

Tumor cell-expressed OX40L enhances the efficacy of PD-1 axis blockade in non-small cell lung cancer

Carmen Fernández-Luna,¹ Javier Baena,^{1,2} Antonio Calles,³ Mónica Antoñanzas-Basa,⁴ Yolanda Lage Alfranca,⁵ Xabier Mielgo,⁶ Maria Sereno,^{7,8} Raquel Cervera,⁹ Luis Cabezon-Gutierrez,^{10,11} Ana López,¹² Judit Rubio,¹³ Esther Conde,^{14,15,16,17} Fernando Lopez-Rios,^{14,15,16,17} Luis Alvarez-Vallina ,^{18,19,20} Karnele Valencia,^{17,21} Luis M Montuenga,^{17,21} Gorka Ruiz De Garibay,¹ Magdalena Molero,¹ Vera Adradas,¹ Daniel Curto-Aguilera,¹⁴ Melina Peressini,¹ Esther Carrera-Elvira ,¹ Daniel Ballesteros,¹ Adrián Portillo-Fernández,¹ Teresa Lopez-Cárdenas,¹ Cristina Serrano-Gómez,² Helena Bote,² Javier Torres- Jiménez,² Alberto Cruz-Bermúdez ,²² Mariano Provencio,²² Luis Paz-Ares,^{2,17} Estela Sánchez-Herrero,¹ Jon Zugazagoitia ,^{1,2,17}

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CF-L and JB contributed equally.

CF-L and JB are joint first authors.

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For numbered affiliations see end of article.

Correspondence to

Dr Jon Zugazagoitia; j.zugazagoitia.imas12@h12o.es

Dr Estela Sánchez-Herrero; esanchez.imas12@h12o.es

ABSTRACT

Background Immune checkpoint inhibitors have transformed non-small cell lung cancer (NSCLC) treatment. Understanding the tumor-immune interactions that dictate sensitivity to PD-1 blockade is essential for improving outcomes and optimizing immunotherapy personalization.

Methods We performed compartment-specific (tumor/immune) spatial proteomic profiling (GeoMx) on a discovery cohort of 104 patients with advanced, PD-L1-high (tumor proportion score ≥50%) NSCLC treated with first-line pembrolizumab. Identified markers were further assessed using orthogonal methods in two independent early-stage NSCLC cohorts treated with neoadjuvant/perioperative chemo-immunotherapy: (a) NADIM trial (n=36) with bulk RNA-seq and (b) NeoITX_H120 cohort (N=41) with quantitative immunohistochemistry. Potential baseline prognostic value without immunotherapy was evaluated in the TCGA dataset (N=940). Functional validation was assessed using in vitro and in vivo (nude and syngeneic) models.

Results We identified tumor-cell OX40L, but not immune-cell OX40L, as the single marker significantly associated with prolonged progression-free survival (PFS) and overall survival under single-agent pembrolizumab in patients with PD-L1-high tumors. Spatially resolved analysis linked tumor-intrinsic OX40L to an active immune microenvironment with increased antigen presentation. In both neoadjuvant/perioperative cohorts, high OX40L levels in residual tumors from patients with non-pathological complete response were associated with superior PFS. Conversely, no significant association with survival was observed in the immunotherapy-naïve TCGA cohort. Functional studies confirmed that OX40L-expressing NSCLC cells directly co-stimulate T cells in vitro and significantly enhance anti-PD-1 efficacy in vivo (p=0.0005), without affecting intrinsic tumor growth.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Immune checkpoint inhibitors have transformed the management of non-small cell lung cancer (NSCLC), yet robust biomarkers and mechanistic determinants of sensitivity to PD-1 axis blockade remain limited. OX40L is a member of the tumor necrosis factor ligand superfamily that provides a critical late-stage co-stimulatory signal to promote T-cell survival, expansion, and memory formation. While its expression is traditionally restricted to professional antigen-presenting cells, evidence of OX40L on lung cancer cells remains limited and functionally uncharacterized.

WHAT THIS STUDY ADDS

⇒ Using compartment-specific spatial proteomics, we demonstrate that OX40L is expressed intrinsically by tumor cells in a subset of NSCLC patients and serves as a predictor of superior clinical benefit from PD-1 blockade, particularly in the PD-L1 high (≥50%) population. Furthermore, we show that high OX40L expression in residual tumors after neoadjuvant therapy correlates with improved survival and does not exhibit a significant prognostic impact in the absence of PD-1 axis inhibition. Functional in vivo syngeneic models confirm that tumor-cell OX40L directly co-stimulates T cells and enhances the antitumor efficacy of PD-1 blockade without altering intrinsic tumor growth kinetics.

Conclusions OX40L expressed by tumor cells contributes to an enhanced efficacy of PD-1 blockade in patients with NSCLC. These findings may open new therapeutic avenues and potentially contribute to improving immunotherapy personalization in this disease.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study combines clinically relevant patient cohorts, advanced spatial profiling technologies, and functional mechanistic validation to identify tumor-cell OX40L as a biomarker that might contribute to refining patient stratification for immunotherapy, particularly in the advanced PD-L1-high population and in the perioperative residual disease setting. Furthermore, it might be informative for the development of novel therapeutic strategies, such as mRNA-based delivery, aimed at inducing local and “natural” co-stimulation to enhance the efficacy of PD-1 axis inhibition.

BACKGROUND

PD-1 axis blockade has transformed non-small cell lung cancer (NSCLC) outcomes. In advanced disease, immune checkpoint inhibition has led to substantial survival improvements, particularly among patients with high PD-L1-expressing tumors, in whom single-agent PD-1 axis inhibition is standard of care and confers a robust overall survival (OS) benefit compared with platinum-based chemotherapy.¹ More recently, these advances have translated into early-stage disease. In this setting, neoadjuvant chemo-immunotherapy has shown consistent improvement in pathological complete response (pCR) rates and progression-free survival (PFS), with long-term OS benefit reported in at least two randomized studies, including a pure neoadjuvant (CheckMate 816)² and a perioperative trial (KEYNOTE-671).³

Despite these advances, the mechanisms underlying sensitivity and resistance to PD-1 axis blockade remain mostly unknown. In the clinic, this translates into a lack of robust biomarkers for appropriate patient selection and treatment personalization. New biomarker discovery studies in academic settings have been mostly conducted in cohorts of patients unselected for PD-L1 expression who had been previously treated with chemotherapy. In such scenarios, the low overall response rates (10%–20%) and the confounding effects of prior cytotoxic therapy make it difficult to discern true signals of immunotherapy efficacy.⁴ The challenge of patient stratification has recently extended to the neoadjuvant setting. The landmark CheckMate 816 trial established the efficacy of neoadjuvant immunotherapy, raising a critical clinical question: which patients are truly cured by surgery after induction therapy and who requires additional treatment? In this context, the study of baseline and residual viable tumor in surgical specimens has emerged as a crucial window to understand resistance and to identify biomarkers that can predict long-term outcomes in patients who do not achieve a pCR.⁵

Beyond patient stratification, understanding the mechanisms that govern response and resistance to PD-1/PD-L1 blockade could also be informative for developing novel immunotherapy strategies. The limited success of multiple novel PD-1/PD-L1 combinations in the clinic likely reflects inappropriate patient selection and an

incomplete understanding of tumor-intrinsic immunobiology that determines sensitivity to immune checkpoint blockade. Deeper mechanistic insight into these tumor-derived signals may therefore enable more effective combinatorial strategies and enhance the clinical impact of PD-1-based therapies.

