Supplementary Materials for

Tumor MHC Class I Expression Associates with Interleukin-2 Response in Melanoma

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Supplementary Figures and Legends: S1 to S15
Supplementary Tables and Legends: S1-S3, S5, S12, S16
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Other Supplementary Materials for this manuscript include the following:

Supplementary Tables: S4, S6-S11, S13-S15, S17-S19 (.xlsx)

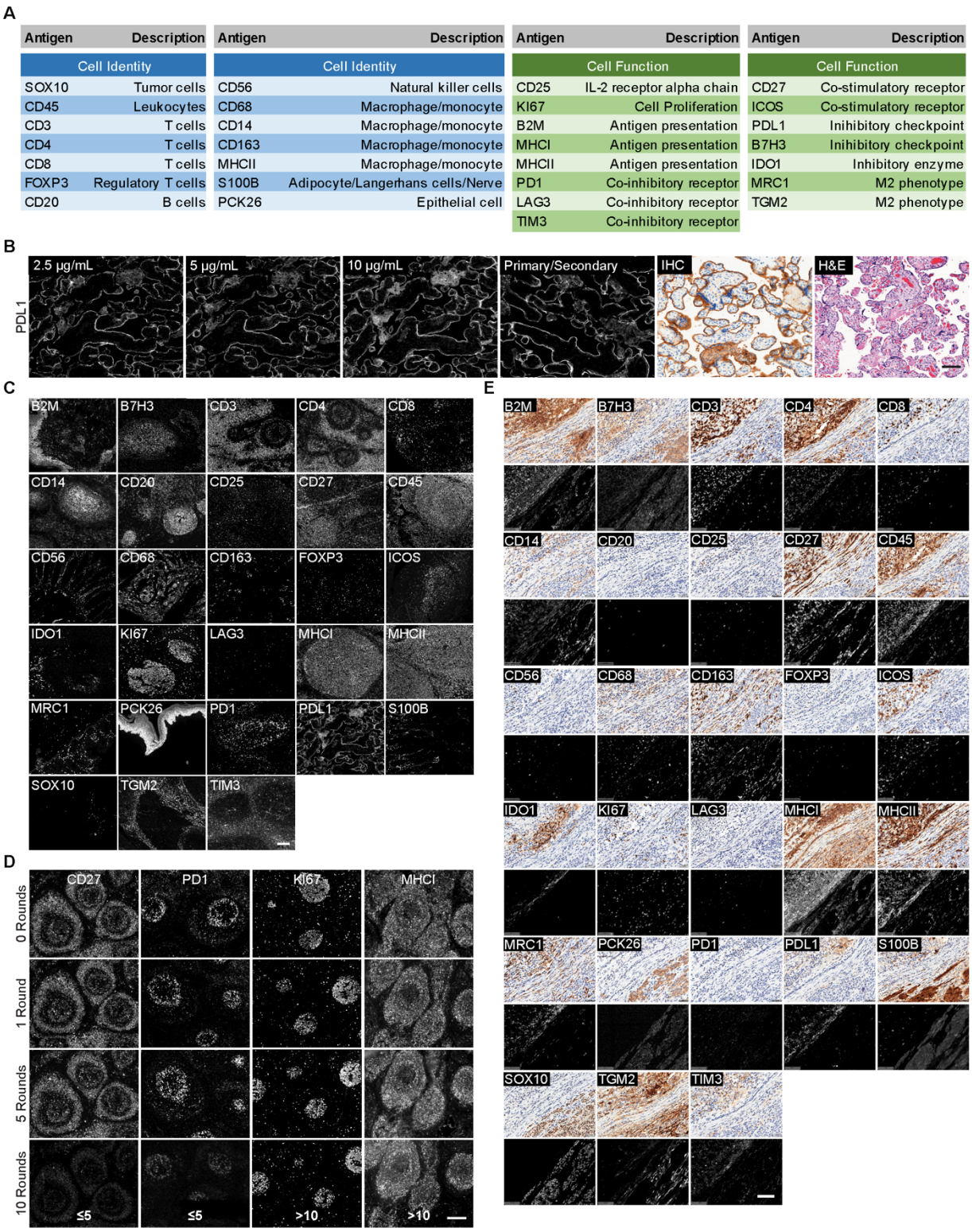
A

Antigen	Description
Cell Identity	
SOX10	Tumor cells
CD45	Leukocytes
CD3	T cells
CD4	T cells
CD8	T cells
FOXP3	Regulatory T cells
CD20	B cells

Antigen	Description
Cell Identity	
CD56	Natural killer cells
CD68	Macrophage/monocyte
CD14	Macrophage/monocyte
CD163	Macrophage/monocyte
MHCII	Macrophage/monocyte
S100B	Adipocyte/Langerhans cells/Nerve
PCK26	Epithelial cell

