

2 **Multi-omics Analysis Reveals Immune Features**  
 3 **Associated with Immunotherapy Benefit in Patients with**  
 4 **Squamous Cell Lung Cancer from Phase III Lung-MAP**  
 5 **S1400I Trial**



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16 **ABSTRACT**

17 **Purpose:** Identifying molecular and immune features to guide  
 18 immune checkpoint inhibitor (ICI)-based regimens remains an  
 19 unmet clinical need.

20 **Experimental Design:** Tissue and longitudinal blood specimens  
 21 from phase III trial S1400I in patients with metastatic lung squa-  
 22 mous cell carcinoma (SqNSCLC) treated with nivolumab mono-  
 23 therapy (nivo) or nivolumab plus ipilimumab (nivo+ipi) were  
 24 subjected to multi-omics analyses including multiplex immunoflu-  
 25 orescence (mIF), nCounter PanCancer Immune Profiling Panel,  
 26 whole-exome sequencing, and Olink.

27 **Results:** Higher immune scores from immune gene expression  
 28 profiling or immune cell infiltration by mIF were associated with  
 29 response to ICIs and improved survival, except regulatory T cells,  
 30 which were associated with worse overall survival (OS) for patients  
 31 receiving nivo+ipi. Immune cell density and closer proximity of  
 CD8+GZB+ T cells to malignant cells were associated with

superior progression-free survival and OS. The cold immune  
 landscape of NSCLC was associated with a higher level of chro-  
 mosomal copy-number variation (CNV) burden. Patients with  
*LRP1B*-mutant tumors had a shorter survival than patients with  
*LRP1B*-wild-type tumors. Olink assays revealed soluble proteins  
 such as LAMP3 increased in responders while IL6 and CXCL13  
 increased in nonresponders. Upregulation of serum CXCL13,  
 MMP12, CSF-1, and IL8 were associated with worse survival  
 before radiologic progression.

**Conclusions:** The frequency, distribution, and clustering of  
 immune cells relative to malignant ones can impact ICI efficacy  
 in patients with SqNSCLC. High CNV burden may contribute  
 to the cold immune microenvironment. Soluble inflammation/  
 immune-related proteins in the blood have the potential to  
 monitor therapeutic benefit from ICI treatment in patients with  
 SqNSCLC.

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## Translational Relevance

Identifying molecular and immune features to guide immune checkpoint inhibitor (ICI) regimens remains an unmet clinical need. We performed multi-omics analysis of biospecimens from a phase III trial LUNG-MAP S1400I that compared ipilimumab combined with nivolumab versus nivolumab monotherapy in patients with metastatic lung squamous cell carcinoma. An overall cold tumor immune microenvironment correlated with high chromosomal copy-number variant burden and was associated with inferior benefit from ICIs. In addition to the immune cell density, the proximity and local neighborhood clustering of a subset of immune cells to tumor cells also impacted the benefit from ICI therapy. Interestingly, patient survival was decreased with *LRP1B*-mutant tumors, but not with *LRP1B*-wild type tumors. Many soluble proteins related to inflammation or T-cell and dendritic cell activation correlated with clinical outcome from ICI therapy. Together, these immune features highlight the potential of biomarker-based strategies to select patients for ICI-based regimens and dynamically monitor their response.

## Introduction

Immune checkpoint inhibitors (ICI) targeting programmed cell death protein 1 (PD-1, e.g., nivolumab, pembrolizumab, cemiplimab) or its ligand PD-L1 (e.g., atezolizumab) have become pillars of treatment in both frontline and salvage settings for patients with advanced non-small cell lung cancer (NSCLC; refs. 1–4). In addition, recent efforts have led to multiple approved frontline regimens incorporating chemotherapy and other ICIs with anti-PD-1/PD-L1 antibodies (5–8). However, in the salvage setting, anti-PD-1/PD-L1 monotherapy remains the treatment of choice for ICI-naïve advanced-stage NSCLC (9, 10).

Ipilimumab is an ICI targeting CTL-associated protein 4 (CTLA-4). Its dual inhibition with PD-1/PD-L1 may have synergistic effects on the anticancer immune response, given the complementary functions of these two pathways. The combination of nivolumab with ipilimumab (nivo+ipi) was demonstrated to have superior efficacy than nivolumab alone in patients with advanced melanoma (11, 12). For patients with metastatic NSCLC, ipilimumab plus nivolumab has been approved by the FDA in the frontline setting with or without concurrent chemotherapy (7, 8, 13, 14). In the salvage setting, a recent phase III study, S1400I, evaluated the efficacy of nivo+ipi versus nivolumab monotherapy (nivo) in patients without previous ICI treatment for squamous NSCLC (SqNSCLC; ref. 15). The study did not show that ipilimumab plus nivolumab improved clinical outcomes. However, progression-free survival (PFS) and overall survival (OS) curves separated during later follow-up, suggesting that a subset of patients may benefit from combination treatment with ipilimumab and nivolumab.

Understanding the mechanisms underlying response and resistance to ICIs and establishing predictive molecular and immune features to identify patients who will benefit the most from ICI therapy remain unmet clinical needs. High PD-L1 expression is associated with improved outcomes in patients receiving ICI monotherapy (1, 8). However, the geographical heterogeneity of PD-L1 expression between primary tumors and metastatic sites and even between different regions within the same tumors—as well as the potential dynamic changes in PD-L1 expression over time—have raised questions about

its reliability as a predictive biomarker (16, 17). Although tumor mutational burden (TMB) has been approved as a predictive marker for anti-PD-1/PD-L1 treatment for melanoma and NSCLC, and several other cancer types (18), one study found no correlation between TMB or PD-L1 with anti-PD-1 plus anti-CTLA-4 therapy in patients with NSCLC (19). Furthermore, the predictive value of PD-L1 and TMB becomes less clear when chemotherapy is added. These findings underscore the complexity of molecular determinates of the tumor immune microenvironment and response to ICIs.

In this study, we sought to elucidate the immune and molecular mechanisms that affect benefit from ICIs in patients with advanced SqNSCLC. Toward this end, we integrated immune and multi-omics profiling platforms supported by Cancer Immune Monitoring and Analysis Centers (CIMAC) in the current study. Specifically, we performed multiplex immunofluorescence (mIF), gene expression profiling (ncounter PanCancer Immune Profiling Panel), whole-exome sequencing (WES), and Olink proteomics on tissue and blood specimens from the S1400I trial to identify molecular or immune factors associated with better prognoses in patients treated with anti-PD-1 monotherapy versus anti-PD-1/CTLA-4 dual combination.

## Material and Methods

### Study population and human tissue samples

Lung-MAP (S1400I, NCT02785952) was a multicenter, open-label, phase III randomized clinical trial. The substudy Lung-MAP-I (S1400I) was conducted from December 18, 2015, to April 23, 2018, through the National Clinical Trials Network and led by the SWOG Cancer Research Network. The study was conducted in accordance with the Declaration of Helsinki and the Lung-MAP design has been described previously (15). Briefly, the trial compared nivo+ipi with nivo in patients with chemotherapy-pretreated, immunotherapy-naïve, advanced sqNSCLC. Two hundred fifty-two patients were randomly assigned to receive nivo+ipi ( $n = 125$ ) or nivo ( $n = 127$ ). The clinical efficacy endpoints were OS, PFS, duration of response, and best objective response by RECIST 1.1. Each site required approval by the U.S. NCI central Institutional Review Board or approval by their local Institutional Review Board. Written, informed consent was required for all patients prior to registration.

Available tumor tissue samples and blood samples ( $N = 160$ , Supplementary Fig. S1) submitted for Lung-MAP screening were provided by the SWOG tissue bank. The clinical information for correlative studies in collaboration with the CIMAC–Cancer Immunologic Data Commons (CIDC) Network is shown in Supplementary Table S1 across the different assays.

