Efficacy, safety, and biomarker analyses of bintrafusp alfa, a bifunctional fusion protein targeting TGF-β and PD-L1, in patients with advanced non-small cell lung cancer

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SUPPLEMENTARY MATERIALS

Section A. Details of the treatment procedures and statistical analyses.

Section B. Details of biomarker methodologies.

List of Supplementary Tables and Figures

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patients who were ICI-naïve and those who were ICI-experienced before treatment with bintrafusp alfa.

Supplementary Figure 2. The peripheral immune profile of patients pre- and on-treatment with bintrafusp alfa associates with clinical response as measured by BOR. Baseline levels (A) and early changes after (B) 1 cycle (day 15 vs. pre), (C) 2 cycles (day 29 vs. pre), and (D) 3 cycles (day 43 vs. pre) of bintrafusp alfa in soluble analytes and complete blood counts that associate with BOR.

Supplementary Figure 3. Representative hematoxylin and eosin showing fibrosis on-treatment with bintrafusp alfa. (A) Pre-treatment biopsy shows a moderately differentiated adenocarcinoma. (B) On-treatment tumor biopsy from the same patient highlights areas of fibrosis and inflammation. H&E images were scanned at 20X resolution.

Supplementary Figure 4. Changes in immune cell profiles in the tumor microenvironment of patients pre- and on-treatment with bintrafusp alfa were grouped by PFS (<3 months vs. >3 months). (A) Each pie chart shows percentages of various immune subsets in biopsies from each patient pre-treatment (designated as A for each patient number) and on-treatment (designated as B for each patient number) with bintrafusp alfa. (B) Representative 20X images of paired biopsy tissues from two patients (#507 and #510) immuno-stained with the multiplex IF-panel.

Section A. Details of the treatment procedures and statistical analysis

Treatment procedures

Premedication with an antihistamine and acetaminophen (administered 30–60 min before each dose of bintrafusp alfa) was optional for all infusions to mitigate potential infusion-related reactions; steroids were not permitted as premedication. Dose reduction was not permitted, and interruption or discontinuation of bintrafusp alfa was allowed if treatment-related adverse events, infusion-related reactions of grade ≥2 severity, or severe or life-threatening adverse events (AEs) occurred.

Tumor response was assessed by radiographic imaging using RECIST version 1.1 6 weeks after starting treatment, then every 6 weeks for the first year and every 12 weeks thereafter. Response was confirmed by repeated radiographic assessment 4 weeks or longer from the first documented response. To evaluate the safety of bintrafusp alfa, AEs were monitored

throughout treatment and assessed using CTCAE version 4.03 at 28 days after the last study dose, at 10 weeks post-treatment, and every 12 weeks thereafter.

Statistical analysis

The uncertainty of the estimates was assessed by calculating a 95% exact (Clopper–Pearson) confidence interval. The disease control rate was defined as the proportion of patients with a confirmed best overall response (BOR) of complete response (CR), partial response (PR), stable disease (SD), or non-CR/nonprogressive disease. The duration of response was analyzed using Kaplan–Meier analysis as were progression-free survival (PFS) and overall survival. Safety and pharmacokinetic parameters were analyzed using descriptive statistics. PK parameters after the first dose, including concentration after the end of infusion (C_{eoi}), area under the serum concentration–time curve, clearance, and half-life, were derived via noncompartment analysis. Furthermore, C_{eoi} and C_{trough} were obtained throughout the treatment period (reported as geometric mean with geometric coefficient variation percentage).

Section B. Details of biomarker methodologies

Programmed death ligand 1 tumor cell expression was determined using different immunohistochemistry assays, such as antibody clone 73-10, E1L3N, 22C3 (DAKO FDA-approved pharmDx^{TM} protocol using the DAKO automated link 48 platform) [1-3], VENTANA PD-L1 (SP263) [4], and clone SP142 from Abcam (catalog ab228463) using the Leica Bond Max platform. The peripheral immunome of patients (n = 14) with non-small cell lung cancer enrolled at the National Cancer Institute (NCI) was analyzed before treatment with bintrafusp alfa and at 2 (after 1 cycle), 4 (after 2 cycles), 6 (after 3 cycles), and 12 (after 6 cycles) weeks after therapy initiation, where samples were available. Patients were analyzed for levels and

changes in (a) multiple serum and plasma soluble factors, including analytes reflecting immune stimulatory and inhibitory status, (b) complete blood counts, and (c) 158 immune cell subsets. For the analyses of peripheral immune parameters with clinical response, patients with a PFS of >3 months (n = 8) were compared with those with a PFS of <3 months (n = 6). Analyses were also performed by comparing patients with a BOR of CR or PR (n = 3) with those with a BOR of SD (n = 9) or progressive disease (PD) (n = 2). Analyses were also performed by comparing the peripheral immune profile of immune checkpoint inhibitor (ICI)-naïve patients (n = 6) with that of ICI-experienced patients (n = 8) and of patients with a history of heavy smoking (n = 10) with that of nonsmokers (n = 4).