In this study, we used spatial proteomics as a discovery approach in NSCLC patients with PD-L1-high expression treated with single-agent PD-1 blockade, identifying OX40 ligand (OX40L, CD252, TNFSF4) expressed in the tumor compartment as a marker associated with durable clinical benefit. We subsequently extended these findings to the neoadjuvant/perioperative chemo-immunotherapy setting, showing that OX40L in residual tumors from patients with incomplete response to neoadjuvant therapy was associated with longer PFS. We observed no prognostic relevance of OX40L expression in the immunotherapy-untreated TCGA cohort. Finally, functional validation in immunocompetent syngeneic mouse models demonstrated that OX40L expression in cancer cells enhances anti-PD-1 therapy efficacy.

MATERIALS AND METHODS

Patient cohorts

The ITX_ILM cohort was used as the primary discovery set and included 104 patients with advanced NSCLC (stage III–IV) and PD-L1-high expression (tumor proportion score (TPS) $\geq 50\%$). Patients were recruited from 10 university hospitals in the Madrid Region between 2017 and 2022. To ensure clinical homogeneity, only individuals with Eastern Cooperative Oncology Group (ECOG) performance status 0–1 who received first-line pembrolizumab monotherapy were included. Baseline clinical-pathological characteristics of the ITX_ILM cohort are summarized in [table 1](#). Clinical follow-up was collected retrospectively, with median PFS and OS of 9.7 and 16.3 months, respectively (online supplemental figure 1A,B), consistent with real-world outcomes in high PD-L1 NSCLC treated with anti-PD-1 monotherapy.⁶

To further assess the role of identified biomarkers in a clinically relevant setting, we used two independent cohorts of stage II–IIIB NSCLC treated with neoadjuvant/perioperative chemo-immunotherapy: (a) the NADIM cohort, comprising 36 patients with available tumor RNA-seq data that were enrolled in the phase II, open-label, multicenter NADIM clinical trial (NCT03081689), that includes treatment-naïve patients with resectable stage IIIA NSCLC and an ECOG performance status of 0–1. Patients received three cycles of neoadjuvant paclitaxel and carboplatin plus nivolumab, followed by 1 year of adjuvant nivolumab monotherapy. The demographic and clinical profile of the cohort has been summarized in Provencio *et al* study⁷; and (b) the NeoITX_H12O, comprising 41 consecutive NSCLC patients treated with neoadjuvant or perioperative platinum-based chemotherapy plus PD-1 axis inhibition at Hospital Universitario 12 de Octubre (H12O). Eligible patients had resectable

Table 1 Baseline clinical and demographic characteristics of ITX_1LM cohort

Characteristic	N=104*
Age (years)	69 (44–91)
Age group	
<65 years	39 (38%)
≥65 years	65 (63%)
Sex	
Female	29 (28%)
Male	75 (72%)
Smoking history	
Active smoker	37 (36%)
Former smoker	64 (62%)
Never smoker	3 (3%)
ECOG performance status	
0	25 (24%)
1	79 (76%)
Histology	
Adenocarcinoma	67 (64%)
Squamous cell carcinoma	31 (30%)
Others	6 (6%)
Stage at diagnosis	
Stage III	8 (8%)
Stage IV	96 (92%)
PD-L1 expression (TPS)	
TPS 50%–89%	70 (67%)
TPS ≥90%	34 (33%)
Liver metastases	13 (13%)
CNS metastases	16 (15%)
dNLR ≥3	37 (36%)
LDH >ULN	40 (48%)
LIPI score	
Good (0)	26 (31%)
Intermediate (1)	45 (54%)
Poor (2)	13 (15%)
KRAS mutation status	
WT	80 (77%)
Mutated	9 (9%)
Unknown	15 (14%)

*Median (min–max); n (%).

CNS, central nervous system; dNLR, derived neutrophil-to-lymphocyte ratio; LDH, lactate dehydrogenase; LIPI, Lung Immune Prognostic Index; TPS, tumor proportion score; ULN, upper limit of normal; WT, wild-type.

stage II–IIIB disease or oligometastatic stage IV disease deemed suitable for surgery. The clinico-pathological characteristics of the cohort are summarized in online supplemental table 1. The included population is

representative of the routine clinical practice scenario for early-stage NSCLC, capturing the full spectrum of PD-L1 expression levels, including 18% of patients with negative PD-L1 expression (TPS <1%). The cohort has a median follow-up of 33.9 months and has not reached the median for PFS and OS (online supplemental figure 1C,D).

To assess prognostic relevance in the absence of immunotherapy, we used TCGA lung adenocarcinoma (LUAD, n=472) and lung squamous cell carcinoma (LUSC, n=468) cohorts, consisting of NSCLC stage I–III patients not exposed to immunotherapy, retrieved from the cBioPortal for Cancer Genomics (online supplemental table 2). Only tumor samples with available OX40L RNA-seq data and complete clinical annotation were included in the analyses.

Patient samples and analytical platforms

Tumor specimens were obtained as formalin-fixed paraffin-embedded (FFPE) tissue across the four independent datasets. In the ITX_1LM cohort, pre-treatment specimens from advanced NSCLC patients (N=104) were analyzed using the GeoMx Digital Spatial Profiler (DSP) platform (Bruker Spatial Biology). For the NADIM cohort (N=36), RNA was extracted from baseline (n=11) and surgically resected specimens (n=36) and profiled using the Ion AmpliSeq platform (Thermo Fisher Scientific).⁷⁸ In the NeoITX_H12O cohort (N=41), pre-treatment (Bx, n=26), and post-neoadjuvant surgical specimens (Qx, n=41) were assessed by conventional immunohistochemistry (IHC) and quantified using digital image analysis in QuPath software. Finally, the TCGA cohort (N=940) provided an immunotherapy-naïve reference dataset, using whole-transcriptome bulk RNA sequencing (HiSeq 2000, Illumina) from samples with available OX40L expression and complete clinical annotation.

GeoMx DSP analysis

Spatially resolved protein expression in FFPE tumor samples represented as whole tissue sections (ITX_1LM cohort) or under tissue microarray (TMA) format from mice tumors after anti-PD-1 experiment was assessed using the 36-plex human antibody panel (online supplemental table 3) or the 37-plex mouse antibody panel (online supplemental table 4) and morphology markers (PanCK-Alexa Fluor 532, CD45-Alexa Fluor 594) in the GeoMx DSP platform (Bruker Spatial Biology) according to manufacturer instructions (figure 1A). Regions of interest (ROIs) (max circular diameter 660 μm) from ITX_1LM samples were selected based on high-resolution digital images, with guidance from a pathologist, to include tumor regions that also included adjacent stroma or evidence of immune cell infiltration. ROIs were segmented into tumor (PanCK+) and immune (CD45+) compartments using the GeoMx DSP software pixel classifier algorithm. Oligonucleotide tags were released by UV photocleavage and quantified using the nCounter platform. Data were normalized to housekeeping targets or negative controls and averaged per patient to obtain