### mIF staining and analysis

mIF staining was performed in 82 screening tumor tissue samples (nivo+ipi = 38, and nivo = 42; Supplementary Table S1). Unstaining slides from formalin-fixed, paraffin-embedded (FFPE) tissue were received from the SWOG bank and stained using methods previously described and validated (20). Briefly, 4- $\mu$ m-thick FFPE tumor sections were stained using an automated staining system (Leica Microsystems) and two mIF panels with the following antibodies: Panel 1, cytokeratin (CK), CD3, CD8, PD-1/PD-L1, and CD68 and Panel 2, CK, CD3, CD8, CD45RO, granzyme B (GZB), and FOXP3. Antibody clones, dilutions, and RRIDs are included in Supplementary Table S2 and have been described previously (20). All the markers were stained in sequence using their respective fluorophore contained in the Opal 7-Color Automation IHC Kit (catalog no. NEL821001KT; Akoya Biosciences). The slides were scanned using the Vectra/Polaris 3.0.3 (Akoya

148	Biosciences) at low magnification, 10× (1.0 μm/pixel) through the full	
149	emission spectrum and positive tonsil controls from the run staining to	
150	calibrate the spectral image scanner protocol (21). A pathologist	
151	selected representative areas inside the tumor using regions of interest	
152	for scanning in high magnification by the Phenochart Software image	
153	viewer 1.0.12 (931 × 698 μm size at resolution 20× = 0.5 μm/pixel) to	
154	capture various elements of tissue heterogeneity. Marker coexpression	
155	was employed to identify malignant cells (CK+), malignant cells	
156	expressing PD-L1 (CK+PD-L1+), and the cellular subsets of	
157	tumor-associated immune cells (TAIC) listed in Supplementary	
158	Table S3. Densities of each cell phenotype were quantified as the	
159	number of cells/mm <sup>2</sup> in the tumor compartment characterized by	
160	group or nests of malignant cells, in the stroma compartment char-	
161	acterized by the fibrous tissue present between the tumor nets, and in	
162	both compartments described as a total. PD-L1+ malignant cells were	
163	also expressed in percentages. The data were consolidated using R	
164	studio 3.5.3 (Phenopter 0.2.2 packet; Akoya Biosciences).	
165	<b>Spatial point pattern distribution analysis</b>	
166	Using the point pattern distribution of the cell phenotypes relative	
167	to malignant cells, we measured the distance from malignant cells	
168	(CK+) to TAICs included in each mIF panel using R studio 3.5.3	
169	(Phenopter 0.2.2 packet). We applied the median nearest neighbor	
170	function from malignant cells (CK+) to different cell phenotypes to	
171	determine where these TAICs were located; specifically, whether the	
172	TAICs were close to (i.e., equal to or less than the median distance) or	
173	far from (i.e., more than the median distance) the malignant cells	
174	(CK+) and associated with clinical outcomes.	
175	<b>Spatial organization of cells by type</b>	
176	Cells were subset by phenotype using the markers in the mIF panels	
177	and examined as the following: Tumor/PD-L1+ (CK+PD-L1+), Tumor	
178	(CK+), Other-Tcells (CD3+), Other-Tcells/PD-1+ (CD3+PD-1+),	
179	Macrophages(CD68+), Macrophages/PD-L1+(CD68+PD-L1+),	
180	CTLs(CD3+CD8+), CTLs/PD-1+(CD3+CD8+PD-1+), CTLs/GB+	
181	(CD3+CD8+GB+), and Tregs (CD3+CD8-Foxp3+). The above	
182	phenotypes were used to visualize the spatial organization of cells by	
183	type. This analysis was carried out in R version 4.2.0 (R studio 2022.07.2).	
184	<b>Spatial neighborhood</b>	
185	Using the marked planar point pattern representations of each mIF	
186	image, we calculated the spatially varying probabilities for each of the	
187	phenotypes (described above). We used the spatstat toolbox (22)	
188	which provides the relrisk function to identify areas of segregation	
189	for a multitype (markers >2) marked point pattern. This function	
190	estimates for each phenotype, the spatially varying probability or the	
191	ratios of the probabilities, using kernel smoothing. The output of this	
192	function was used to plot the contour of the spatially segregated	
193	neighborhoods for each phenotype.	
194	<b>Identifying cell clusters in the local neighborhood</b>	
195	We identified cell clusters in each image using Euclidean distance	
196	and a hierarchical clustering method. A minimum cluster size of 10	
197	cells and distance ≤ 20 μm was the requirement for clustering. The	
198	distance-based hierarchical clustering yielded the neighborhood	
199	information in a matrix. The cells that did not form clusters were	
200	labeled "Free_cell". The relative percentages of cells in each phe-	
201	notype within a cluster were used to generate the heat map. We used	
202	the SPIAT library (SPIAT version 1.0.4) to identify cell clusters and	
203	made additions to the SPIAT functions as required for our analysis	
204	using R version 4.2.0.	
	<b>NanoString gene expression profiling</b>	206
	DNA and RNA were coextracted from FFPE specimens received	207
	from the SWOG bank (Supplementary Table S1) and subjected to WES	208
	and gene expression. The RNA from a total of 38 FFPE samples (nivo =	209
	23 and nivo+ipi = 15) passed the quality control (QC) and was run on	210
	the nCounter platform using the PanCancer Immune Profiling Panel	211
	(730 immune-related and 40 housekeeping genes) per the manufac-	212
	turer's instructions. Briefly, samples were hybridized overnight at 65°C	213
	to probes, excess probes were washed using the automated prep station	214
	and then imaged on the digital analyzer. All runs included a Human	215
	Reference RNA control for batch correction. Data were processed and	216
	normalized with NanoString's nSolver analysis software (23). All	217
	samples passed the post-run QC metrics, and no batch effects were	218
	evident in the runs. In addition, gene expression profiles were decon-	219
	voluted by TIMER and nSolver advanced analysis tools to infer	220
	immune cells correlated to clinical outcomes.	221
	<b>WES data analysis</b>	222
	WES analysis was conducted using the CIDC WES pipeline on	223
	tumor DNA from 50 tumors (nivo = 28 and nivo+ipi = 22, Supple-	224
	mentary Table S1) that passed the QC. DNA from paired peripheral	225
	blood mononuclear samples was used as germ line control. WES	226
	implements Gene Analysis Toolkit (24) best practices and identifies	227
	somatic variants using Sentieon TNScope and Haplotyper algo-	228
	rithms (25), respectively. Somatic variants are annotated using the	229
	Variant Effect Predictor software (26). The pipeline uses an ensemble	230
	of three callers, CNVkit (27), Sequenza (28), and Facets (29), to	231
	characterize tumor copy-number variation (CNV), and the CNV	232
	segments called by at least two callers were used to generate a high-	233
	confidence consensus set. Sequenza and FACETS were used to estimate	234
	tumor purity and also PyClone-VI was utilized to infer clonal status of	235
	mutations (30). PyClone v 0.13.1 (31) was used to perform mutation	236
	clonality analysis. It is a Bayesian clustering method that enables	237
	mutations to be grouped into putative clonal clusters by integrating	238
	copy number, tumor purity (obtained from Sequenza), and variant	239
	allele frequency data.	240
	<b>Olink serum soluble analyte assay</b>	241
	We performed circulating serum analyte measurements using	242
	proximity extension assay (Olink) in 561 serum samples collected	243
	longitudinally from 160 patients (Supplementary Table S1). A series of	244
	92 proteins, such as cytokines and soluble immune checkpoints	245
	included in the "immuno-oncology" panel, was measured as described	246
	previously (32). Protein levels were normalized using internal positive	247
	and negative controls and quantified as log <sub>2</sub> protein expressions	248
	(NPX), which were subsequently used as input for downstream	249
	analysis.	250
	<b>Correlative analysis and statistical methods</b>	251
	To evaluate whether the baseline biomarkers are prognostically	252
	associated with survival, we dichotomized biomarker data by the	253
	median and performed univariate survival analysis with the log-	254
	rank test. OS and PFS were evaluated. The Cox proportional hazards	255
	regression model was used for multivariate survival analysis (R	256
	package Survival, <a href="https://CRAN.R-project.org/package=survival">https://CRAN.R-project.org/package=survival</a> ;	257
	ref. 33). We included TMB (≥10 or <10 mutations per Mb), PD-L1	258
	(≥5 or <5%), and other statistically significant biomarkers identified	259
	from univariate analysis in Cox models. Thresholds for TMB and PD-	260
	L1 were determined from previous clinical studies (18). To assess	261
	whether continuous biomarker data are associated with response and	262
	other clinical variables, we used nonparametric tests: Spearman rank	263