Complete blood counts with differential analysis were performed at the NCI's Center for Cancer Research, and the neutrophil-to-lymphocyte ratio was subsequently calculated. For serum assays, blood was collected in serum separator tubes, centrifuged, and stored at −80°C before analysis. For plasma assays, blood was collected in ethylenediaminetetraacetic acid tubes, centrifuged, and stored at −80°C before analysis. For the analysis of peripheral blood mononuclear cells (PBMCs), blood was collected in sodium heparin tubes, and PBMCs were isolated after Ficoll–Hypaque density gradient separation. Cells were cryopreserved in 90% heat-inactivated human AB serum and 10% dimethyl sulfoxide at a concentration of 1×10^7 cells/mL before analysis.

The serum levels of IL-8, sCD27, sCD40L, and sPD-L1 and plasma levels of sPD-1, sCD73, TGF-β1, and Granzyme B were analyzed using commercially available kits according to the manufacturers' instructions. IL-8 was measured using AlphaLISA (PerkinElmer, Waltham, MA, USA); sCD27 and sCD40L were measured using instant enzyme-linked immunosorbent assay (ELISA) kits (Life Technologies, Carlsbad, CA, USA); sPD-1, sPD-L1, and sCD73 were

measured using ELISA kits from Abcam (Cambridge, UK); and TGF-β1 and Granzyme B were measured using ELISA kits from R&D Systems (Minneapolis, MN, USA). Plasma samples were also analyzed using Olink Target 96 Immuno-Oncology panel for biomarker discovery (Olink, Watertown, MA, USA).

Cryopreserved PBMCs collected before and after bintrafusp alfa therapy were examined via multicolor flow cytometry using 30 markers in four panels to identify 158 peripheral immune cell subsets[5] following methods previously described.[6,7] The subsets evaluated included 10 parental cell types ($CD4^+$ and $CD8^+$ T cells, regulatory T cells, NK cells, NKT cells, conventional dendritic cells, plasmacytoid dendritic cells, B cells, myeloid-derived suppressor cells, and monocytes) and 148 refined subsets related to the maturation/function of the parental cell types. Flow cytometry files were acquired on an LSR Fortessa equipped with five lasers and analyzed using FlowJo v.9.9.6 for Macintosh, with nonviable cells excluded and negative gates based on fluorescence-minus-one controls. The frequency of all subsets was calculated as a percentage of PBMCs to eliminate any bias that might occur in the smaller populations with fluctuations in parental leukocyte populations.

Multiplex immunofluorescence staining and multispectral imaging: Formalin-fixed paraffin-embedded 5-µm sections from tumor biopsies were immunostained using Opal multiplex 6-plex kits according to the manufacturer's protocol (Akoya Biosciences) for a panel of DAPI, CD4, CD8, FOXP3, CD56, CD68, and CD163. Deparaffinization, rehydration, epitope retrieval, and staining of slides were performed using Leica BOND RX Autostainer (Leica). The optimum staining conditions for each antibody were determined using IHC and single immunofluorescence before combination. Details regarding the antibodies, protocols, and opals used in this panel are described in Supplementary Table 1. Hematoxylin and eosin and multiplex

immunofluorescence whole-slide scans were captured using Vectra Polaris (PerkinElmer) at 20x magnification. Selected regions of interest (ROI) were scanned at 40x magnification for multispectral (MS) imaging. MS images were unmixed and analyzed using InForm, version 2.5 (Akoya Biosciences). Using training ROI images, an algorithm was built and applied for cell segmentation and phenotyping. All immune cell infiltrates were measured as cell counts/mm² of tissue (density).

T-cell receptor (TCR) sequencing: TCR sequencing of biopsy tissue obtained before and during treatment (~7 weeks after treatment initiation) was performed using the Adaptive Biotechnologies immunoSEQ assay where adequate samples were available (n = 9). Eight samples were fresh-frozen cores; one was formalin-fixed, paraffin-embedded tissue. TCR-seq was also performed on the corresponding PBMCs. The number of clones comprising the top 25% of the T-cell repertoire were analyzed, and their associations with the BOR were investigated.