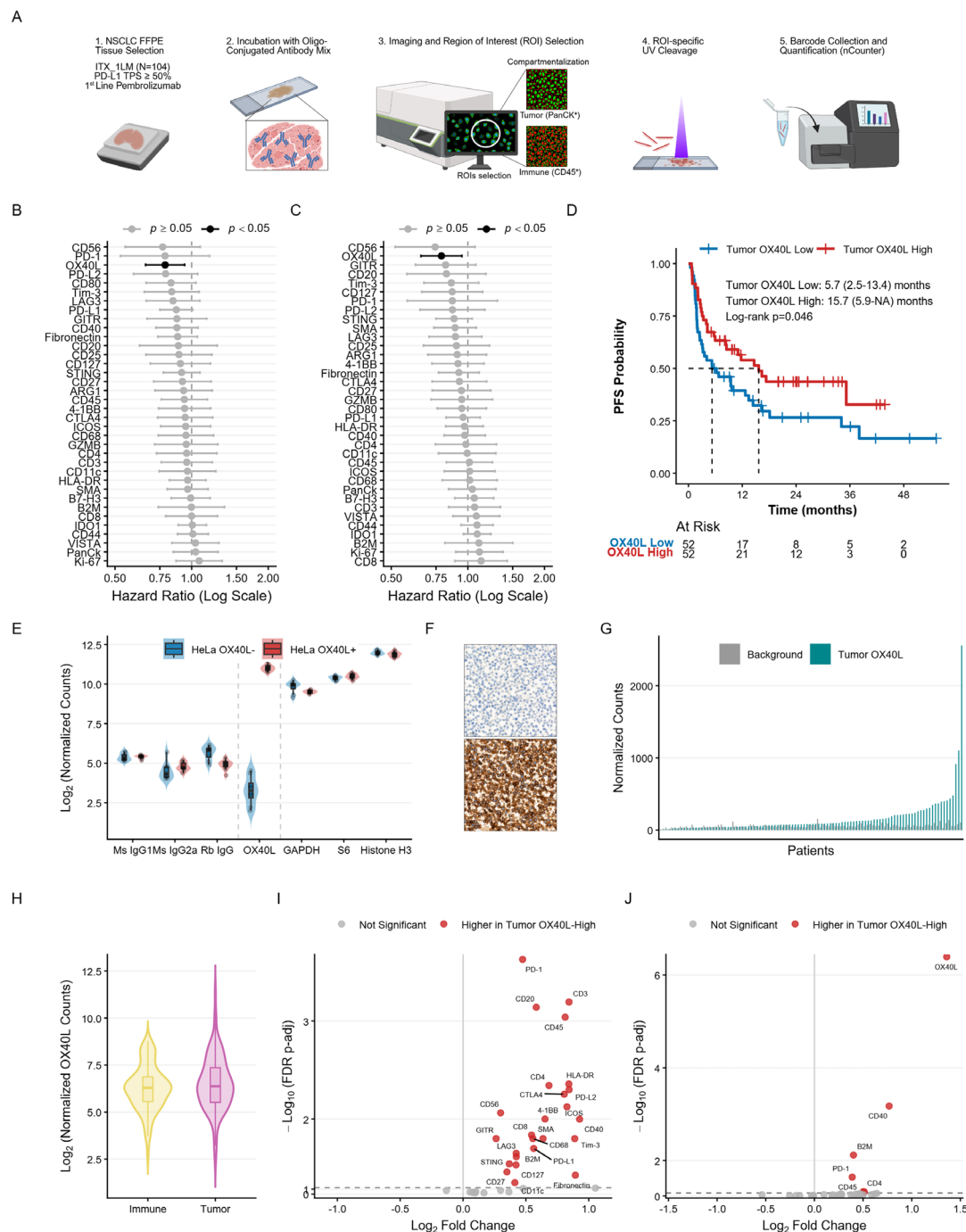


Figure 1 Spatial proteomic profiling of high PD-L1 NSCLC patients treated with first-line pembrolizumab. (A) Schematic representation of the GeoMx Digital Spatial Profiler (DSP) pipeline using PanCK and CD45 morphology markers for compartment-specific (tumor vs immune) quantification in FFPE NSCLC sections. (B) Univariate Cox analysis for progression-free survival (PFS) and (C) overall survival (OS) based on $\log_2$ -transformed protein expression in the tumor compartment (pan-cytokeratin (PanCK)⁺). Forest plots show HRs and 95% CIs. (D) Kaplan-Meier estimates of PFS stratified by median tumor-compartment OX40L expression; p value by log-rank test. (E) Validation of α -OX40L antibody (clone D6K7R) using engineered HeLa cells (red OX40L+ vs blue OX40L-). Plots show $\log_2$ -normalized DSP counts for OX40L, isotype negative controls (Ms IgG1, Ms IgG2a, Rb IgG), and housekeeping proteins (GAPDH, S6, histone H3). (F) Representative immunohistochemistry (IHC) images of H1581 cell pellets (OX40L+ vs ExNeg) stained with α -OX40L. Nuclei were stained with hematoxylin (blue). (G) Compartmental distribution and absolute expression levels of OX40L in the ITX_1LM cohort. (H) Density distribution of normalized OX40L counts in the tumor compartment (PanCK⁺) versus the immune compartment (CD45⁺). (I) Volcano plots illustrating differential protein expression between OX40L-high and OX40L-low subgroups (stratified by median tumor-OX40L expression) within tumor and (J) immune compartments. Positive $\log_2$ FC indicates protein enrichment in the OX40L-high group. OX40L was excluded from (I) to avoid circularity. Dashed lines indicate false discovery rate (FDR) < 0.05 (Wilcoxon rank-sum test). FFPE, formalin-fixed, paraffin-embedded; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NSCLC, non-small-cell lung cancer; ROI, region of interest; SMA, smooth muscle actin; TPS, tumor proportion score.

compartment-specific expression values. The detailed protocol is provided in online supplemental methods.

IHC and quantitative digital image analysis

OX40L protein expression was evaluated by IHC (clone D6K7R) on an automated Leica BOND system following manufacturer guidelines. Digital quantification was performed using QuPath (V.0.5.1) with a deep-learning-based cell segmentation (StarDist). Specific staining parameters and software settings are detailed in online supplemental methods.

Cell lines development and functional assays

Human NSCLC cell lines (H1581, H1781, H520), HEK293T cells, and mouse NSCLC lines (KLN205, LACUN3) were utilized for in vitro experiments. To investigate the biological impact of OX40L, stable OX40L overexpression was achieved via lentiviral transduction using the pReceiver-Lv156 vector (Genecopoeia), with an empty vector (ExNeg) as a negative control. Successful integration was confirmed by flow cytometry on a DxFLEX cytometer (Beckman Coulter).

To assess OX40L intrinsic effects on tumor behavior, we performed WST-1 proliferation and wound-healing migration assays comparing OX40L-overexpressing cells against controls. Additionally, to identify potential upstream regulators of OX40L expression, tumor cells were stimulated for 72–96 hours with a panel of inflammatory cytokines and mediators, including factors previously reported to induce PD-L1 expression (IL-2, IL-9, IFN- α , IFN- γ , TNF- α , and LPS). OX40L surface expression was subsequently assessed by flow cytometry, while PD-L1 expression was measured in parallel as a positive control.

For histological validation and spatial analysis, FFPE cell pellets were generated. Briefly, human NSCLC cell lines and HeLa cells were expanded and fixed in 10% neutral-buffered formalin, embedded in 2% low-melting agarose, and processed into paraffin blocks. Detailed protocols regarding culture conditions, viral production, and functional assays are provided in online supplemental methods.

OX40L-mediated co-stimulation assay

OX40L-mediated co-stimulation was assessed using the OX40 Bioassay kit (Promega), which contains genetically engineered Jurkat cells expressing a luciferase reporter under the control of an OX40 receptor construct (OX40 Effector Cells). The assay was performed following the manufacturer's protocol for the OX40L/Fc γ RIIb-independent format, with minor modifications. Different numbers of OX40L-overexpressing or control human NSCLC cells were co-cultured with Jurkat cells in 96-well plates. After 5 hours, Bio-Glo luciferase reagent was added, and luminescence was measured after 10 min using a VICTOR Nivo plate reader (PerkinElmer). Background from Jurkat-only wells was subtracted. Assays were performed in technical triplicate.