266	correlation for continuous clinical variables, Mann–Whitney U test for	328
267	categorical clinical variables with two groups, and Kruskal–Wallis test	329
268	for categorical variables with more than two groups. In parallel, we also	330
269	dichotomized biomarker data and used the $\chi^2$ test for a robust	331
270	assessment with responders. The Benjamini-Hochberg method (34)	332
271	was used for multiple testing adjustment of <i>P</i> values. The analysis was	333
272	performed on all samples and on samples in two treatment arms	334
273	separately.	335
274	To explore the association of each baseline protein level with clinical	
275	outcomes from the Olink data, we used logistic regression models	336
276	for best objective response and Cox proportional hazards models	337
277	for PFS and OS (R package Survival, <a href="https://CRAN.R-project.org/package=survival">https://CRAN.R-project.org/</a>	338
278	<code>package=survival</code> , RRID: SCR_021137; ref. 33). In separate regression	339
279	models, univariate analyses included only the protein expression	340
280	values, while the multiple variable analyses adjusted for additional	341
281	covariates (i.e., treatment, age, sex, race, smoking). Then, to investigate	
282	the longitudinal changes in serum protein associated with treatment,	
283	we used mixed linear models (R package Dream and lme4; refs. 35, 36)	342
284	and the timepoints baseline, cycle 2 week 3, cycle 4 week 7, and cycle	343
285	5 week 9 to quantify the effect of these variables and additional relevant	344
286	clinical parameters. These were analyzed with the treatment arms	345
287	nivo+ipi and nivo. In our models, each protein NPX was considered an	346
288	independent variable. In contrast, phase, timepoints, and treatments	347
289	were considered dependent variables and other covariates as random	348
290	effects. This approach allowed us to quantify the variance across	349
291	proteins and approximate degrees of freedom of the hypothesis test	350
292	for each protein, thereby minimizing false-positive results. We used F-	351
293	tests for multiple coefficient comparisons and moderate <i>t</i> tests for	
294	single coefficient comparisons.	
295	To identify the differences between responders and nonresponders	352
296	at each timepoint and longitudinally, we used time as a dependent	353
297	variable. We jointly modeled survival with cytokine expression [R	354
298	packages lme4, rstanarm: Bayesian applied regression modeling via	355
299	Stan (RRID:SCR_024605), bayestestR, bayesplot: Plotting for Bayesian	356
300	Models ( <a href="https://mc-stan.org/bayesplot/">https://mc-stan.org/bayesplot/</a> , RRID: SCR_024588] (36, 37)	357
301	to investigate the association of longitudinal protein levels with	358
302	survival outcomes. The model used Cox proportional hazards and	359
303	linear mixed regression and assessed the association of dynamic	360
304	biomarker changes with survival outcomes. In the random intercept,	361
305	the independent variable was the number of months from baseline to	362
306	biomarker collection, set as a natural spline with three knots (at most	363
307	three changing timepoints between baseline and progression/death).	364
308	The dependent variable was the Olink analyte NPX value. In the	365
309	survival analysis component, the independent variable includes the	366
310	treatment arms. The convergence of the Markov chain Monte Carlo	367
311	samples was assessed using several diagnostics: potential scale reduc-	368
312	tion factor, autocorrelation and trace plots, adequate sample size, and	369
313	Monte Carlo standard error (32, 38–40). Finally, we used the FDR as	370
314	the preferred method to correct for multiple hypothesis testing. The	371
315	thresholds for significance in the mixed linear models for differential	372
316	expression tests were a $\log_2$ fold change of at least 0.5 and an FDR <	373
317	0.05. The joint model's threshold for significance was at least 1 unit	374
318	increase in $\log_2$ NPX expression and FDR < 0.05.	375
319	For integrative analysis, we applied recursive partitioning tree	376
320	analysis (RPART, rpart library in R, <a href="https://cran.r-project.org/web/packages/rpart/vignettes/longintro.pdf">https://cran.r-project.org/web/</a>	377
321	<code>packages/rpart/vignettes/longintro.pdf</code> ) and random forest (refs. 41, 42;	378
322	RF, randomForest and randomForestSRC libraries in R) on Olink	379
323	( <i>N</i> = 159) and mIF ( <i>N</i> = 82) data. We fitted RPART tree using	380
324	responder status as the dependent variable, 92 baseline level Olink	381
325	proteins and 17 mIF markers as predictors. We also created decision	382
326	tree survival prediction model. Separate RPART trees were fitted for	383
	mIF markers from different compartments along with Olink proteins.	384
	The minimum number of observations in a node for a split was set to be	
	15; 10-fold cross-validation was carried out and results used for tree-	
	pruning. For RF, we used 81 samples with both Olink and mIF data.	
	Bootstrap the data to create bootstrap samples; grow a survival tree for	
	each bootstrap sample with split criteria based on the log-rank	
	statistics; continue the recursive partition; and calculate importance	
	of each predictor by averaging over the forest.	
	<b>Data availability</b>	
	In conjunction with the clinical study principal investigator/chair,	
	the NCI-sponsored network and CIDC, we will make available de-	
	identified data publicly available by request under the dbGaP PHS	
	accession number: phs003412.v1.p1. Questions and requests for addi-	
	tional data can be directed to the corresponding author.	
	<b>Results</b>	
	<b>Clinical characteristics</b>	
	There were 31 responders (19.4%), including complete responses,	
	partial responses, and unconfirmed partial/complete response; 63	
	patients with stable disease (39.4%); and 61 patients (38.1%) with	
	progressive disease (i.e., increasing disease and symptomatic deter-	
	ioration). Overall, 31 patients (19.4%) were alive at the end of the study,	
	and the median OS was 10.02 months (range: 0.3–40.3). One hundred	
	and forty-eight patients (92.5%) had disease progression, with a	
	median PFS of 3.4 months (range: 0.3–36.6; Supplementary Table S1).	
	<b>An active immune infiltration is associated with benefit from ICI</b>	
	<b>treatment</b>	
	We first analyzed the mIF and gene expression profiling data from	
	baseline tissue samples taken before ICI treatment to identify immune	
	features associated with clinical benefit. mIF data revealed higher	
	densities of various immune cells in the stroma compartment com-	
	pared with the tumor compartment across the whole cohort (nivo+ipi	
	and nivo arms), with no significant differences between the nivo+ipi	
	and nivo arms (Supplementary Table S4). The overall immune cell	
	densities were higher in the responders across both arms, although	
	the difference did not reach statistical significance. In the whole	
	cohort, higher median densities of PD-1+ cytotoxic T cells (CTL;	
	CD3+CD8+PD-1+) in the stroma (>4.1 cells/mm <sup>2</sup> , <i>P</i> = 0.042),	
	presence of GZB+ CTLs in the tumor compartment (>0 cells/mm <sup>2</sup> ,	
	<i>P</i> = 0.011), and higher median densities of memory T cells	
	(CD3+CD45RO+; >23.4 cells/mm <sup>2</sup> , <i>P</i> = 0.041) and PD-1+ T cells	
	(CD3+PD-1+; >16.0 cells/mm <sup>2</sup> , <i>P</i> = 0.023) in the total compartment	
	(tumor plus stroma) were associated with longer PFS (Supplementary	
	Table S5). Similarly, transcriptomic analysis demonstrated that	
	patients having tumors with a higher expression of genes associated	
	with myeloid infiltration, immune recruitment, and inflammation had	
	superior clinical outcomes in the whole cohort (Table 1; Supplemen-	
	tary Table S6). The associations between higher expression of CD163,	
	BLNK, IRF1, FCGR2A with better OS ( <i>P</i> < 0.05) and higher expression	
	of MAPK11 with worse OS remained significant in subsequent	
	multivariate analyses after adjustments for known predictive biomar-	
	kers, including TMB and PD-L1.	
	In the nivo arm, higher densities of memory T cells	
	(CD3+CD45RO+) in the total compartment (median > 24.6 cells/mm <sup>2</sup> ,	
	<i>P</i> = 0.028) and memory/regulatory T cells (Treg; CD3+CD8-	
	CD45RO+FOXP3+) in the total compartment (median > 4.6 cells/mm <sup>2</sup> ,	
	<i>P</i> < 0.001) and the stroma compartment (median > 12.0 cells/mm <sup>2</sup> ,	
	<i>P</i> = 0.049) were associated with longer PFS (Table 2; Fig. 1A and B).	



Q6 **Table 1.** Associations of genes with outcomes by arm using NanoString.

Arm	Gene	Outcome	HR	CI	P
nivo	FADD	OS	3.63	1.32-9.93	0.002
	CLEC4C	OS	3.06	1.16-8.09	0.009
	DNAJC14	OS	2.96	1.13-7.79	0.010
	CREB5	PFS	4.04	1.41-11.56	<0.001
	FADD	PFS	2.79	1.08-7.19	0.007
	IL19	PFS	2.65	1.04-6.75	0.009
nivo+ipi	PIN1	PFS	0.36	0.15-0.90	0.005
	CCL22	OS	4.26	1.16-15.66	0.006
	CD163	OS	0.21	0.06-0.71	0.007
	CXCL10	OS	0.22	0.06-0.74	0.009
	CXCL11	OS	0.22	0.06-0.74	0.009
	IFI27	OS	0.22	0.07-0.76	0.010
	ITGB3	OS	0.17	0.05-0.62	0.002
	MAPK11	OS	4.48	1.20-16.68	0.004
	MAPK8	OS	0.19	0.06-0.67	0.004
	CIR	PFS	0.22	0.07-0.75	0.010
	C1S	PFS	0.21	0.06-0.71	0.007
	CD163	PFS	0.18	0.05-0.64	0.002
	ETS1	PFS	0.19	0.06-0.67	0.004
	FCGR2A	PFS	0.20	0.06-0.69	0.004
	IL15RA	PFS	0.22	0.07-0.75	0.010
	IL32	PFS	0.19	0.06-0.67	0.004
	ITGB3	PFS	0.17	0.05-0.60	0.001
	MAPK8	PFS	0.20	0.06-0.71	0.006
	PRKCD	PFS	0.19	0.05-0.66	0.004
	STAT2	PFS	0.22	0.06-0.74	0.009

Abbreviations: nivo, nivolumab; nivo+ipi, nivolumab plus ipilimumab; OS, overall survival; PFS, progression-free survival.

