collection (Fig. 1B and C). In both cohorts, the levels of T-cell PD-1, LAG-3, and TIM-3 protein were significantly correlated with each other (Spearman R =
The lowest correlation coefficients were between PD-1 and TIM-3. Comparable results were obtained when measuring the expression in the total tissue compartment (e.g., outside T cells) and between the levels of PD-1 (PDCD1), LAG-3, and TIM-3 (HAVCR2) mRNA transcripts in 406 cases from the NSCLC datasets of TCGA (cohort #3, Fig. 2C).

**Target expression in individual immune cell populations and functional impact**

To determine the expression of the targets and functional associations in specific immune cell subpopulations, we used mass cytometry by time-of-flight (CyTOF) to analyze freshly isolated leukocytes obtained from a collection of 20 primary...
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Figure 4.
Association of LAG-3, PD-1, TIM-3, and CD3 with major driver mutations and tumor mutational burden in NSCLC. A, Levels of PD-1, LAG-3, and TIM-3 in lung adenocarcinoma cohort #3 comprising cases wild type for EGFR and KRAS driver mutations and with oncogenic KRAS or EGFR variants. B, Association between the marker mRNA levels (FPKMs) and tumor mutational burden (e.g., number of nonsynonymous mutations) in the TCGA NSCLC cohort (cohort 3; N = 406). *P < 0.01; **P < 0.001; ***P < 0.0001; *R Spearman correlation coefficient.

resected NSCLCs (cohort #4). A panel of 35 phenotypical and functional markers was costained (Supplementary Table S2) and SPADE was used to identify distinct immune cell populations (Supplementary Fig. S3). PD-1 was predominantly expressed on cytotoxic CD8\(^+\) T cells, CD4\(^+\)/CD25\(^-\)/Foxp3\(^-\) helper cells, regulatory CD4\(^+\)/CD25\(^+\)/Foxp3\(^+\) T cells (Tregs), and CD3\(^+\)/CD56\(^+\) NK cells (Fig. 3A, left). Virtually, no PD-1 signal was detected in B lymphocytes, NK cells, and myeloid cell subsets. LAG-3 expression was seen in all studied T-cell groups with the highest levels in CD8\(^+\) T cells. LAG-3 was also present on NK cells, NKT cells, and granulocytes, but it was low or absent in antigen-presenting cells (APC) and B lymphocytes (Fig. 3A, middle). TIM-3 was broadly expressed in adaptive and innate immune cells, with detectable levels in all T-lymphocyte subsets, NK cells, NKT cells, and dendritic cells. Notably, the highest TIM-3 levels were seen in macrophages/NK/NKT cells, whereas low/absent expression was found in granulocytes (Fig. 3A, right).

Overall, simultaneous coexpression of PD-1, LAG-3, and TIM-3 was seen in 5.4% of CD3\(^+\) TILs. PD-1 and LAG-3 were coexpressed in 15.8% of cells, PD-1 and TIM-3 in 21% of cells, and LAG-3 and TIM-3 in 10.5% of TILs (Fig. 3B, green chart area). The proportion of TILs lacking all 3 markers was 29.4%. Notably, 8.6% of LAG-3\(^+\) and 16.8% of TIM-3\(^+\) TILs showed absence of PD-1 expression; and 15.8% showed LAG-3 positivity in the absence of TIM-3 (Fig. 3B, red chart area).

To evaluate the impact of the markers, we analyzed the expression of functional indicators in individual T-cell populations. Coexpression of PD-1, LAG-3, and TIM-3 was prominently associated with high levels of markers of T-cell activation (CD69, 4-1BB), cytotoxic/effectector function [Granzyme-B (GZB)] and proliferation (Ki-67), but also with higher levels of the apoptotic signal receptors (FAS or CD95) and proapoptotic proteins (BIM; Fig. 3C and D). TILs with high LAG-3, but low PD-1 and TIM-3 expression (e.g., PD-1\(^-\)LAG-3\(^+\)TIM-3\(^-\)) showed higher cytotoxic potential (GZB, CD69, and CD137) than T cells with elevated TIM-3 alone (e.g., PD-1\(^-\)LAG-3\(^-\)TIM-3\(^+\)) or those expressing only PD-1 (e.g., PD-1\(^+\)LAG-3\(^-\)TIM-3\(^-\); Supplementary Fig. S4A and S4B). Notably, coexpression of two or more of the immune-inhibitory receptors was associated with higher levels of all functional markers (Supplementary Fig. S4C).