Supplementary references:

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166 **Supplementary Table 1:** Details of the antibodies, protocols, and Opal kits used

Antibody	Clone	Catalog#	Company	Dilution	ER (Leica)	Secondary antibody (Vector labs)	OPAL (Akoya Biosciences)
CD68	D4B9C	76437	Cell signaling	1:400	ER1	Immpress Mouse/Rabbit	520 (FP1487001KT)
CD4	EPR6855	ab133616	Abcam	1:250	ER1	Immpress Mouse/Rabbit	570 (FP1488001KT)
CD8	EPR10640	ab215041	Abcam	1:100	ER1	Immpress Mouse/Rabbit	540 (FP1494001KT)
FOXP3	SP97	MA5-16365	Thermo-Fisher	1:200	ER2	Immpress Mouse/Rabbit	620 (FP1495001KT)
CD163	EPR19518	ab182422	Abcam	1:200	ER2	Immpress Mouse/Rabbit	650 (FP1496001KT)
CD56	MRQ-42	156R-94	Sigma-Aldrich	1:200	ER2	Immpress Mouse/Rabbit	690 (FP1497001KT)

167 CD4/8/56/68/163; cluster of differentiation 4/8/56/68/163; FOXP3, forkhead box P3

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Supplementary Table 2. Baseline and disease characteristics in biomarker-evaluable patients

	Biomarker-evaluable cohort	
Characteristics, n (%)	**ICI-naïve (n = 6)**	**ICI-experienced (n = 8)**
Sex		
Male	2 (33.3)	5 (62.5)
Female	4 (66.7)	3 (37.5)
Age		
Median (range), years	60.5 (50–73)	58.5 (52-73)
<65 years	4 (66.7)	5 (62.5)
≥65 years	2 (33.3)	3 (37.5)
ECOG performance status		
0	1 (16.7)	0
1	5 (83.3)	8 (100.0)
Tumor cell PD-L1 expression[a]		
<1%	2 (33.0)	1 (12.5)
1-49%	1 (16.7)	1 (12.5)
50-75%	0	1 (12.5)
>75%	1(16.7)	1 (12.5)
Not available	2 (34.0)	4 (50.0)
***EGFR* mutation status**[b]		
Wild type	4 (66.6)	6 (85.7)
Mutated	1 (16.7)	1 (14.3)
Not available	1 (16.7)	0
Tumor histology		
Adenocarcinoma	5 (83.3)	6 (75.0)
Squamous cell carcinoma	0	1 (12.5)
Other	1 (16.7)	1 (12.5)
Number of prior anticancer regimens		
0	3 (50.0)	0
1	1 (16.7)	0
2	0	5 (62.5)
3	0	2 (25.0)
≥4	2 (33.3)	1 (12.5)
Type of prior anticancer therapy for metastatic or locally advanced disease		
Anti-PD-(L)1	0	7 (87.5)
Cytotoxic therapy	2 (33.3)	5 (62.5)
Endocrine therapy	0	0
Monoclonal antibody therapy	0	3 (37.5)
Small molecules	2 (33.3)	1 (12.5)
Immunotherapy other than anti-PD-(L)1	1 (16.7)	1 (12.5)
Other	0	0

ECOG, Eastern Cooperative Oncology Group; ICI, immune checkpoint inhibitor; PD-L1, programmed cell death ligand 1.
Data are presented as n (%) or median (range)
[a] PD-L1 immunohistochemistry data were obtained using one of the following clones: 73-10, clone E1L3N, clone SP263, and clone 22C3.
[b]The percentage is calculated based on the number of subjects with nonsquamous histology.

Supplementary Table 3: Baseline patient PD-L1 expression based on various cut-off points in non-biomarker evaluable patients

Tumour cell PD-L1 expression[a,b]	**ICI-naïve (n = 12)**	**ICI-experienced (n = 15)**
≥10%	3 (25.0)	5 (33.3)
<10%	7 (58.3)	9 (60.0)
Not available	2 (16.7)	1 (6.7)
≥20%	3 (25.0)	5 (33.3)
<20%	7 (58.3)	9 (60.0)
Not available	2 (16.7)	1 (6.7)
≥50%	2 (16.7)	4 (26.7)
<50%	8 (66.7)	10 (66.7)
Not available	2 (16.7)	1 (6.7)
≥80%	1 (8.3)	2 (13.3)
<80%	9 (75.0)	12 (80.0)
Not available	2 (16.7)	1 (6.7)

ICI, immune checkpoint inhibitor; PD-L1, programmed cell death ligand 1; TPS, tumor proportion score.