387 Higher densities of memory/Tregs (CD3+CD8-CD45RO+FOXP3+) 388 in the total compartment (median > 4.6 cells/mm<sup>2</sup>, P = 0.026) were 389 associated with better OS (Table 2; Fig. 1C). In the nivo+ipi arm, higher 390 densities of PD-1+ T cells (CD3+PD-1+) in the total compartment 391 (median > 16.0 cells/mm<sup>2</sup>, P = 0.0347) and the presence of GZM+ 392 CTLs (CD3+CD8+GZB+) in the tumor compartment (>0 cells/mm<sup>2</sup>, 393 P = 0.0154) were associated with longer PFS (Table 2; Fig. 1D and E). 394 Conversely, higher densities of Tregs (CD3+CD8-FOXP3+) in the 395 total compartment (median > 12.4 cells/mm<sup>2</sup>, P = 0.0418) were 396 associated with worse OS (Table 2; Fig. 1F). In the nivo+ipi arm, 397 deconvolution of transcriptomic profiling data by TIMER and nSolver 398 demonstrated significantly higher total immune cells (CD45+), a 399 higher exhausted CD8+ T-cell score, and a higher neutrophil score 400 in responders versus nonresponders (P < 0.05, Fig. 1G-I), further 401 supporting that overall higher immune infiltration is associated with 402 superior clinical benefit from ICI treatment.

**High infiltration of CTLs is associated with exceptional response to ICIs**

Next, we specifically investigated exceptional responders, defined as patients who had no progression for at least 18 months and were still alive by 24 months, versus early progressors, who survived more than 1 month but had progressive disease and died within 6 months after initiating ICI treatment. By these definitions, there were 11 exceptional responders and 44 early progressors across the total trial cohort (Fig. 2A). There were more exceptional responders in the nivo+ipi arm than in the nivo arm (7 of 73 vs. 4 of 87, P = 0.35). Among these patients, 8 exceptional responders and 21 early progressors had tissues available for mIF, and 6 exceptional responders and 8 early progressors had tissues available for gene expression analysis.

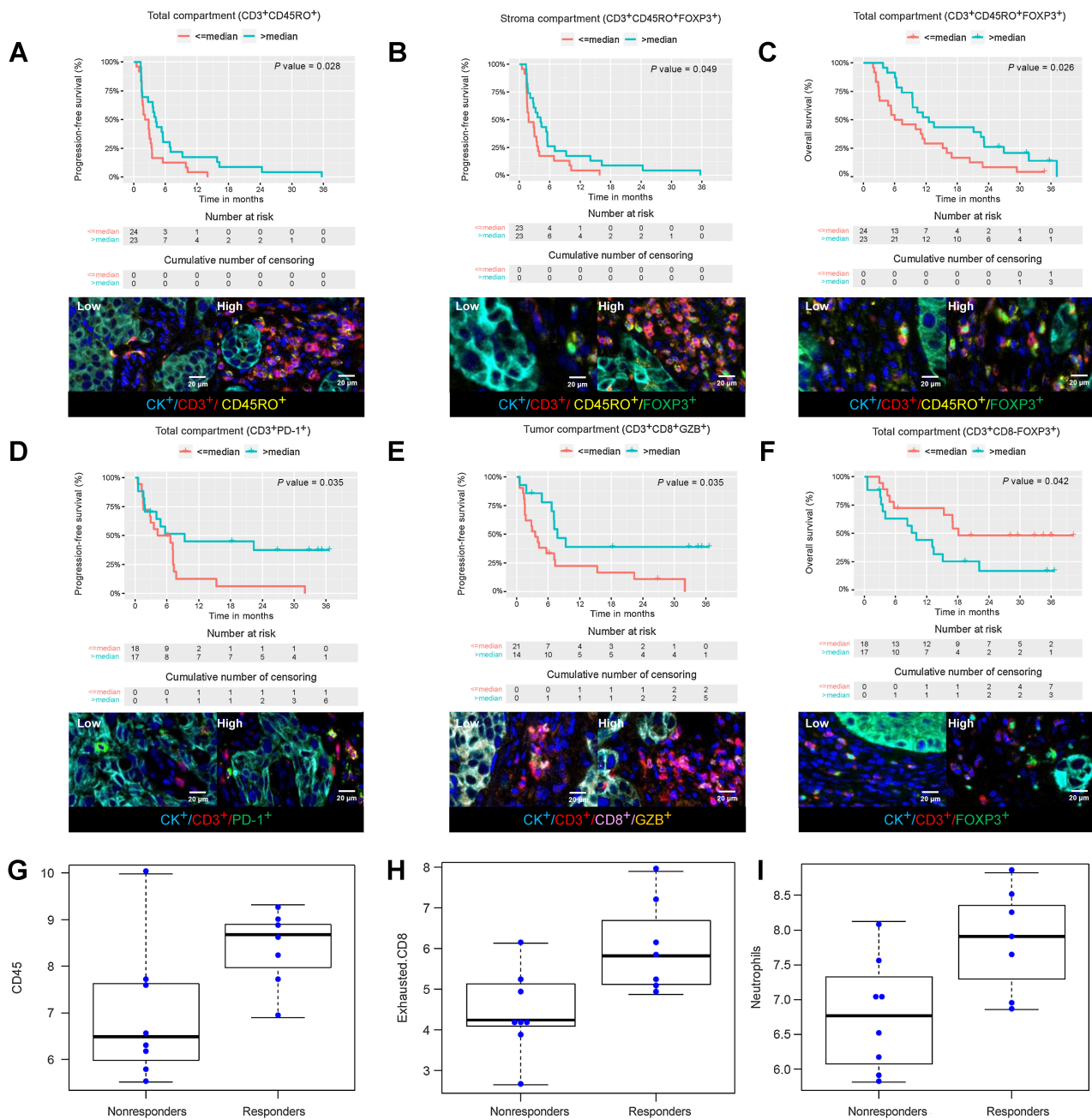
By mIF, we observed higher densities of CTLs (CD3+CD8+) and memory CTLs (CD3+CD8+CD45RO+) in the total compartment in exceptional responders than in early progressors (CTLs: median, 152.1 vs. 27.3 cells/mm<sup>2</sup>; P = 0.032; memory CTLs: median, 31.2 vs. 2.1 cells/mm<sup>2</sup>; P = 0.040; Supplementary Table S7). Representative images from an exceptional responder are shown in Fig. 2B and C. Moreover, in the tumor compartment, we observed higher densities of GZB+ CTLs (CD3+CD8+GZB+) in the exceptional responders than the early progressors (median, 3.6 vs. 0 cells/mm<sup>2</sup>, P = 0.027; Fig. 2D). Representative images from an early progressor showing a lower density of immune infiltration are shown in Fig. 2E and F.

Furthermore, distinctive spatial neighborhoods and cell organization in tumor microenvironment (TME) were observed in exceptional responders (n = 6) relative to early progressors (n = 6; Fig. 2G-J; Supplementary Figs. S2 and S3). Shown in Fig. 2G; Supplementary Figs. 2A and 3A is the distribution of different cell subsets relative to each other, with higher immune infiltration and higher CTL densities in the TME of exceptional responders versus early progressors. We then used spatially varying probabilities of different cell phenotypes to determine the segregation among immune subsets and malignant cells, and a contour plot to represent the neighborhoods within the TMEs. These analyses revealed a higher spatial segregation of immune cell subsets relative to malignant cells in the early progressors as compared with the exceptional responders (Fig. 2H; Supplementary Figs. S2B and S3B) in line with above observation that higher densities of CTLs in the tumor region positively associated with survival. Through distance-based hierarchical clustering, we identified local cell clusters within the TME and observed distinct compositions in exceptional responders versus early progressors. The clusters (cells ≥ 10 within interacting distance 20 μm) in exceptional responders often consisted of CTLs and other T-cell populations in the proximity to tumor cells (Fig. 2I and J; Supplementary Figs. S2C, S2D, S3C, and S3D). Finally, infiltration of neutrophils inferred from gene expression profiling data was significantly higher in exceptional responders than early progressors (P = 0.029; Supplementary Fig. S4).