In vivo tumor models

The tumor growth was evaluated in both immunodeficient and immunocompetent mouse models: (a) immunodeficient athymic nude mice (Hsd:Athymic Nude-Foxn1nu, Inotiv, USA) and (b) immunocompetent syngeneic mice (BALB/cOlaHsd, Inotiv, USA). Subcutaneous tumors were established by injecting 0.5×10^6 Lacun3 cells (OX40L-overexpressing or control) into the right flank of 6–8-week-old mice.⁹ Tumor growth was monitored three times per week using digital calipers, and tumor volumes were calculated using the formula $V = \pi/6 \times (\text{short diameter})^2 \times \text{long diameter}$.

For anti-PD-1 efficacy experiments, the Lacun3-derived BALB/c syngeneic model was used. At day 6 post-implantation, mice were randomized, and treatment was initiated, with all baseline tumor volumes $< 50 \text{ mm}^3$. Mice received 200 μg of anti-PD-1 antibody (clone RMP1-14, Bio X Cell) or isotype control antibody (rat IgG2a, clone 2A3, Bio X Cell) intraperitoneally on days 7, 10, and 13 post-implantation. Following sacrifice, FFPE tumors were represented in TMA format tumor microenvironment (TME) characterization. Detailed anesthetic protocols, as well as specifications for reagents and TMA construction, are provided in online supplemental methods.

Statistical analysis

Statistical analyses and visualizations were performed in R. Survival outcomes were evaluated using univariate Cox proportional hazards models, with patients stratified into OX40L-high/low groups based on cohort-specific cutpoints. Survival curves were estimated using the Kaplan-Meier method and compared via log-rank tests (*survival*, *survminer*, and *ggsurvfit* libraries).

Biological associations within the TME were assessed using two-tailed Spearman rank correlations. Differential expression between groups was performed using two-sided Mann-Whitney U tests, reporting $\log_2$ -fold changes ($\log_2\text{FC}$). P values were adjusted using the Benjamini-Hochberg false discovery rate method.

In vivo tumor kinetics were analyzed using linear mixed-effects models (LMMs) (*lme4*, *lmerTest*). For untreated models, volume was modeled as a function of time, group, and their interaction. Immunotherapy studies used a three-way interaction (time $\times$ cell line $\times$ treatment), including random intercepts and slopes per mouse. P values were computed using Satterthwaite's, with significance set at $p < 0.05$.

RESULTS

OX40L expressed in the tumor compartment is associated with prolonged survival under single-agent PD-1 axis blockade in patients with PD-L1-high NSCLC

We first profiled 104 FFPE tumor samples from the ITX_ILM cohort using GeoMx DSP protein assay. An average of 5.59 ROIs per patient were selected (range 2–16). For each patient, normalized protein expression values were averaged across all ROIs to generate a

single representative value per protein and compartment (tumor/immune) for downstream analyses. As an initial biological quality control, differential expression analysis confirmed accurate compartmental segregation. Tumor-associated markers, including pan-cytokeratin (PanCK), PD-L1, and Ki-67, were significantly enriched in the tumor compartment, whereas immune-related proteins such as CD3, CD4, CD8, CD68, or CD27 were preferentially expressed in the immune compartment, validating the accuracy and robustness of the spatial segmentation strategy (online supplemental figure 2A).

Within the immune compartment, higher expression of CD45 and CD80 was individually associated with improved PFS (HR=0.75, $p=0.049$ and HR=0.78, $p=0.042$, respectively; online supplemental figure 2B and online supplemental table 5). However, no immune compartment markers showed a statistically significant association with OS (online supplemental figure 2C and online supplemental table 6).

In the tumor compartment, OX40L emerged as the only protein consistently associated with clinical outcome. Higher tumor-specific OX40L expression was significantly associated with both prolonged PFS (HR=0.79, $p=0.008$) (figure 1B and online supplemental table 7) and OS (HR=0.79, $p=0.011$) (figure 1C and online supplemental table 8). Using the cohort-specific median tumor OX40L expression, OX40L-high patients exhibited a significantly longer PFS compared with OX40L-low patients (15.7 vs 5.7 months, $p=0.046$) (figure 1D). This association remained significant after adjusting for tumor stage, histology, and the presence of baseline central nervous system or liver metastasis ($p=0.037$; online supplemental table 9). Notably, OX40L expression within the immune compartment was not associated with treatment outcome.

OX40L has traditionally been reported to be predominantly expressed by immune cells, particularly antigen-presenting cells (APCs).¹⁰ Since we unexpectedly observed consistent tumor-compartment OX40L expression associated with clinical outcomes, we performed rigorous antibody validation to confirm the specificity of OX40L detection following recommended guidelines.¹¹ OX40L-overexpressing HeLa cells (OX40L⁺) showed OX40L signal levels like housekeeping controls (GAPDH, Histone H3, S6) using the GeoMx DSP protein assay, while ExNeg-HeLa cells (OX40L⁻) had OX40L at background levels defined by IgG controls (figure 1E). Additionally, IHC staining of H1581 FFPE cell blocks ($\pm$ OX40L) using the same antibody clone (D6K7R) included in the GeoMx DSP protein assay visually confirmed the specificity of the anti-OX40L antibody (figure 1F).

After confirming the specificity of tumor compartment OX40L detection, we next characterized the distribution of tumor-cell OX40L expression in the TME. Intratumoral OX40L heterogeneity was generally modest, although greater dispersion was observed in tumors with higher OX40L expression (online supplemental figure 2D and online supplemental table 10). Overall, patient-level mean values adequately captured the principal differences in

OX40L expression between patients. OX40L expression in the tumor compartment showed a continuous distribution, with a subset of tumors showing signal levels clearly above background (figure 1G). Notably, OX40L levels in the tumor compartment were frequently comparable to, and in a subset of cases exceeded, the immune compartment (figure 1H).

We then examined the TME according to tumor-cell OX40L expression. OX40L expression in the tumor compartment was significantly associated with key immune markers (PD-1, CD20, CD45), consistent with increased immune cell infiltration (figure 1I and online supplemental figure 2E). In addition, within the immune compartment, tumor-OX40L showed strong associations with OX40L, CD40, and β 2-microglobulin (B2M) (figure 1J and online supplemental figure 2F). No significant differences in tumor-cell OX40L expression were observed according to *KRAS* mutational status (online supplemental table 11).

Finally, we explored the relationship between tumor-cell OX40L and PD-L1 expression in greater detail. Spearman correlation analysis using continuous DSP-derived protein expression values showed a weak-to-moderate but statistically significant positive correlation between both markers ($R=0.33$, $p=0.00078$; online supplemental figure 2G). However, when PD-L1 was categorized according to TPS subgroups, OX40L expression did not differ across clinically defined PD-L1 TPS categories ($p=0.650$; online supplemental figure 2H). In multivariable Cox regression models including both biomarkers, the association between high tumor-cell OX40L expression and improved clinical outcome remained independent of PD-L1 status, whether evaluated by DSP ($p=0.030$) or TPS ($p=0.009$), whereas PD-L1 status was not independently associated with outcome (DSP: $p=0.348$; TPS: $p=0.462$), suggesting that the association between tumor-cell OX40L and clinical benefit is not driven by PD-L1 expression (online supplemental table 12).