[a]For 27 patients the calculated scorings for TPS ≥10%, ≥20%, ≥50% and ≥80% are based on a continuous reading of the TPS for 1+, 2+ and 3+ tumor cells. A TPS has been calculated by summing up the TPS scores for 1+, 2+ and 3+ tumor cells. Based on the calculated TPS score the samples were classified as negative/positive for TPS ≥10%, ≥20%, ≥50% and ≥80% based on the respective cut-point.

[b]PD-L1 immunohistochemistry data were obtained using the clone 73-10.

Supplementary Table 4. Efficacy according to RECIST 1.1 as assessed by the investigator

	ICI-naïve (n = 18)	ICI-experienced (n = 23)
Confirmed BOR		
Complete response (CR)	1 (5.6)	0
Partial response (PR)	4 (22.2)	0
Stable disease (SD)	5 (27.8)	5 (21.7)[a]
Progressive disease (PD)	4 (22.2)	13 (56.5)[b]
Not evaluable	4 (22.2)	5 (21.7)
Overall response rate (CR+PR), (95% CI)	5 (27.8) (9.7–53.5)	0 (0.0–14.8)
Disease control rate (95% CI)	10 (55.6) (30.8–78.5)	5 (21.7)[a] (7.5–43.7)
Median progression-free survival, months (95% CI)	2.8 (1.4–6.5)	1.4 (1.3–1.4)
Median duration of response, months (95% CI)	16.8 (2.8–NE)	NE (NE–NE)

BOR, best overall response; CI, confidence interval; ICI, immune checkpoint inhibitor; IRC, independent review committee; NE, not estimable; RECIST, Response Evaluation Criteria in Solid Tumors

Data are presented as n (%) and n (%; 95% CI), unless specified otherwise.

[a]Among the five patients with SD, three had stable disease at the initial tumor evaluation in week 6, which changed to progressive disease in the subsequent evaluation. The sum of the longest diameter at the initial tumor evaluation showed an increase of 19.9%, 19.5%, and 19.7% for these patients compared with the baseline measurements.

[b]Two cases of radiological pseudoprogression showed progressive disease at the first restaging, and on subsequent scans, they showed stable disease or partial response.

Supplementary Table 5. Patients reporting adverse events of special interest

	ICI-naïve (n = 18)		ICI-experienced (n = 23)	
	Any grade, n (%)	**Grade ≥3, n (%)**	**Any grade, n (%)**	**Grade ≥3, n (%)**
Immune-related AESI	3 (16.7)	0	4 (17.4)	1 (4.3)*
Immune-related endocrinopathies: adrenal insufficiency	1 (5.6)	0	1 (4.3)	1 (4.3)*
Adrenal insufficiency	1 (5.6)	0	1 (4.3)	1 (4.3)*
Immune-related rash	2 (11.1)	0	2 (8.7)	0
Lichen planus	1 (5.6)	0	-	-
Pruritus	2 (11.1)	0	1 (4.3)	0
Rash	-	-	1 (4.3)	0
Immune-related endocrinopathies: thyroid disorders	-	-	1 (4.3)	0
Hypothyroidism	-	-	1 (4.3)	0
Skin AESI	4 (22.2)	-	5 (21.7)	-
Keratoacanthoma	3 (16.7)	-	3 (13.0)	-
Squamous cell carcinoma of skin	1 (5.6)	-	0	-
Hyperkeratosis	0	-	1 (4.3)	-
Actinic keratosis	0	-	1 (4.3)	-

*Also reported as grade ≥4 events.

AESI, adverse events of special interest; ICI, immune checkpoint inhibitor

Supplementary Table 6. Changes in the peripheral immune profile of patients after one, three, and six cycles of therapy

A

Soluble Analyte	Change	Median D1	Median D15	P value	% with ≥25% increase	% with ≥25% decrease	Median D1	Median D43	P value	% with ≥25% increase	% with ≥25% decrease	Median D1	Median D85	P value	% with ≥25% increase	% with ≥25% decrease
sCD27	↑	62.3	77.6	0.068	**50**	0	62.3	86.8	0.077	50	8	62.4	95.4	**0.020**	**78**	0
sCD27/ sCD40L	↑	5.1	6.3	**0.042**	**57**	14	4.5	5.8	0.151	50	8	4.4	8.3	0.055	**78**	11
TGFβ	↓	68247	1673	**<0.001**	**0**	**100**	63510	1385	**0.001**	0	**100**	71714	1444	**0.002**	0	**100**
CCL17	↑	11.27	11.88	**0.049**	**57**	7										