**Table 2.** Associations between cell phenotypes by compartment and by treatment arm.

Arm	Compartment	Cell phenotype	Outcome	HR	CI	P
nivo+ipi	Tumor	CD3+CD8+GZB+	PFS	0.38	0.18-0.81	0.015
	Total	CD3+CD8-FOXP3+	OS	2.33	0.99-5.51	0.042
		CD3+PD-1+	PFS	0.45	0.21-0.97	0.035
nivo	Stroma	CD3+CD45RO+FOXP3+	PFS	0.58	0.32-1.05	0.049
	Total	CD3+CD45RO+	PFS	0.55	0.31-1.00	0.028
		CD3+CD45RO+FOXP3+	OS	0.52	0.28-0.96	0.026
		CD3+CD45RO+FOXP3+	PFS	0.42	0.23-0.78	<0.001

Abbreviations: nivo, nivolumab; nivo+ipi, nivolumab plus ipilimumab; GZB, granzyme B; Total, tumor plus stroma; PFS, progression-free survival.



**Figure 1.**

Kaplan-Meier survival curves of cellular densities and immune signatures. In the nivo arm, Kaplan-Meier survival curves show high cellular densities (>the median value used as cutoff) of memory T cells (CD3+CD45RO+; **A**) in the total compartment and CD45RO+ Tregs (CD3+CD45RO+FOXP3+; **B**) in the stroma compartment were associated with better PFS. **C**, CD45RO+ Tregs (CD3+CD45RO+FOXP3+) in the total compartment were associated with better OS. Representative multispectral images show low and high cell phenotype densities for **A-C**. In the nivo+ipi arm, the Kaplan-Meier survival curves show that high cellular densities of PD-1+ T cells (CD3+PD-1+; **D**) in the total compartment and GZB+ CTLs (CD3+CD8+GZB+; **E**) in the total compartment were associated with better PFS. Conversely, (**F**) Tregs (CD3+CD8-FOXP3+) in the total compartment were associated with poor OS. Representative multispectral images show low and high cell phenotype densities for **D-F**. Cell scoring derived from gene expression profiling using nSolver shows higher scores for CD45+ immune cells (**G**), CD8+ T cells (**H**), and neutrophils (**I**) in responders compared with nonresponders in the nivo+ipi arm.

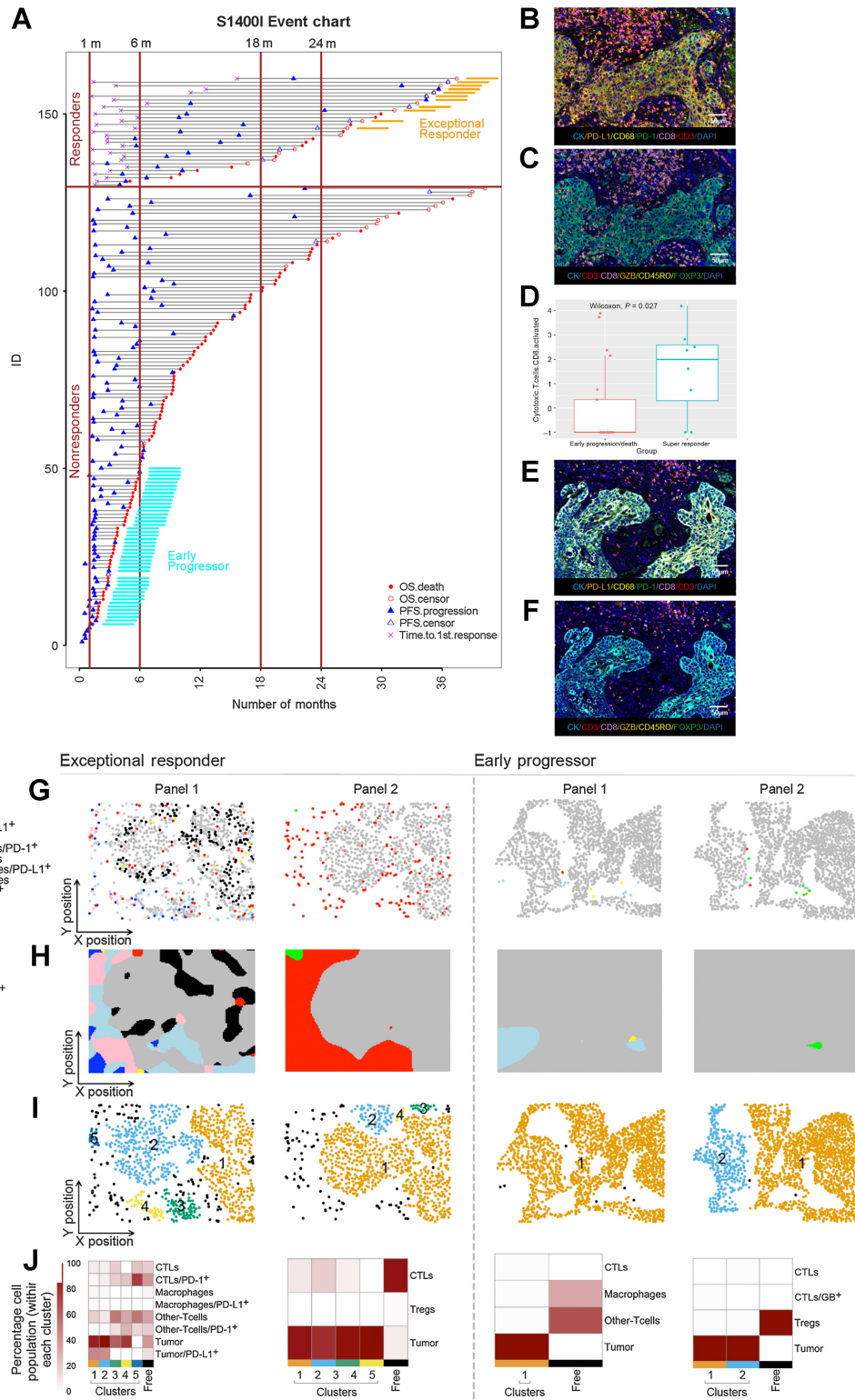
Q7

454 **Close proximity of T cells and malignant cells is associated with**  
 455 **benefit from ICIs**

456 Given the recognized importance of distance between different cells  
 457 and the clustering of CTLs and tumor cells observed in exceptional

responders, we expanded our analysis to understand the spatial  
 relationship between the cell types associated with clinical outcome  
 described above with other cells within the TME (43, 44). In the  
 whole cohort, shorter distances from malignant cells (CK+) as well as

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**Figure 2.** Immune infiltration in exceptional responders and early progression across the arms. **A**, The upper level of the event chart shows the exceptional responders, and the lower level shows the early progression/death group. The solid red circles represent deaths in the OS analysis, the open red circles indicate OS-censored patients, the solid blue triangles indicate progression in the PFS analysis, the open blue open triangles indicate PFS-censored patients, and the violet X indicates the time to the first response. (Continued on the following page.)

465 PD-L1+ malignant cells (CK+PD-L1+) to CTLs (CD3+CD8+; median, 139 and 148  $\mu\text{m}$ ) were associated with better PFS ( $P = 0.045$  and  
466  $P = 0.027$ , respectively); and shorter distances from malignant cells  
467 (CK+) to GZB+ CTLs (CD3+CD8+GZB+) were associated with  
468 significantly longer PFS ( $P = 0.035$ ) and a trend toward longer OS  
469 ( $P = 0.054$ ; **Fig. 3A–E**). In the nivo+ipi arm, shorter distances of CTLs  
470 (CD3+CD8+) as well as GZB+ CTLs (CD3+CD8+GZB+) from  
471 malignant cells (CK+;  $P = 0.045$  and,  $P = 0.026$ , respectively) and  
472 shorter distances between CTLs (CD3+CD8+) from PD-L1+ malignant  
473 cells (CK+PD-L1+;  $P = 0.033$ ) were associated with longer  
474 PFS (**Fig. 3F–H**). In addition, shorter distances of GZB+ CTLs  
475 (CD3+CD8+GZB+; median, 245  $\mu\text{m}$ ) from malignant cells (CK+)  
476 were associated with longer OS ( $P = 0.045$ ; **Fig. 3I**). Taken together,  
477 these results suggest that the immune cells' density and spatial distribution  
478 may impact response to ICI therapy.  
479