OX40L expression in residual tumors after neoadjuvant chemioimmunotherapy is associated with improved event-free survival

To evaluate the clinical relevance of our findings in early-stage disease, we first assessed the role of *TNFSF4* (OX40L) mRNA expression in predicting outcomes from perioperative chemo-immunotherapy in the NADIM cohort. In the limited baseline sample set with suitable RNA-seq data ($n=11$), no significant correlation was found between OX40L expression and pCR ($p=0.84$; online supplemental figure 2I). We then investigated the role of OX40L expression in resected tumor specimens following neoadjuvant therapy. In the entire cohort ($n=36$), higher OX40L levels in post-treatment biopsy specimens (median cutpoint) were significantly associated with prolonged event-free survival ($p=0.046$; figure 2A). This benefit was more pronounced when excluding patients with PCR (those with no viable tumor cells in post-neoadjuvant specimens) ($p=0.021$; figure 2B), potentially supporting a role for

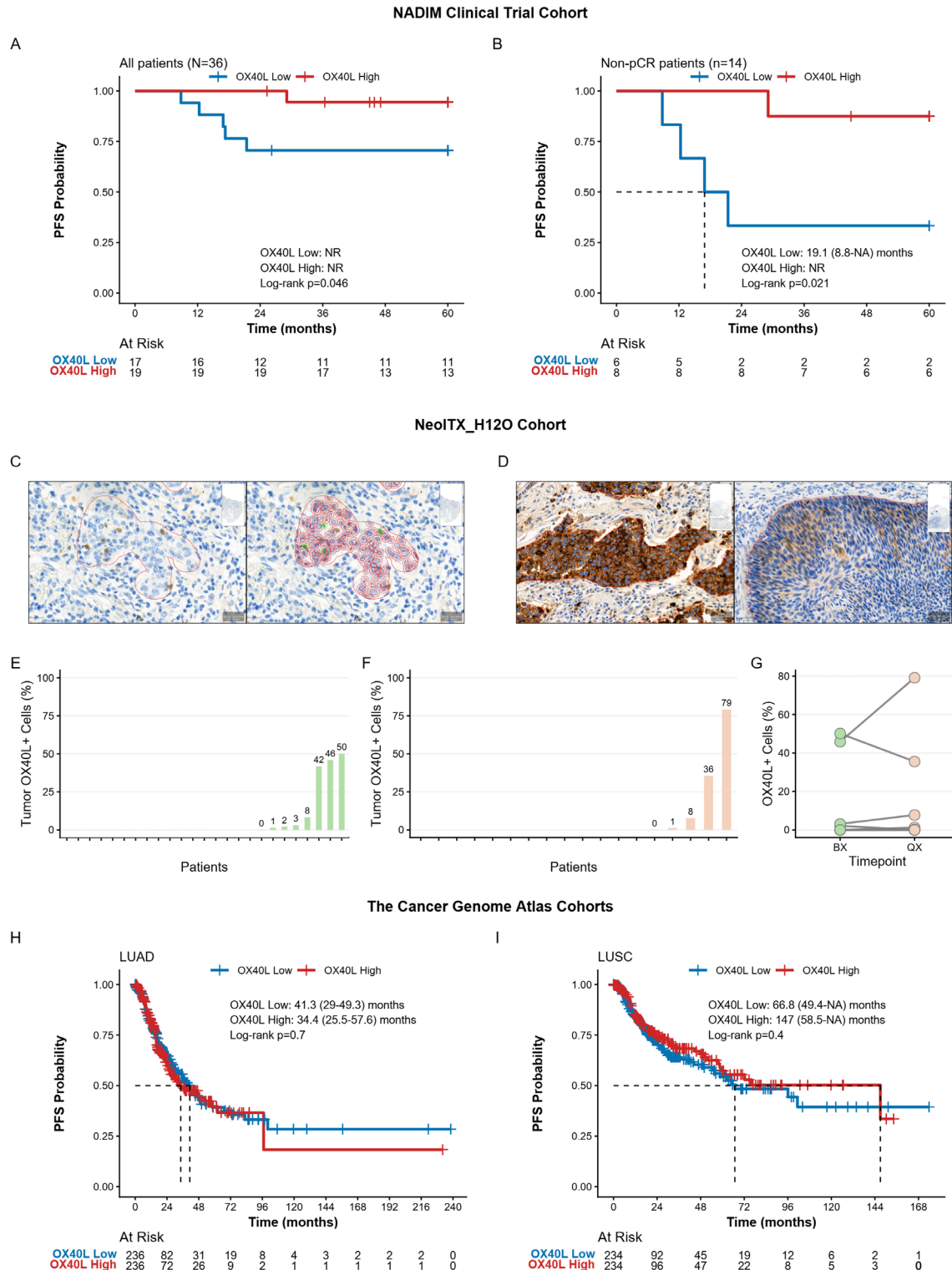


Figure 2 Clinical significance of OX40L in early-stage non-small-cell lung cancer across neoadjuvant immunotherapy-treated and untreated cohorts. (A) Kaplan-Meier estimates of PFS in the overall NADIM cohort (N=36) and (B) in the subgroup of patients with residual viable tumor (non-pCR, n=14), stratified by median OX40L mRNA expression in surgical specimens. (C) QuPath workflow showing tumor annotation (red outline), StarDist-based cell detection, and OX40L+ classification (green) (right). (Scale bar: 50 μ m). (D) Representative immunohistochemistry images from NeolTX_H120 cohort showing strong (left) or diffuse (right) OX40L distribution across tumor nests. (Scale bar: 50 μ m.) (E) Distribution of OX40L+ tumor cell percentages in NeolTX_H120 baseline biopsy samples (Bx). (F) Distribution of OX40L+ tumor cell percentages in NeolTX_H120 surgical samples (Qx), ranked by expression level. (G) Longitudinal dynamic analysis of OX40L expression in paired pre- (Bx) and post-treatment (Qx) samples (n=11). Gray lines connect baseline and surgical time points. (H) Kaplan-Meier estimates of PFS in the immunotherapy-naïve patients from TCGA lung adenocarcinoma (LUAD, n=472) and (I) lung squamous cell carcinoma (LUSC, n=468) cohorts, stratified by median OX40L expression. pCR, pathological complete response; PFS, progression-free survival.

OX40L expressed specifically in residual tumor cells as a driver of enhanced efficacy. In line with our observations in the discovery cohort, continuous RNA-seq analysis in the entire cohort ($n=36$) revealed no significant correlation between *OX40L* and *CD274* (PD-L1) expression levels ($R=0.14$, $p=0.420$; online supplemental figure 2J), and *OX40L* expression was homogeneously distributed across clinical PD-L1 TPS strata ($p=0.89$; online supplemental figure 2K).

Then, we assessed the role of OX40L protein expression in predicting outcomes from neoadjuvant/perioperative chemo-immunotherapy in a second real-world cohort (NeoITX_H12O, $n=41$) using quantitative IHC. To confirm that the indicative OX40L signal originated primarily from tumor cells, quantitative IHC analysis was restricted to morphologically annotated tumor cells, deliberately excluding OX40L expression in adjacent immune cells (figure 2C). Out of the total cohort, 25 baseline samples were fully evaluable for quantitative IHC analysis. This analysis confirmed detectable OX40L protein expression localized in the membrane and cytoplasm of tumor cells (figure 2D) in 8 of these 25 (32%) evaluable baseline samples (median percentage of OX40L⁺ tumor cells: 19.1% (0.2%–50.1%)) (figure 2E).