B

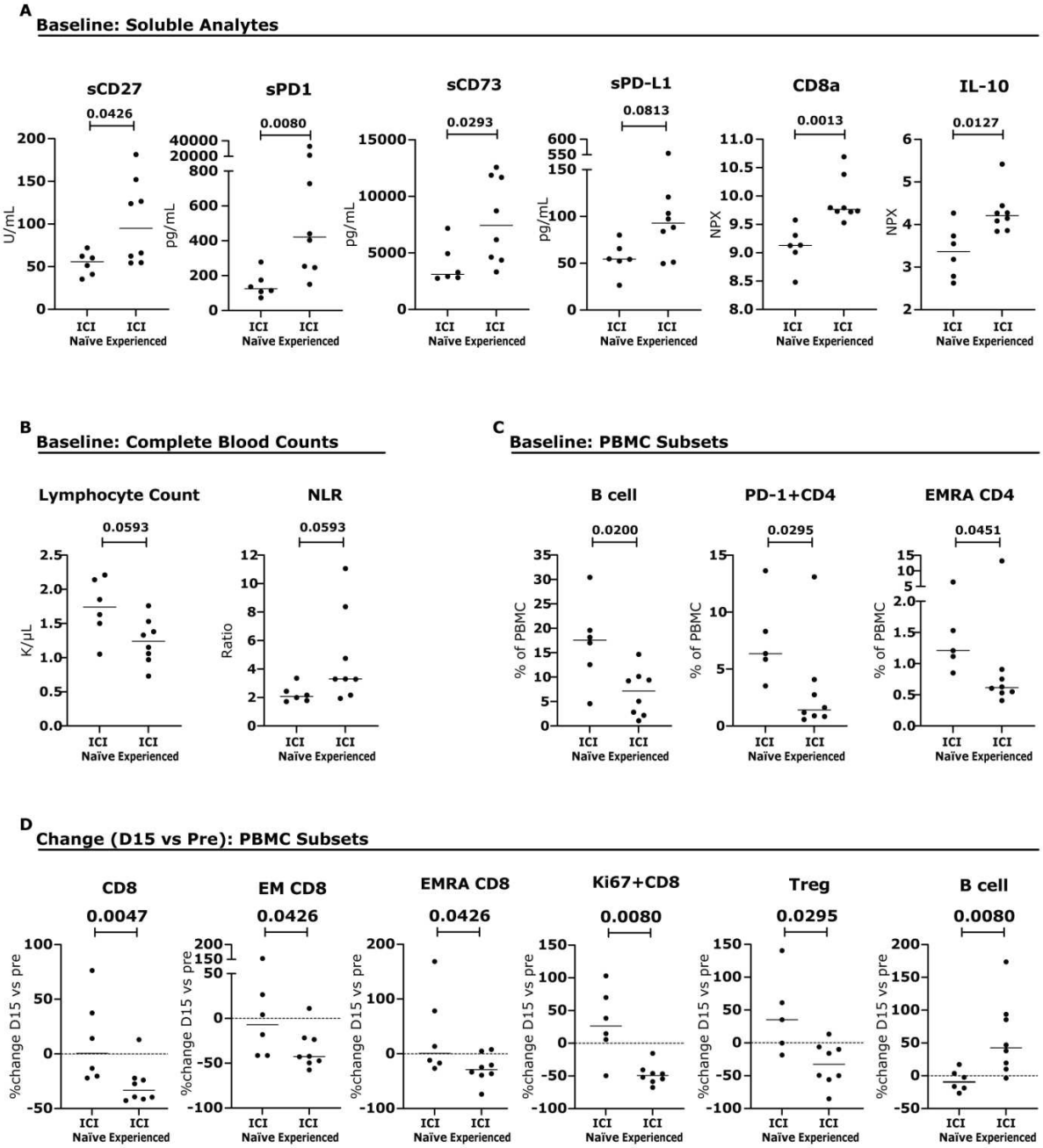
Complete Blood Count	Change	Median D1	Median D15	P value	% with ≥25% increase	% with ≥25% decrease	Median D1	Median D43	P value	% with ≥25% increase	% with ≥25% de-crease	Median D1	Median D85	P value	% with ≥25% increase	% with ≥25% decrease
White Blood	↑	7.2	8.4	**0.006**	**57**	0	7.2	7.6	0.080	31	0	7.2	6.7	0.123	**55**	9
Neutrophil	↑	4.5	5.9	**0.002**	**57**	0	4.4	4.9	0.092	46	8	3.8	4.5	0.206	**55**	9