### 480 High CNV burden is associated with cold immune infiltration

481 We next performed WES ( $n = 50$ ) with the intent of identifying the  
482 genomic basis underlying the immune features associated with benefit  
483 from nivo+ipi versus nivo in these metastatic squamous cell carcinomas.  
484 A total of 30,081 nonsilent mutations were detected with transversions,  
485 particularly C>A, as the predominant substitutes, which was expected  
486 because most patients were smokers (Supplementary Fig. S5A). The  
487 commonly mutated cancer genes included *TP53*, *LRP1B*, *CDKN2A*,  
488 *ARID1A*, and *PIK3CA* (Supplementary Fig. S5B). High CNV burden  
489 was associated with a colder tumor immune microenvironment, as  
490 evidenced by lower infiltration levels of overall CD3<sup>+</sup> T cells from mIF  
491 and lower levels of various immune signatures derived from gene  
492 expression profiling (Supplementary Fig. S6A and S6B). Importantly,  
493 CNV burden was not associated with estimated tumor purity, suggesting  
494 the correlation between high CNV burden and cold tumor immune  
495 microenvironment was not due to relative high tumor cell density  
496 leading to dilution of immune cells. Taken together, these results indicate  
497 that chromosomal instability may be an underlying genomic feature  
498 associated with immune evasion in metastatic SqNSCLC. Among the  
499 commonly mutated cancer genes, mutations in *LRP1B*, a recently  
500 recognized potential regulator of the inflammatory response, was associated  
501 with less infiltration of GZB+ CTLs (CD3+CD8+GZB+; Supplementary  
502 Fig. S7A; refs. 45, 46). Interestingly, *LRP1B* mutations  
503 were enriched in nonresponders but not in responders (18/19 vs.  
504 2/11,  $P = 0.049$ ). Furthermore, patients with *LRP1B* mutations had  
505 significantly ( $P = 0.008$ ) shorter PFS (Supplementary Fig. S7B) and  
506 numerically shorter OS in the overall cohort (Supplementary Fig. S7C).  
507 *LRP1B*-mutant tumors were not associated with short PFS in the  
508 nivo+ipi arm (Supplementary Fig. S7D) but were in the nivo arm  
509 ( $P = 0.033$ ; Supplementary Fig. S7E).

### 510 Dynamic changes in peripheral blood cytokines are associated 511 with benefit from ICIs

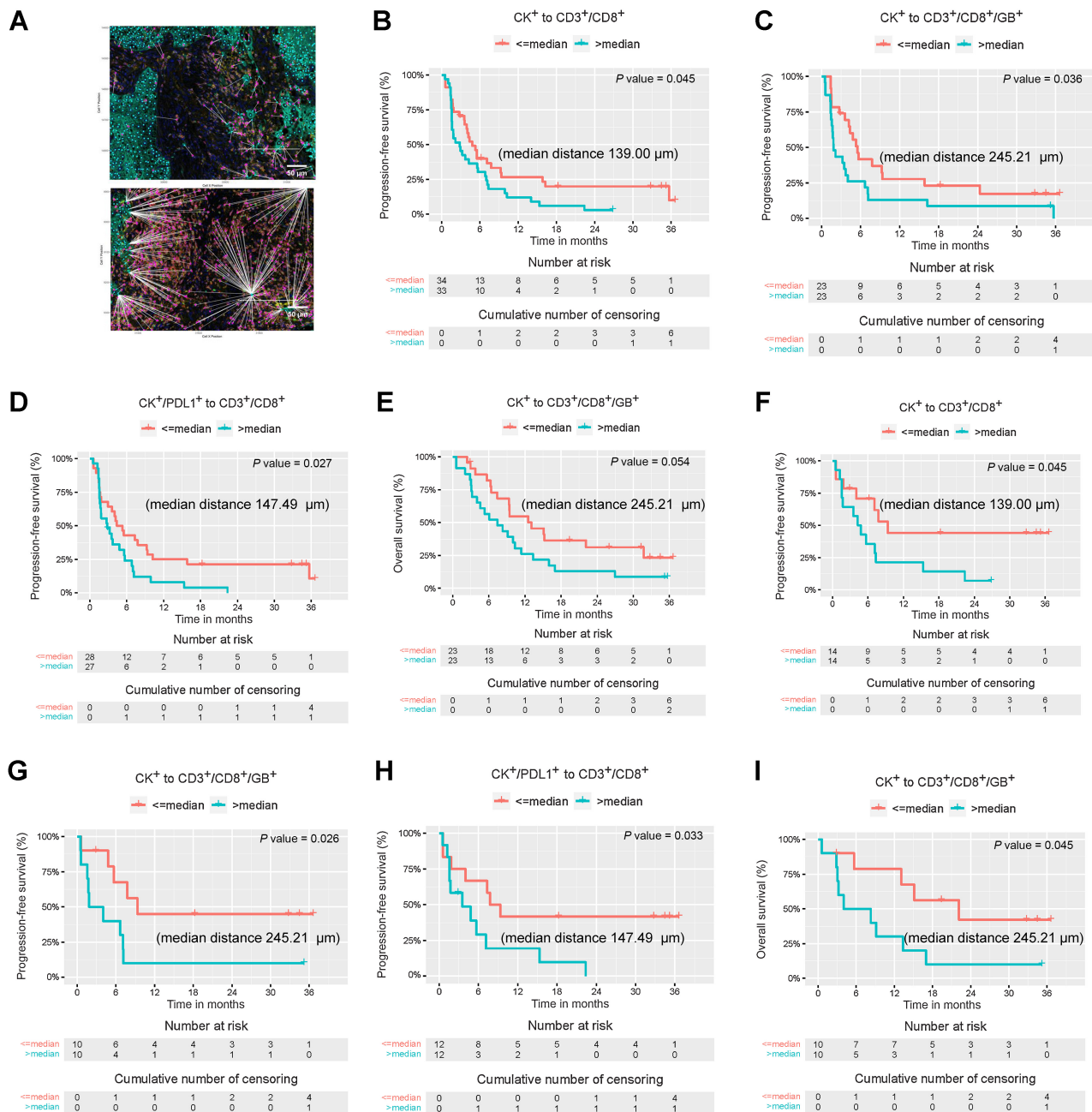
512 Blood-based biomarkers are attractive because they are non-  
513 invasive, dynamic, and less impacted by intratumor heterogeneity

515 than tissue-based markers (47). We performed Olink proximity  
516 extension assay using the immuno-oncology panel assaying a series  
517 of 92 proteins in 561 serum samples collected longitudinally from  
518 160 patients. Using mixed models to account for demographic  
519 and relevant clinical covariates with multiple testing adjustments,  
520 several serum chemokines (CXCL9, CXCL10, CXCL13, CCL19) and  
521 activated T-cell markers (PD-1, IFN $\gamma$ , IL12, IL10) were found  
522 durably increased from baseline with either nivo or nivo+ipi,  
523 (**Fig. 4A and B**) indicating the ICIs' immune regulating effect.  
524 Multiple markers of immune activation and priming (ICOS-L,  
525 LAMP3/DC-LAMP, IL4, IL13, NRC1, CD5) were found increased  
526 at baseline or early during treatment in responders, regardless of  
527 treatment type ( $P < 0.05$ ; **Fig. 4C and D**), in line with associations of  
528 these important immune processes with clinical response to ICI.  
529 Conversely, macrophage-derived and hyperinflammation markers,  
530 such as IL6, IL8, CXCL13, CSF-1, TNFSF14/LIGHT, and CCL23, as  
531 well as likely stromal or tumor-derived markers, such as VEGFA,  
532 HGF, and HO-1, were significantly upregulated in nonresponders  
533 at baseline or after ICI preceding radiologic progression, with  
534 some differences based on treatment received for CXCL13 and  
535 CSF-1 ( $P < 0.05$ ; **Fig. 4C and D**). Joint modeling of survival with  
536 Olink analytes showed an increased risk of death (HR > 1) with  
537 higher longitudinal serum levels of CXCL13, MMP12, CSF-1, and  
538 IL8, which was confirmed with independent Kaplan–Meier analyses  
539 based on median protein levels at baseline (**Fig. 4E**). Similar results  
540 were generally observed in the subset of patients with extreme  
541 outcomes (exceptional responders and early progressors), where  
542 LAMP3/DC-LAMP was higher while CXCL13, CCL23, and  
543 TNFSF14 were lower in exceptional responders at baseline compared  
544 with nonresponders (**Fig. 4F**,  $P < 0.05$ ). Together with the  
545 above-described data, and considering only baseline markers, these  
546 results suggest that an activated T-cell signature (cytotoxic effector  
547 T cells and DC-LAMP) was important for responsiveness to treatment  
548 with either nivo or nivo+ipi, while a hyperinflammatory milieu  
549 (IL6, IL8, CXCL13, CCL23, TNFSF14/LIGHT, CSF-1, MMP12) had an  
550 adverse impact on response and OS.