In line with our previous observations in the NADIM cohort, no correlation was found between baseline tumor-cell OX40L expression and pathological response to neoadjuvant chemo-immunotherapy ($p=0.59$; online supplemental figure 2L). Similarly, among the surgical specimens, 18 samples contained evaluable residual tumor cells (patients who did not achieve a pCR). Within this subgroup, patients with the highest levels of OX40L expression in residual tumor cells ($\geq 10\%$ OX40L⁺ cancer cells) showed a trend toward improved PFS ($p=0.4$; online supplemental figure 2M). Notably, the two patients with the highest levels of tumor cell OX40L experienced prolonged recurrence-free survival (22 and 58 months, respectively) despite the presence of residual viable disease following neoadjuvant therapy (online supplemental figure 2M). Paired analysis of baseline and surgical specimens ($n=11$) revealed that tumor-cell OX40L protein levels did not augment following neoadjuvant chemo-immunotherapy (figure 2F,G). Tumor-cell OX40L protein expression showed no significant association across PD-L1 TPS categories ($p=0.61$; online supplemental figure 2N).

OX40L expression shows no significant association with survival in immunotherapy-naïve TCGA NSCLC cohorts

To determine whether OX40L acts as an indicative biomarker of immunotherapy response rather than a general prognostic factor, we evaluated its impact in immunotherapy-untreated early-stage NSCLC cohorts from TCGA, including LUAD ($n=472$) and LUSC ($n=468$). In contrast to immunotherapy-treated cohorts, OX40L expression failed to stratify PFS ($p=0.7$ LUAD figure 2H, and $p=0.4$ LUSC figure 2I). Collectively, while acknowledging the clinical discrepancies in disease stage

between datasets, our results suggest that the clinical benefit associated with OX40L is prominent under PD-1 blockade, and that this effect is primarily driven by its expression in tumor cells and not in immune cells.

Lung cancer cells expressing OX40L mediate functional co-stimulation of OX40-positive immune cells in vitro

Given the observed indicative value of tumor-OX40L expression for PD-1 axis blockade efficacy in human tumors, we sought to elucidate the biological mechanisms underlying this association. Specifically, we aimed to determine: (a) whether NSCLC tumor cells expressing OX40L can engage and co-stimulate OX40-expressing immune cells; (b) whether OX40L exerts cell-intrinsic effects on NSCLC tumor cells; and (c) whether inflammatory cytokines can directly regulate OX40L expression in cancer cells. To address this, we generated human and murine NSCLC cell line models with stable OX40L overexpression for subsequent in vitro and in vivo functional analyses (online supplemental figure 3A). Because surface expression alone does not ensure OX40 receptor engagement, we next assessed whether tumor-expressed OX40L could activate OX40 signaling in immune cells. Using a bioluminescent OX40 reporter assay in engineered Jurkat T cells (figure 3A), OX40L-overexpressing human NSCLC cell lines (H520, H1581, and H1781) elicited robust cell-number-dependent activation of OX40-driven NF- κ B signaling relative to controls ($p<0.01$) (figure 3B–D). These results demonstrate that OX40L expressed on tumor cells is functionally competent to deliver co-stimulatory signals to OX40⁺ immune cells.

We then assessed the impact of OX40L overexpression on intrinsic tumor cell properties using in vitro assays. OX40L overexpression did not induce a uniform effect on tumor cell proliferation or migration across the human and murine NSCLC cell lines tested. LMM analyses revealed that OX40L expression induced only minor, non-reproducible changes in proliferation and migration across NSCLC cell lines, with no consistent biological effect on intrinsic tumor cell behavior (online supplemental figure 3B–D).

Finally, we investigated potential upstream regulators of tumor-cell OX40L expression. Human NSCLC cells were stimulated with IL-2, IL-9, IFN- α , IFN- γ , TNF- α , or LPS for 72 and 96 hours. While IFN- γ robustly upregulated PD-L1 expression, with IFN- α inducing a characteristically weaker upregulation, none of the inflammatory stimuli induced changes in OX40L surface levels (online supplemental figure 3E), suggesting that acute exposure to individual inflammatory cytokines, including those known to upregulate PD-L1, is insufficient to drive its expression in cancer cells.

OX40L expression on tumor cells improves response to anti-PD-1 therapy in vivo

To evaluate the functional relevance of tumor-cell OX40L expression in vivo, we first assessed tumor growth kinetics. No differences in tumor growth were observed between