Clinical significance and molecular associations of PD-1, LAG-3, and TIM-3 expression in NSCLC
To explore the clinical role of the markers, we studied their association with major clinicopathologic variables and survival in the retrospective cohorts #1 and #2. There was a significant association between the levels of the markers and TIL abundance; and all the markers showed a positive association with each other (Supplementary Tables S3 and S4). There were no consistent associations between expression of PD-1, LAG-3, or TIM-3 and major clinicopathologic features including age, gender, smoking status, clinical stage, and tumor histology variant.

As shown in Fig. 4A, the level of PD-1 and TIM-3 were significantly lower in tumor harboring KRAS mutations than in cases with wild-type EGFR and KRAS. EGFR-mutated lung adenocarcinomas showed significantly lower TIM-3 than tumors lacking mutations in both oncogenes. In the TCGA lung cancer datasets, there was a positive correlation between tumor mutational burden and LAG-3, but this association was not evident for the other markers (Fig. 4B).

To explore the survival effect of dominant expression of each marker, we assessed the 5-year overall survival in cases with scores...
above or below the top 15th percentile of the cohort. As shown in Fig. 5A–F, prominent T-cell PD-1, LAG-3, and TIM-3 were significantly associated with longer 5-year overall survival in the first cohort, but this was not evident in the second population. Similar associations with survival were seen when stratifying the markers by quartiles (Supplementary Fig. S5).

**PD-1, LAG-3, TIM-3, and sensitivity/resistance to PD-1 axis blockers in NSCLC**

We then studied the association between the baseline level of the markers and survival after treatment with PD-1 axis blockers in 90 patients. Cases with prominent T-cell LAG-3 expression (top 15th percentile of the cohort) showed a significantly shorter progression-free survival (log-rank \( P = 0.03 \); Fig. 6A–C). In contrast, elevated levels of T-cell PD-1 or TIM-3 were not significantly associated with survival in the cohort. Notably, cases with high LAG-3 and low tumor PD-L1 expression (<50% tumor proportion score [TPS]) showed a markedly lower progression-free survival than cases with low LAG-3 and high (≥50% TPS) tumor PD-L1 expression (Fig. 6D).

**Discussion**

Using QIF and mass cytometry, we determined the expression, functional associations, and clinical significance of PD-1, LAG-3, and TIM-3 in human NSCLC. Specifically, we found that all three receptors display variable expression in NSCLC, distinct immune cell distribution, and association with T-cell activation and proapoptotic markers. Notably, TILs with elevated LAG-3 showed the most prominent activated and proapoptotic phenotype and elevated T-cell LAG-3 (but not PD-1 or TIM-3) was significantly associated with shorter survival after PD-1 axis blockade. Taken together, our findings support a distinct and independent role of these immune-inhibitory receptors in lung cancer and show that tumors with dominant LAG-3 expression are less sensitive to PD-1 axis blockers.

Detectable expression of PD-1, LAG-3, and TIM-3 was seen in 55%, 41.5%, and 25.3% of NSCLCs, respectively; and was associated with T-cell–inflamed tumors, but not consistently associated with other clinicopathologic variables. A recent study detecting PD-1 and LAG-3 using single-marker IHC in 139 surgically resected lung carcinomas reported PD-1 expression in 43.4% and LAG-3 in 26.9% of cases (32). Here, LAG-3 detection was associated with higher PD-1, nonsquamous tumor histology, and worse survival. Although our study showed no consistent association between elevated LAG-3 and specific tumor histology or with survival in cases not receiving immunotherapy, we found similar frequencies and patterns of tumor LAG-3 and PD-1 protein expression. The primary source explaining the differences between this study and our findings is uncertain, and diverse methodologic considerations may account for this, including the cases analyzed, modality of marker testing, cut-points used for stratification, and assay-specific variables. In our study, we evaluated four different LAG-3 antibodies using control FFPE samples including clones 17B4, D2G40, EPR4392, and 11E3; and selected clone 17B4 for its high specificity and broader dynamic range.
Figure 6. Association of PD-1, LAG-3, and TIM-3 expression with survival in patients with NSCLC treated with PD-1 axis blockers. A–C, Charts showing the Kaplan–Meier survival estimates of patients treated with PD-1 axis blockers (cohort #6, n = 90). The scores of T-cell PD-1 (A), LAG-3 (B), and TIM-3 (C) were measured using multiplex QIF in pretreatment samples and stratified using top 15th percentile as cut-point. The differences between groups were compared using the log-rank test. D, Kaplan–Meier survival estimates of patients treated with PD-1 axis blockers stratified by LAG-3 expression (high, above top 15th; low, below or equal to top 15th) and PD-L1 expression (high, above 50% tumor proportion score; low, below or equal to 50% tumor proportion score).