### 551 Integrative analysis of immune features across different 552 platforms

553 The antitumor immunity and response to ICIs is often determined  
554 at different molecular levels. The multiomics profiling in this study  
555 provided a unique opportunity for integrative analysis to understand  
556 the molecular and immune features associated with ICI benefit. We  
557 first performed recursive partitioning on Olink, mIF, NanoString, and  
558 WES data for classification of responders (Supplementary Fig. S8A and  
559 S8B). We identified that proteins from Olink provide good prediction  
560 on response. However, mIF markers did not contribute significantly in  
561 the decision tree, which might be due to relatively small sample size  
562 for mIF ( $n = 159$  for Olink and  $n = 82$  for mIF). We next created  
563 a decision tree survival prediction model and observed that  
564

(Continued.) Representative multispectral images of panels 1 (**B**) and 2 (**C**) show high levels of inflammatory cells in a sample from an exceptional responder patient. **D**, Box plot shows GZB+ CTLs (CD3+CD8+GZB+) in patients with exceptional response compared with patients with early progression/death. Representative multispectral images of panels 1 (**E**) and 2 (**F**) show reduced immune infiltration in a progression/death patient sample. **G**, The spatial organization of immune and malignant cell phenotypes for the two mIF panels is shown with an example each from exceptional responders and early progressors. The colors for the different subpopulations are indicated under panel phenotype legend (on the left). **H**, For the above images, segregation of different cell phenotypes based on their spatially varying probabilities is shown as a contour plot. The colors of different neighborhoods are same as the panel phenotypes (above). **I**, For the above images, Euclidean distance-based clusters of cells (10 or more) within 20  $\mu\text{m}$  are identified. The clusters are represented by numbers and distinct colors. **J**, The relative percentage composition of cell types within each cluster (above) is indicated in the heat map. The corresponding cluster colors are indicated below the heat map for reference. The color scale representing percentage composition (0–100) is shown on the left.



**Figure 3.**

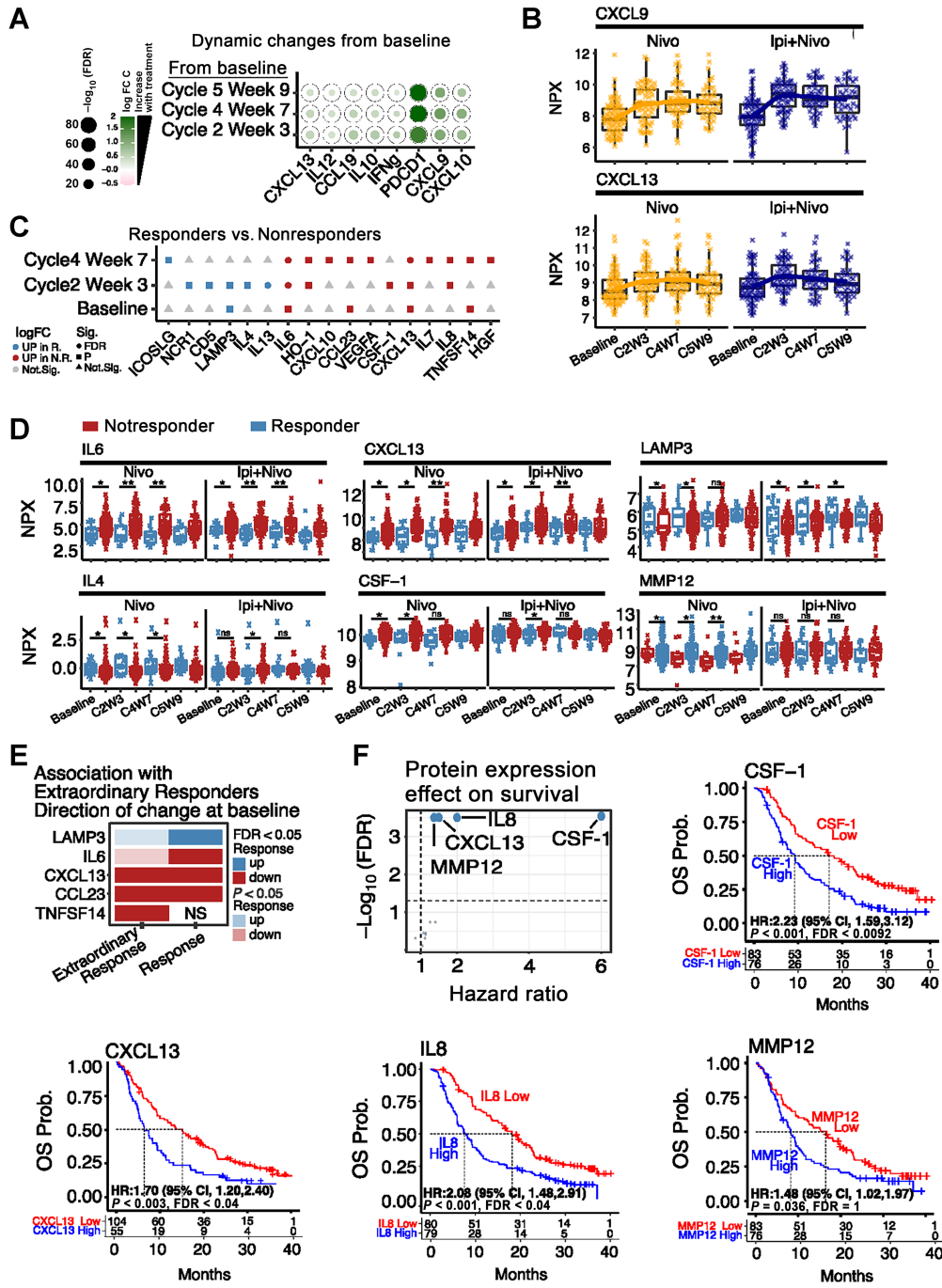
Kaplan-Meier survival curves of nearest neighbor distance from both arms. **A**, Upper and lower image showing proximity map overlay, where cyan dots represent malignant cells (CK+) and red dots represent T cells (CD3+). White lines display distances from all malignant cells (CK+) to neighboring T cells (CD3+). Kaplan-Meier survival curves show that distances (≤the median value used as cutoff) from malignant cells (CK+) to CTLs (CD3+CD8+; **B**) and GZB+ CTLs (CD3+CD8+GZB+; **C**), and malignant cells expressing PD-L1 (CK+PD-L1) to CTLs (CD3+CD8+; **D**) were associated with better PFS when combining both treatment arms. **E**, Kaplan-Meier OS curve for distances from malignant (CK+) to GZB+ CTLs (CD3+CD8+GZB+) in both arms. In the nivo+ipi arm, Kaplan-Meier survival curves show that close distances (≤the median value used as cutoff) from malignant cells (CK+) to CTLs (CD3+CD8+; **F**) and GZB+ CTLs (CD3+CD8+GZB+; **G**), and PD-L1+ malignant cells (CK+PD-L1+) to CTLs (CD3+CD8+; **H**) were associated with better PFS. **I**, Close distances from malignant cells (CK+) to GZB+ CTLs (CD3+CD8+GZB+) was associated with OS.

567 Cytotoxic.T.cells.antigen.experienced (CD3+CD8+PD-1+) together  
 568 with IL6, LAG3 and MICA.B separate patients into sub-populations  
 569 with different survival. Furthermore, we applied random forest  
 570 classifier, which identified Cytotoxic.T.cells.antigen.experienced

(CD3+CD8+PD-1+) and IL6 as important variables. NanoString  
 and WES did not contribute to the association between omics  
 markers and outcomes likely due to insufficient samples with data  
 from these platforms.

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**Figure 4.** Olink serum soluble analyte assessment. **A**, Heat map of dynamic changes in protein expression. The x-axis shows the protein names, while the y-axis shows the comparisons between timepoints and progression. The color represents the logFC. Green represents increase from baseline while pink represents decrease. The size of the circle indicates the statistical significance expressed as  $-\log_{10}(\text{FDR})$ . **B**, Boxplots and median rend lines showing the expression over time by cohort for CXCL9 and CXCL13. **C**, Heat map for differential protein expression between responders and nonresponders. The x-axis shows the protein names, while the y-axis shows each timepoint. The symbol in the heat map represents the statistical significance: circles for  $FDR < 0.05$  or adjusted  $P$  values, squares for  $P < 0.05$ , and triangles for nonsignificant or  $P > 0.05$ . The color represents the change relative to upregulation in responders (blue) or nonresponders (red). **D**, Boxplots corresponding to significant markers in **C** over time, stratified by treatment arm for the indicated proteins. Comparisons for individual baseline, cycle 2, and cycle 4 timepoints are shown for  $P < 0.05$  and  $FDR < 0.05$  with (\*) and (\*\*), respectively. **E**, Heat map showing the concordance in directionally of differentially expressed proteins significant between exceptional responders and all responders. The direction of the protein changes was identical between both groups of responders, but only CXCL13 and CCL23 reached statistical significance (FDR, darker colors) for exceptional responders due the decreased numbers. Nominal significance is shown as transparent colors, indicating proteins with  $P < 0.05$ . **F**, Volcano plot showing the proteins significantly associated with OS when jointly modeling cytokine expression over time. The proteins labeled in blue are associated with increased HR or decreased survival. Kaplan-Meier OS curves for CSF-1, IL8, CXCL13, and MMP12 stratified on the basis of their expression from the average expression (higher values from the mean as blue, lower values from the mean as red).