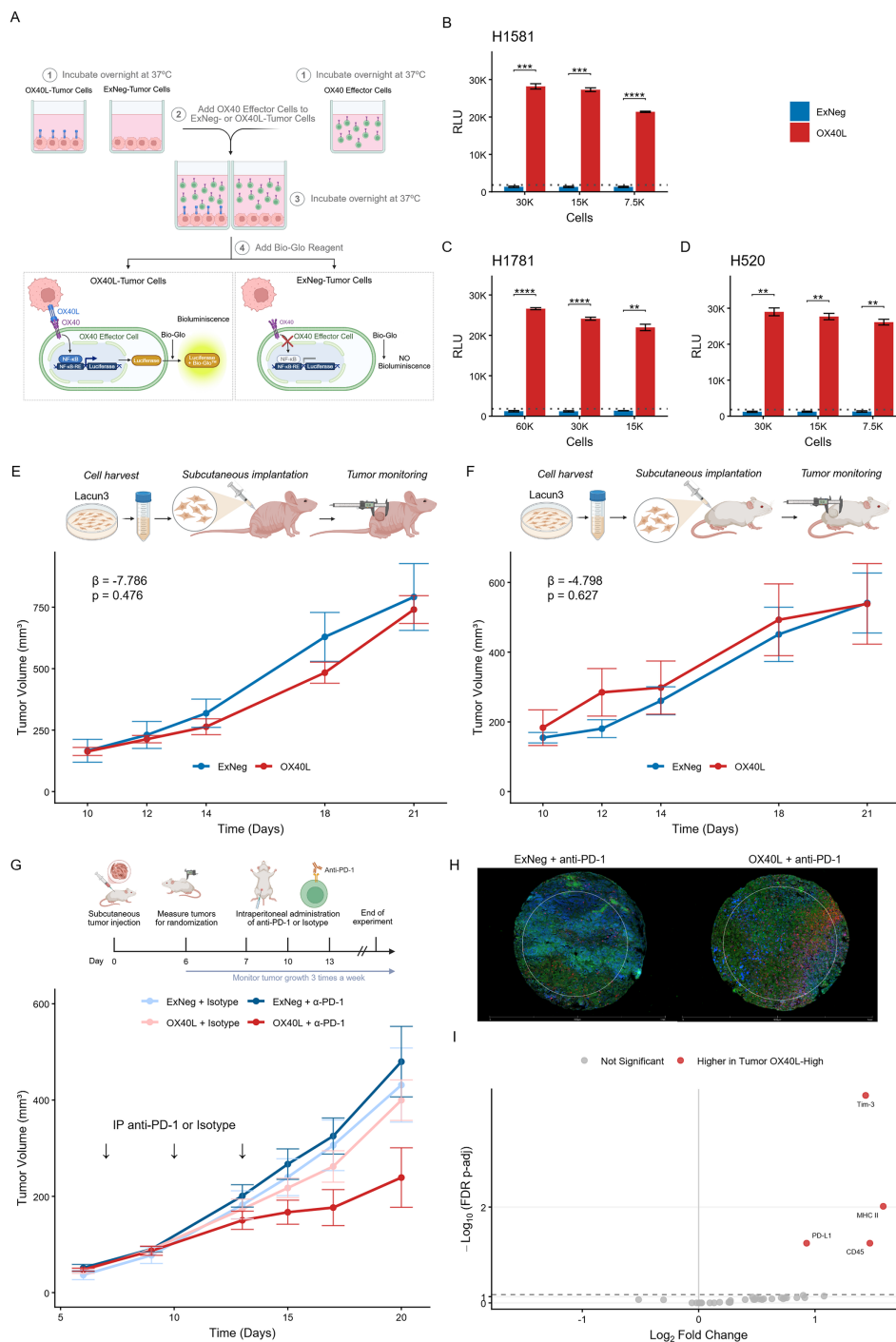


Figure 3 Functional validation of tumor-cell OX40L and its impact on immunotherapy response. (A) Schematic of the OX40 bioassay: Jurkat effector cells (OX40+/NF-κB-Luciferase) co-cultured with NSCLC cells to evaluate surface-expressed OX40L bioactivity. (B) NF-κB-mediated luciferase activity in reporter cells co-cultured with H1581, (C) H1781, and (D) H520 cells transduced with OX40L (red) or empty vector (ExNeg, blue). Bars represent mean relative light units (RLU) ± SEM (n=3). Dotted line indicates background. Significance by multiple t-tests with Bonferroni correction. (E) Longitudinal tumor volume in immunodeficient nude mice and (F) immunocompetent BALB/c mice implanted with Lacun3-OX40L (red) or Lacun3-ExNeg (blue) cells (n=5/group). (G) Therapeutic efficacy in BALB/c mice bearing Lacun3-ExNeg or Lacun3-OX40L tumors treated with α -PD-1 or isotype control (n=8/group). Arrows indicate intraperitoneal (IP) injections. All in vivo data are presented as mean volume ± SEM. Growth rate differences were determined by a linear mixed-effects model (LMM); the interaction coefficient (β) represents the slope difference (with $\beta < 0$ indicating slower growth in the OX40L group). (H) Representative GeoMx DSP immunofluorescence images (regions of interest) of Lacun3-ExNeg (left) and Lacun3-OX40L (right) tumors from BALB/c mice treated with α -PD-1. Images show the spatial distribution of immune cells (CD45+, red), tumor cells (PanCK+, green), and nuclei (SYTO13, blue). (I) Volcano plot illustrating differential protein expression between Lacun3-OX40L and Lacun3-ExNeg tumors under α -PD-1 treatment. The x-axis represents the log₂ fold change and the y-axis represents the -log₁₀ p value. Highlighted proteins indicate significant upregulation (red) in the OX40L-expressing tumors.

OX40L-overexpressing and ExNeg Lacun3 tumors in either immunodeficient (Nude-Foxn1^{nu}) ($p=0.476$) (figure 3E and online supplemental figure 4A) or immunocompetent (BALB/c) ($p=0.627$) (figure 3F and online supplemental figure 4B) murine models, indicating that OX40L expression does not confer a basal growth disadvantage or intrinsic antitumor activity in the absence of immune pressure, consistent with our findings in patient cohorts (figure 2H,I) and in vitro experiments (online supplemental figure 3B-D).

We next assessed the impact of tumor cell OX40L expression on response to immune checkpoint blockade. In the syngeneic BALB/c model, anti-PD-1 treatment (α -PD-1) induced a pronounced reduction in tumor burden selectively in mice bearing OX40L-overexpressing tumors compared with the control group (ExNeg+ α -PD-1) that exhibited minimal response ($\beta=-16.93$, $p<0.001$; figure 3G, online supplemental figure 4C) and online supplemental table 13). Notably, ExNeg tumors failed to achieve a statistically significant response to α -PD-1 monotherapy relative to the isotype control ($p=0.51$). These findings were independently validated in a biological replicate (online supplemental figure 4D and E) and online supplemental table 14). Spatial proteomic analysis using the GeoMx DSP system revealed a robust increment in the levels of intratumoral CD45 and MHC-II in OX40L⁺ tumors treated with anti-PD-1 compared with controls (ExNeg+ α -PD-1) (figure 3H,I). Collectively, these findings demonstrate that tumor-cell OX40L enhances immune-mediated tumor regression in the context of PD-1 axis blockade in NSCLC.

DISCUSSION

In this study, we identified tumor-cell OX40L as a marker of enhanced single-agent PD-1 blockade efficacy in PD-L1-high NSCLC. This association appeared to extend to patients with incomplete tumor response in the neoadjuvant/perioperative chemo-immunotherapy setting. These observations were further supported by functional in vitro and in vivo experimental models.

OX40L is a member of the tumor necrosis factor (TNF) ligand superfamily that provides a critical late-stage co-stimulatory signal to promote T-cell survival, expansion, and memory formation.¹² While its expression is traditionally restricted to professional APCs,^{13,14} evidence of OX40L on cancer cells remains limited and functionally uncharacterized. Our findings indicating OX40L expression on tumor cells in a fraction of human NSCLC cases align with Porciuncula *et al.*¹⁵ who also observed OX40L tumor cell positivity in about 10% of unselected NSCLC patients. The apparently higher prevalence of tumor-cell OX40L in our metastatic cohort likely reflects the selection of PD-L1-high (TPS $\geq$ 50%) tumors, which represents a more inflamed microenvironment.

The clinical significance of OX40L remains controversial. While associated with improved prognosis in some melanoma¹⁶ and pancreatic adenocarcinoma,¹⁷ Feldman

et al found no significant outcome association in melanoma when the analysis was restricted specifically to OX40L⁺ melanocytes.¹⁸ Our data, integrating multiple independent NSCLC cohorts with complementary in vitro and in vivo functional studies, suggest that tumor-cell OX40L has little impact on baseline prognosis but may contribute to more robust and durable responses following PD-1 axis blockade. This interpretation is further supported by our analysis of the TCGA cohort, which showed no prognostic association in a predominantly early-stage, immunotherapy-naïve population. However, because PD-1 blockade is now the standard first-line treatment for advanced NSCLC, we were unable to include an appropriate untreated comparator cohort or perform the formal statistical interaction analysis required to definitively establish OX40L as a predictive biomarker. Accordingly, we have adopted more cautious terminology describing tumor-cell OX40L as indicative of clinical benefit from PD-1 blockade rather than definitively predictive. Importantly, although tumor-cell OX40L is associated with features of an inflamed TME, its only modest correlation with PD-L1 suggests that it captures biological information beyond that reflected by PD-L1 alone. This is further supported by its persistent association with clinical benefit after adjustment for PD-L1 expression.