The single-cell studies revealed distinct expression and functional impact of PD-1, LAG-3, and TIM-3 in immune cell subpopulations. Although PD-1 and LAG-3 were predominantly localized in NKT and CD8⁺ T cells, TIM-3 was commonly seen in macrophages and NK cells. The elevated expression of PD-1 and LAG-3 in cytotoxic T CD8⁺ and NKT cells is consistent with their expected immune regulatory function in effector cells. Notably, both targets were also highly expressed in Tregs, suggesting that PD-1 and LAG-3 pathways can have additional and cell-specific suppressive functions constituting complex internal systems mediating immune tolerance. Prominent expression of TIM-3 in innate immune cells such as monocytes, dendritic cells, and NK cells has been previously reported in nontumor tissues such as peripheral blood (33–35), but this is the first time to our knowledge it is reported in lung cancer.

KRAS and EGFR are the most commonly mutated driver oncogenes in lung adenocarcinoma. EGFR-mutant tumors display commonly lower TILs, PD-L1 expression, and tumor mutational burden than EGFR-wild-type tumors (36–38). Consistent with this, patients with EGFR-mutated carcinomas derive less clinical benefit from PD-1 axis blockade (39, 40). In our study, we found lower levels of PD-1, LAG-3, and TIM-3 in KRAS- and EGFR-mutant tumors than in cases lacking mutations in both genes. This supports lower overall immune activation and regulation mediated by PD-1, LAG-3, and TIM-3 in these malignancies and suggests limited therapeutic potential of targeting these receptors. However, a fraction of NSCLCs with KRAS driver mutations may also harbor other genomic variants which could alter the tumor-immune microenvironment (41).

Despite being traditionally considered as exhaustion T-cell markers (42, 43), PD-1, LAG-3, and TIM-3 are expressed preferentially in activated TILs. This is consistent with a model where coinhibitory receptors are upregulated upon T-cell stimulation in order to limit exaggerated responses and potential tissue damage. In this regard, it has been reported that the expression of these three markers is associated and could be used to identify antigen-experienced T cells in patients with cancer (44). However, previous studies have also shown that prominent T-cell activation is associated with a dysfunctional phenotype characterized by engagement of apoptotic programs (45). Consistent with this notion, expression of PD-1, LAG-3, and TIM-3 was associated with elevated levels of key proapoptotic targets in lung carcinomas. Additional studies are ongoing to refine the phenotype of cells expressing each immune-inhibitory receptor combination and determine how to use this information therapeutically. Studies simultaneously measuring key ligand(s) for PD-1, LAG-3, and TIM-3 are also warranted because functional consequences probably require coexpression of the ligands and receptors in close proximity within the tumor microenvironment.

An intriguing finding of our study is the negative association between LAG-3 overexpression and survival benefit in patients with NSCLC treated with PD-1 axis blockers. These suggest that tumors in which immune evasion is mediated predominantly by LAG-3 are less sensitive to PD-1 axis blockade and opens the possibility of eventually using LAG-3 for selection of patients for immunotherapy. Early results from LAG-3 inhibitors in the CA224-020 clinical trial (NCT01968109) show promising results in patients with advanced melanoma with resistance to PD-1 blockers. Here, LAG-3 positivity by IHC in pretreatment tumor samples is associated with higher response rate supporting a predictive role of LAG-3 expression. In recent work, we also found LAG-3 upregulation by protein and mRNA measurements in TILs from 5 of 8 patients with NSCLC with acquired resistance to immune checkpoint blockers, suggesting a possible role of LAG-3 in this setting (38).
Our study has limitations. The evaluation of three retrospective cohorts (cohorts #1, #2, and #4) was performed using TMAs that analyze relatively small sample fragments and may over- or underestimate the marker measurements. Although not the standard method to measure proteins in tumor tissues clinically, diverse reports from our group and others using TMAs have shown consistent results and significant association with clinical, pathologic variables and outcome (46). In addition, (i) each case was represented two or three times in the TMA to account for possible marker variations across different tumor areas; (ii) the data obtained in the TMA cohorts were consistent with results in the TCGA collection (cohort #3) that was conducted using whole tissue sections and mRNA analysis; and (iii) the marker scores and distribution obtained using TMAs were similar to those in whole tissue sections used for the cohort #6 analysis. Finally, the collection of patients treated with PD-1 axis blockers (cohort #6) included cases treated with different PD-1 axis blockers and samples collected at different time points before treatment initiation and with multiple previous lines of treatment. These factors are common limitations of retrospective cohort studies and their possible impact in the results are uncertain.