C

PBMC Subset	Change	Median D1	Median D15	P value	% with ≥25% increase	% with ≥25% decrease	Median D1	Median D43	P value	% with ≥25% increase	% with ≥25% de-crease	Median D1	Median D85	P value	% with ≥25% increase	% with ≥25% decrease
CD8	↓	11.5	9.4	0.049^	14	36	11.3	7.5	**0.021**	8	**67**	13.1	6.3	**0.049**	10	**70**
NK-T	↓	1.7	0.9	**0.025**	7	**64**	1.7	1.1	**0.021**	8	**67**	1.7	1.0	0.193	20	**50**
B cells	↑	9.8	11.3	0.268	36	7	11.4	15.4	**0.021**	**58**	0	11.4	13.7	0.131	**50**	10
cDC	↑	0.3	0.4	0.217	64	14	0.3	0.5	**0.001**	**75**	0	0.3	0.4	**0.020**	**70**	10
pDC	↑	0.10	0.13	**0.030**	**57**	14	0.10	0.12	**0.021**	**75**	0	0.1	0.1	0.275	**60**	30
MDSC	↑	7.8	8.9	**0.030**	**64**	7	6.7	5.6	0.733	42	33	7.8	12.0	0.084	**50**	10
Monocytes	↑	22.0	26.8	0.035^	43	0	18.7	23.2	0.052	33	0	22.0	28.8	**0.049**	**50**	0
EM CDB	↓	4.07	3.05	**0.030**	14	**50**	3.46	2.18	**0.005**	8	**92**	4.07	1.69	0.065	10	**80**
Ki67+ CD8	↓	0.26	0.17	**0.020**	21	**57**	0.26	0.10	**0.021**	17	**67**	0.30	0.18	0.301	33	**67**

Changes in (A) soluble analytes, (B) complete blood counts, and (C) PBMC immune subsets before and during treatment 15, 43, and 85 days after the initiation of bintrafusp alfa. Changes in immune parameters between two timepoints were calculated using Wilcoxon signed-rank test. Units shown in A are U/mL for sCD27, pg/mL for TGFβ, and NPX for CCL17. Units shown are K/μL in B and % of PBMC in C. ^Despite significant *p*-values, most patients did not exhibit a >25% change. CD, cluster of differentiation; CCL17, CC motif chemokine ligand 17; cDC, conventional dendritic cells; D, days; EM, effector memory; MDSC, myeloid-derived suppressor cells; NKT, natural killer T cells; NPX, normalized protein expression; PBMC, peripheral blood mononuclear cells; pDC, plasmacytoid dendritic cells; TGF-β, transforming growth factor-beta; sCD27/sCD40L, soluble CD27/soluble CD40 ligand.

Supplementary Figure 1. Differences in the peripheral immune profile of patients who were ICI-naïve vs. ICI-experienced. Baseline levels of (A) soluble analytes (measured via enzyme-linked immunosorbent and Olink assays), (B) complete blood counts, and (C) PBMC immune subsets (measured via flow cytometry) that were different between patients who were ICI-naïve (n = 6) and those who were ICI-experienced (n = 8) before treatment with bintrafusp alfa. (D) Changes in PBMC immune subsets after one cycle of therapy (D15) vs. baseline that were different between patients who were ICI-naïve and those who were ICI-experienced before treatment with bintrafusp alfa. Graphs display the median frequency of analytes, and *p*-values were calculated using Mann–Whitney test. B cell, bursa-derived cells; CD, cluster of differentiation; D, day; EM, effector memory; EMRA, terminally differentiated effector memory; ICI, immune checkpoint inhibitor; IL-10, interleukin-10; NPX, normalized protein expression; NLR, neutrophil-to-lymphocyte ratio; PBMC, peripheral blood mononuclear cell; sPD-1/sPD-L1, soluble programmed death-1/ligand-1; Treg, regulatory T cells.