Our findings are in line with evidence from the mechanistic literature indicating that for T cells to acquire full antitumor effector and cytotoxic programs they need to receive, after initial T-cell priming in the lymph nodes, additional localized co-stimulatory signals in the TME.¹⁹ We propose a model where OX40L⁺ tumor cells themselves could actively contribute to this second stage, acting in an APC-like state.²⁰ This concept is supported by Ribas *et al.*, who proposed that the clinical activity of immunotherapy with immune checkpoint blocking antibodies requires that cancer cells facilitate the antitumor immune response.²¹

The clinical application of our results may follow two potential avenues: (a) OX40L as a biomarker for patient stratification: while the utility of OX40L as a selection tool in the advanced setting appears limited (PD-1 blockade with/without chemotherapy will remain the standard of care for most patients), it might have utility in the neoadjuvant/perioperative settings. Our observations indicate favorable outcomes for patients with incomplete response who express OX40L in residual tumor cells. If confirmed in larger cohorts, this could serve as a valuable tool to guide adjuvant therapy decisions; and (b) novel therapeutic strategies centered on the OX40/OX40L axis. While systemic agonistic mAbs targeting the OX40 receptor showed preclinical promise,^{22,23} clinical activity in lung cancer has been disappointing.^{24,25} However, the low baseline expression of OX40L in human lung tumors could be potentially reversed by intratumoral delivery of OX40L mRNA. This strategy could provide therapeutic OX40L levels in cancer cells and a more physiologic co-stimulatory engagement in the TME with

OX40 receptor. This approach has shown promising anti-tumor activity in PD-1-resistant preclinical models,^{26, 27} especially when combined with inflammatory cytokines like IL-23 and IL-36γ.²⁷ Clinical proof-of-concept for this strategy was also demonstrated in a phase I/II study using mRNA-2416 (lipid nanoparticle-encapsulated OX40L mRNA). The treatment was safe and well-tolerated as monotherapy or with durvalumab,²⁸ showing evidence of both local immune activation (increased T-cell infiltration) and systemic immune modulation. Furthermore, a phase I trial in ductal carcinoma in situ (DCIS) evaluated the intratumoral triplet mRNA-2752 (encoding OX40L, IL-23, and IL-36γ) in combination with pembrolizumab. The study reported that this approach was not only safe but could induce rapid tumor regression.²⁹

This study must be interpreted in the context of a number of limitations. First, ROI-based spatial profiling cannot completely exclude signal contribution from adjacent immune infiltrates, and therefore the exclusive attribution of OX40L expression and its associated biological and clinical significance to tumor cells cannot be definitively established. Nevertheless, complementary technical, orthogonal, and functional evidence consistently supports a biologically relevant role for tumor-cell OX40L, including antibody validation, tumor-cell-restricted quantitative IHC in an independent cohort, and concordant in vitro and in vivo functional studies. Further orthogonal validation in larger independent cohorts will be important to definitively establish the specificity and clinical significance of tumor-cell OX40L expression. Second, the use of patient-level mean OX40L expression may partially mask intratumoral spatial heterogeneity across individual ROIs. However, averaging multiple representative ROIs reduces the impact of regional sampling variability while providing a representative patient-level summary of OX40L expression that adequately captures the overall differences between patients. Third, our discovery cohort was retrospective and focused on PD-L1-high patients. While this allowed us to study a more immune-inflamed TME where co-stimulatory signals might be more relevant, further prospective studies are needed for low/intermediate PD-L1 patients. Fourth, the sample size and follow-up of our neoadjuvant cohorts, particularly in the sub-analyses restricted to non-pCR patients, are limited. Consequently, these perioperative findings remain exploratory and require rigorous validation of the role of OX40L in residual tumors through larger perioperative trials. Fifth, the lack of a single, harmonized cut-off across our datasets represents a limitation imposed by the distinct technical nature of the platforms used. Establishing a universal, cross-platform threshold in larger, independent validation cohorts remains a critical requirement for future clinical translation. Finally, our preclinical in vivo experiments were limited to a subcutaneous murine model. While this approach allowed us to successfully validate and reproduce the biological observations from our patient cohorts, further studies using orthotopic models would be needed to fully understand the lung-specific

TME and the underlying immunobiology of treatment response, as well as the specific molecular factors that induce intrinsic OX40L expression by cancer cells.

In conclusion, OX40L expression in tumor cells can enhance the efficacy of PD-1 axis blockade in NSCLC, suggesting that OX40L⁺ cancer cells can act as active participants in the immune response under cancer immunotherapy with immune checkpoint inhibitors. These findings might contribute to improving anti-PD-1 treatment personalization and provide a rationale for mRNA-based therapeutic strategies to enhance OX40/OX40L “natural” co-stimulation to expand the benefit of PD-1 axis blockade in NSCLC.

Author affiliations

- ¹Tumor Microenvironment and Precision Oncology Research Group, Instituto de Investigación Hospital 12 de Octubre (i+12), Madrid, Community of Madrid, Spain
- ²Medical Oncology Department, Hospital Universitario 12 de Octubre, Madrid, Spain
- ³Hospital General Universitario Gregorio Marañón, Madrid, Spain
- ⁴Medical Oncology Department, Hospital Universitario Clínico San Carlos, Madrid, Spain
- ⁵Medical Oncology Department, Ramon y Cajal University Hospital, Madrid, Spain
- ⁶Department of Oncology, Hospital Universitario Fundación Alcorcón, Alcorcón, Spain
- ⁷Medical Oncology Department, Infanta Sofía University Hospital, Madrid, Spain
- ⁸European University of Madrid, Villanueva de la Cañada, Madrid, Spain
- ⁹Department of Medical Oncology, Del Henaes University Hospital, Madrid, Spain
- ¹⁰Medical Oncology Department, Hospital Universitario de Torrejón, Madrid, Spain
- ¹¹Facultad de Ciencias de la Salud, Universidad Francisco de Vitoria, Pozuelo de Alarcón, Spain
- ¹²Medical Oncology Department, Hospital Universitario de Severo Ochoa, Leganés, Spain
- ¹³Medical Oncology Department, Hospital Universitario de Móstoles, Madrid, Spain
- ¹⁴Department of Pathology, Hospital Universitario 12 de Octubre, Madrid, Spain
- ¹⁵Universidad Complutense de Madrid, Madrid, Spain
- ¹⁶Molecular and Computational Pathology Group, Research Institute Hospital 12 de Octubre (imas12), Madrid, Spain
- ¹⁷Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain
- ¹⁸Cancer Immunotherapy Unit (UNICA), Department of Immunology, Hospital Universitario 12 de Octubre, Madrid, Spain
- ¹⁹CNIO-HMRIB Cancer Immunotherapy Clinical Research Unit, Spanish National Cancer Research, Madrid, Spain
- ²⁰Banc de Sang i Teixits, Barcelona, Spain
- ²¹Solid Tumors Program, Clínica Universidad de Navarra Cancer Center and CIMA, University of Navarra, Pamplona, Spain
- ²²Medical Oncology Department, Hospital Universitario Puerta de Hierro Majadahonda, IDIPHISA, Majadahonda, Spain

Contributors JZ conceived and supervised the study. CF-L, VA, EC-E, and ES-H performed experiments. CF-L, JB, MPe, ES-H, and AP-F performed data analysis and bioinformatic processing. EC and DC-A performed the pathological assessment and selection of FFPE samples for DSP and IHC analysis. DB was responsible for FFPE tissue sectioning and slide preparation. JB, CS, and JZ provided clinical information and patient specimens. AC, MA-B, JB, YL, XM, MS, RC, LC-G, AL, JR, LP-A, and JZ contributed to patient recruitment and clinical data collection. KV and LMM provided experimental syngeneic models. Manuscript revisions were performed by all authors. All authors approved the final manuscript. JZ is responsible for the overall content of the manuscript as the guarantor. During the preparation of this work, the author(s) used AI tools to assist with language refinement and formulation of texts. The tools were not involved in generating scientific content hypotheses, or analysis strategies. All scientific concepts, methodologies, and objectives were entirely developed by the research team. The final manuscript represents the team's original intellectual work, with AI serving solely as a tool for editorial support. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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ORCID iDs

Luis Alvarez-Vallina <https://orcid.org/0000-0003-3053-6757>

Esther Carrera-Elvira <https://orcid.org/0009-0007-5251-2410>

Alberto Cruz-Bermúdez <https://orcid.org/0000-0001-5136-7011>

Jon Zugazagoitia <https://orcid.org/0000-0001-9352-5882>

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