Overall, we have characterized the expression and significance of PD-1, LAG-3, and TIM-3 in a sizable number of NSCLC cases from six independent tumor collections. Our results demonstrate dissimilar and nonredundant expression of these targets in TILs from primary lung tumors and provide valuable insights about T-cell activation and dysfunction in this setting.

Disclosure of Potential Conflicts of Interest
B.S. Henick holds ownership interest (including patents) in AbBVe and is a consultant/advisory board member for Boehringer Ingelheim. V. Velchetei is a consultant/advisory board member for Bristol-Myers Squibb, Genentech, AstraZeneca, Nektar Therapeutics, Reddy Labs, Celgene, Merck, and Foundation Medicine. M.D. Hellmann reports receiving other commercial research support from Bristol-Myers Squibb, holds ownership interest (including patents) in Shattuck Labs; is listed as an inventor on a patent that has been filed by MSK related to the use of tumor mutation burden to predict response to immunotherapy, which has received licensing fees from PGDx; is a consultant/advisory board member for Merck, AstraZeneca, Bristol-Myers Squibb, Genentech/Roche, Janssen, Nektar, Syndax, Mirati, and Shattuck Labs; and reports receiving other remuneration from Bristol-Myers Squibb and AstraZeneca. J.F. Gainor reports receiving other commercial research support from Novartis, Genentech/Roche, and Ariad/Takeda; and is a consultant/advisory board member for BMS-936558, Genentech/Roche, OncoImmune, Loxo, Pfizer, Takeda, Agen, Agios, Regeneron, Jounce, and Onconova. K. Politi reports receiving commercial research grants from AstraZeneca, Roche, Symphogen, and Kolltan; holds ownership interest (including patents) in MolecularMD/MSDKCC; and is a consultant/advisory board member for AstraZeneca, Tocagen, Maverick Therapeutics, Dynamo Therapeutics, and NCCN. S. Gettinger is a consultant/advisory board member for Bristol-Myers Squibb and Nektar. D.L. Rimm reports receiving commercial research grants from AstraZeneca, Cepheid, NextCure, Lilly, Uliveir, Perkin Elmer, Nanosting, and Navigate/Novartis, and is a consultant/advisory board member for AstraZeneca, Amgen, Daiichi Sankyo, GSK, Bristol-Myers Squibb, Konica Minolta, Biocept, Cell Signaling Technology, Nanosting, Cepheid, and Ventana. R.S. Herbst is an employee of Junshi Bioscience; reports receiving commercial research grants from NextCure and Tayu Biotech; holds ownership interest (including patents) in NextCure; and is a consultant/advisory board member for Boston Children’s Hospital and Vcanbio. K.A. Schalper reports receiving commercial research grants from NextCure, AstraZeneca, Pierre Fabre, and AbBVe.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.F. Sanmamed, T. Badri, M. Toki, J.L. Perez-Gracia, V. Velchetei, M.D. Hellmann, J.F. Gainor, K. Syrigos, K. Politi, D.L. Rimm, R.S. Herbst
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.F. Sanmamed, B.S. Henick, T. Badri, N. Mari, M. Toki, K.A. Schalper
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Other (pathology analysis): L.D. Mejias

Acknowledgments
The authors thank Dr. Paula Kavathas (Yale University) for providing access to key resources and Lori Charette from Yale Pathology Tissue Services for excellent support in histology and TMA sample preparation. The authors acknowledge Lung Cancer Research Foundation (LCRF), Yale SPORE in Lung Cancer (P50CA196530), Department of Defense-Lung Cancer Research Program Cancer Development Award (W81XWH-16-1-0160), sponsored research grant by Tesaro Inc., Yale Cancer Center Support Grant (P30CA163593), a gift by the Granley Family Fund, and a Stand Up To Cancer – American Cancer Society Lung Cancer Dream Team Translational Research Grant (SU2C-AACR-DT17-15 and SU2C-AACR-DT22-17). Stand Up To Cancer is a program of the Entertainment Industry Foundation. Research grants are administered by the American Association for Cancer Research, the scientific partner of SU2C. M.F. Sanmamed was supported by a Miguel Servet contract from Instituto de Salud Carlos III, Fondo de Investigación Sanitaria (Spain).

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Received December 18, 2018; revised March 12, 2019; accepted April 29, 2019; published first May 3, 2019.


Clinical Cancer Research

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