A
Baseline: Soluble Analytes
sCD27
0.0426
U/mL
sPD1
0.0080
pg/mL
sCD73
0.0293
pg/mL
sPD-L1
0.0813
pg/mL
CD8a
0.0013
NPX
IL-10
0.0127
NPX
ICI Naïve
ICI Experienced
B
Baseline: Complete Blood Counts
Lymphocyte Count
0.0593
K/µL
NLR
0.0593
Ratio
C
Baseline: PBMC Subsets
B cell
0.0200
% of PBMC
PD-1+CD4
0.0295
% of PBMC
EMRA CD4
0.0451
% of PBMC
D
Change (D15 vs Pre): PBMC Subsets
CD8
0.0047
%change D15 vs pre
EM CD8
0.0426
%change D15 vs pre
EMRA CD8
0.0426
%change D15 vs pre
Ki67+CD8
0.0080
%change D15 vs pre
Treg
0.0295
%change D15 vs pre
B cell
0.0080
%change D15 vs pre

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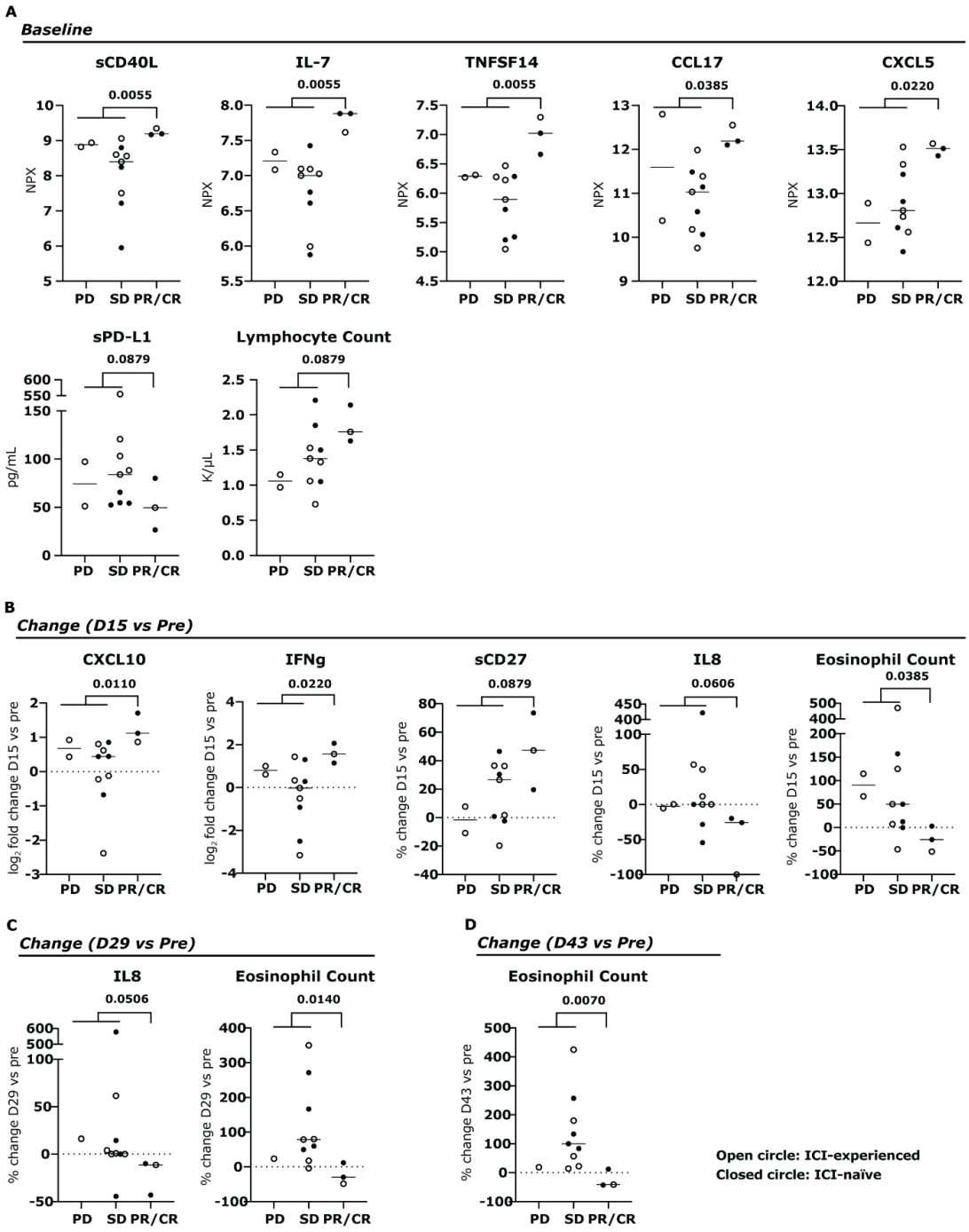
Supplementary Figure 2. The peripheral immune profile of patients pre- and on-treatment with bintrafusp alfa associates with clinical response as measured by BOR. Baseline levels (A) and early changes after (B) 1 cycle (day 15 vs. pre), (C) 2 cycles (day 29 vs. pre), and (D) 3 cycles (day 43 vs. pre) of bintrafusp alfa in soluble analytes and complete blood counts that associate with BOR. Patients with BOR of CR or PR (n = 3) were compared to those with a BOR of PD (n = 2) and SD (n = 9). Graphs display median frequency of analytes and *p* values were calculated using the Mann-Whitney test. BOR, best overall response; CCL17, C-C motif ligand 17; CD, cluster of differentiation; CR, complete response; CXCL5/10, C-X-C motif chemokine ligand 5/10; D, day; IFN-γ, interferon-gamma; IL-7/8, interleukin 7/8; NPX, normalized protein expression; PD, progressive disease; PR, partial response; SD, stable disease; s, soluble; sPD-1/sPD-L1, soluble programmed death-1/ligand-1; TNFSF14, tumor necrosis factor superfamily member 14.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Baseline
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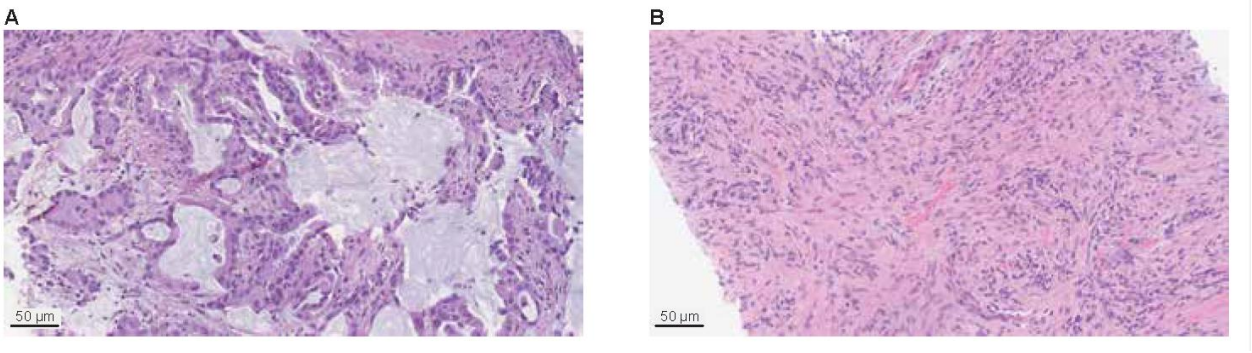
IL-7
0.0055
TNFSF14
0.0055
CCL17
0.0385
CXCL5
0.0220
NPX
PD SD PR/CR
sPD-L1
0.0879
pg/mL
Lymphocyte Count
0.0879
K/µL
B
Change (D15 vs Pre)
CXCL10
0.0110
IFNg
0.0220
sCD27
0.0879
IL8
0.0606
Eosinophil Count
0.0385
log2 fold change D15 vs pre
% change D15 vs pre
C
Change (D29 vs Pre)
IL8
0.0506
Eosinophil Count
0.0140
% change D29 vs pre
D
Change (D43 vs Pre)
Eosinophil Count
0.0070
% change D43 vs pre
Open circle: ICI-experienced
Closed circle: ICI-naïve