## Discussion

Identifying novel biomarkers for ICI response is challenging because the molecular determination of TME and host immune response is complex and heterogeneous across different patients. A large sample size to control interpatient heterogeneity and multi-omics to identify the determinates at different molecular levels are ideal but challenging. Therefore, maximizing the use of clinical, pathologic, molecular data and learning from each patient, particularly from clinical trials and careful analysis is key to pave the way to advance our understanding and ultimately the efficacy of ICI.

In this study, we performed mIF, gene expression profiling, WES, and OLINK on the previous samples from S1400I and identified known and novel molecular features associated with nivo versus nivo+ipi combination. Responders demonstrated higher densities of multiple immune cell types defined by mIF. Analysis of CTL populations revealed that GZB+ CTLs (CD3+CD8+GZB+) located in the tumor compartment were associated with better PFS. This was corroborated by analyzing the spatial organization of cell phenotypes, whereas higher immune cell population in the tumor region was seen in the TME of exceptional responders than that of early progressors. On the other hand, higher densities of Tregs (CD3+CD8-FOXP3+) in the total compartment correlated with worse OS in the nivo+ipi arm. This highlights the emerging dichotomy regarding the impact of ICI therapies on Treg subsets and function (48), as these combinations may not modulate some Treg subsets and dominance of CTLs is needed to overcome local immune suppression. Conversely, higher densities of memory T cells (CD3+CD45RO+) and Treg/memory T cells (CD3+CD8-CD45RO+FOXP3+) were associated with better PFS in the nivo arm. As PD-1 targeting has been shown to result in the reactivation of T cells already present within the tumor immune microenvironment, the presence of Treg/memory T cells at baseline may be an essential biomarker to delineate the need for inclusion of ipilimumab as opposed to a nivolumab alone approach. Estimating the immune subsets using TIMER and nSolver software demonstrated that a higher immune presence was associated with improved outcome. These results emphasize that an active immune response within the TME is required for a favorable clinical outcome in this setting, which is supported by multiple findings identifying mechanisms of response to ICI-based therapeutic strategies across various cancer types (49–51).

The TME is composed of various immune cells and stroma cells entangled with cancer cells. In addition to the densities of different cells, the spatial distribution and proximity among various cell types are also essential features with important impact on the functional status of the tumor immune microenvironment (52, 53). mIF data from this study provided an opportunity to assess the spatial relationship of different cellular components within the tumor immune microenvironment and their association with clinical outcomes from ICI treatment. Using the spatial point metrics through the nearest neighbor analysis, we observed that tumors with higher densities of GZB+ CTLs close to malignant cells were associated with better PFS and OS in the nivo+ipi arm, suggesting that cell-to-cell distribution and specially CTLs play an important role in response to ICIs as showed by others studies in NSCLC (54). The organization of cells into clusters based on distance also demonstrated that the CTLs and malignant cells cluster more frequently in exceptional responders than early progressors suggesting a preformed antitumor response that is aided by the ICI.

We used WES to identify genomic features underlying particular immune features and found that a higher CNV burden was associated with a lower level of immune cell infiltration overall. Similarly, CNV

burden was negatively associated with immune scores derived from immune gene expression profiling. These findings are in line with previous findings in different cancer types suggesting that chromosomal instability may be a common genomic alteration underlying immune evasion across human malignancies (53, 55–58). Interestingly, we also found that patients with *LRP1B*-mutant tumors had a reduced survival compared with patients without *LRP1B* mutations. *LRP1B* has been identified as a putative tumor suppressor and is frequently inactivated in NSCLCs (45). Recently, *LRP1B* mutation was reported to be associated with better prognosis in melanoma and NSCLC after anti-PD-1 therapy (46). However, in our cohort, we observed that *LRP1B* mutation was associated with a worse OS and PFS in both the nivo arm and nivo+ipi combination therapy arms. It is still unclear whether the difference was due to different histology (predominantly adenocarcinoma in the previous study vs. exclusively squamous cell carcinoma in the current study) or different ICI (anti-PD-1 vs. anti-PD-1 with/without anti-CTLA-4) or low sample size. Of note, the impact of *LRP1B* mutations on cancer biology and response to ICIs has not been clearly defined in different cancers. For example, a study on renal clear-cell carcinoma reported worse prognosis and suppressive antitumor immunity when *LRP1B* was overexpressed (59), and another found that *LRP1B* mutations were associated with inferior clinical outcomes after ICI treatment in patients with hepatocellular carcinoma (60).

Although tissue-based assays remain the gold standard for molecular profiling for oncology practice, liquid biopsy, particularly peripheral blood-based assays, have gained more attention for molecular profiling and disease monitoring across various cancers because they are noninvasive, “real-time,” and less affected by intratumor heterogeneity (61, 62). In the era of immune-oncology, the Olink soluble protein detection platform has emerged as a promising tool to assess and monitor host immune response. Using Olink, we identified a high level of protumorigenic factors, such as VEGFA and CCL23, and inflammatory markers, such as IL6, IL8, and MMP12, that were associated with inferior survival in this cohort of patients. These findings suggest that general inflammation is detrimental in the context of cancer and ICI therapy. In contrast, proteins involved in T-cell and natural killer cell activation, such as LAMP3/DC-LAMP, IFN $\gamma$ /IL4/IL13, and NRC-1, were associated with improved outcomes after ICI therapy. It was unexpected that a high level of CXCL13 was associated with poor response to ICI therapy and shorter survival, given the recent studies reporting this chemokine working together with DC-LAMP and playing essential roles in the establishment of tertiary lymphoid structures in NSCLC (63). It is possible that the relatively high levels of circulating CXCL13 in the serum do not reflect relatively rare CD4<sup>+</sup> T cell-derived tumor tissue-specific expression of CXCL13, and this emphasizes the limitations of soluble analytes as a surrogate for local tumor events. Some analytes, such as CXCL9/10 and soluble PD-1, were dynamically increased with treatment and marginally associated with outcomes in exceptional responders, in line with previous reports (64). Of particular interest, other markers showed the strongest association with objective response during ICI treatment, for example, lower CSF-1 or IL6 or higher IL13 at cycle 2. This is reminiscent of findings from melanoma studies in which the on-treatment biopsy was more informative for long-term benefit from ICIs than the baseline biopsy, as the actual changes after treatment reflect the host immune system’s response to ICIs more accurately (65, 66). Although soluble analytes are not ideal predictive biomarkers to select an optimal initial treatment regimen, if validated, these markers will be extremely helpful in switching ineffective therapy to effective alternatives to save time and potential toxicity, which is



critically important for patients with stage IV lung cancers, for whom time and quality of life are essential attributes. In addition, these on-treatment markers are also valuable in distinguishing pseudoprogression from real progression—another critical clinical dilemma that the oncologists face in the era of immuno-oncology.

As a *post hoc* profiling of samples from a completed clinical trial, our study has several inherent limitations, including inadequate tumor specimen availability, which precluded us from generating comprehensive data integration from all platforms; imbalanced distribution of samples from the nivo versus nivo+ipi arms or responders versus nonresponders; inadequate tissues for multiomic analysis and cross-platform integrative analyses; and lack of detailed information regarding the time and anatomic sites of tumor specimens, which limited our ability to perform in-depth, organ-specific analysis. In spite of these challenges, integration of peripheral cytokine profiling and cellular profiling within the TME confirmed our single platform findings highlighting the negative association with hyperinflammation with reduced PFS and the presence of PD-1+ CTLs in the TME with increased survival. Finally, while we presented several candidates in this study, we recognize the need for additional validation and replication of our findings. Specifically, several circulating serum proteins, such as IL6, IL8, CSF-1, MMP12, and CXCL13 are promising candidates for future prospective or *post hoc* confirmatory studies due to their ease of collection and quantification from blood. In addition, investigation of tissue composition using spatial profiling technologies to better understand the complex interplay between tumor tissue and immune infiltrating cells may shed light on the mechanisms of immune-tumor cell-cell interactions and identify key biomarkers that can identify patients who will have the most benefit from ICIs. As a proof-of-principle study, using the S1400I trial as an example, we showcased that multi-omics, multi-institutional analyses of patient samples are feasible and can provide valuable insights for future trial development, which is one of the major goals of the CIMAC-CIDC Network.

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