294

295

296 **Supplementary Figure 3.** Representative hematoxylin and eosin showing fibrosis on-treatment
297 with bintrafusp alfa. (A) Pre-treatment biopsy shows a moderately differentiated
298 adenocarcinoma. (B) On-treatment tumor biopsy from the same patient highlights areas of
299 fibrosis and inflammation. H&E images were scanned at 20X resolution. H & E, hematoxylin
300 and eosin.

301



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Supplementary Figure 4. Changes in immune cell profiles in the tumor microenvironment of patients pre- and on-treatment with bintrafusp alfa were grouped by PFS (<3 months vs. >3 months). (A) Each pie chart shows percentages of various immune subsets in biopsies from each patient pre-treatment (designated as A for each patient number) and on-treatment (designated as B for each patient number). (B) Representative 20X images of paired biopsy tissues from two patients (#507 and #510) immuno-stained with the multiplex IF-panel (CD4, CD8, CD68, CD56, CD163, FOXP3 and DAPI) are shown. CD, cluster of differentiation 4/8/56/68/163; DAPI, 4',6-diamidino-2-phenylindole; FOXP3, forkhead box P3; M1, inflammatory macrophages; M2, tumor-trophic macrophages; PFS, progression-free survival; IF-panel, immunofluorescence-panel.