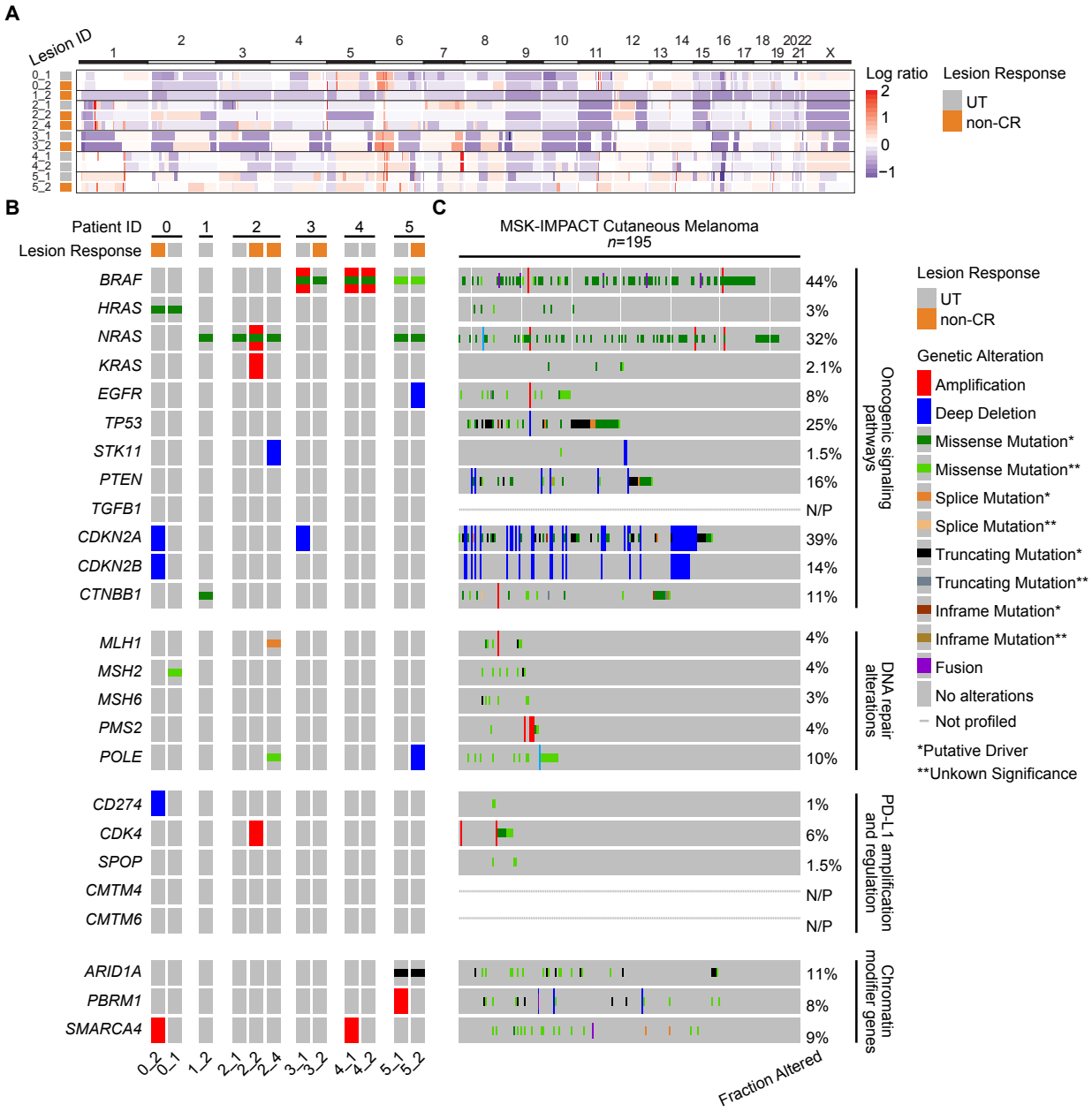
Antigen	Description
Cell Function	
CD25	IL-2 receptor alpha chain
KI67	Cell Proliferation
B2M	Antigen presentation
MHCI	Antigen presentation
MHCII	Antigen presentation
PD1	Co-inhibitory receptor
LAG3	Co-inhibitory receptor
TIM3	Co-inhibitory receptor

Antigen	Description
Cell Function	
CD27	Co-stimulatory receptor
ICOS	Co-stimulatory receptor
PDL1	Inhibitory checkpoint
B7H3	Inhibitory checkpoint
IDO1	Inhibitory enzyme
MRC1	M2 phenotype
TGM2	M2 phenotype

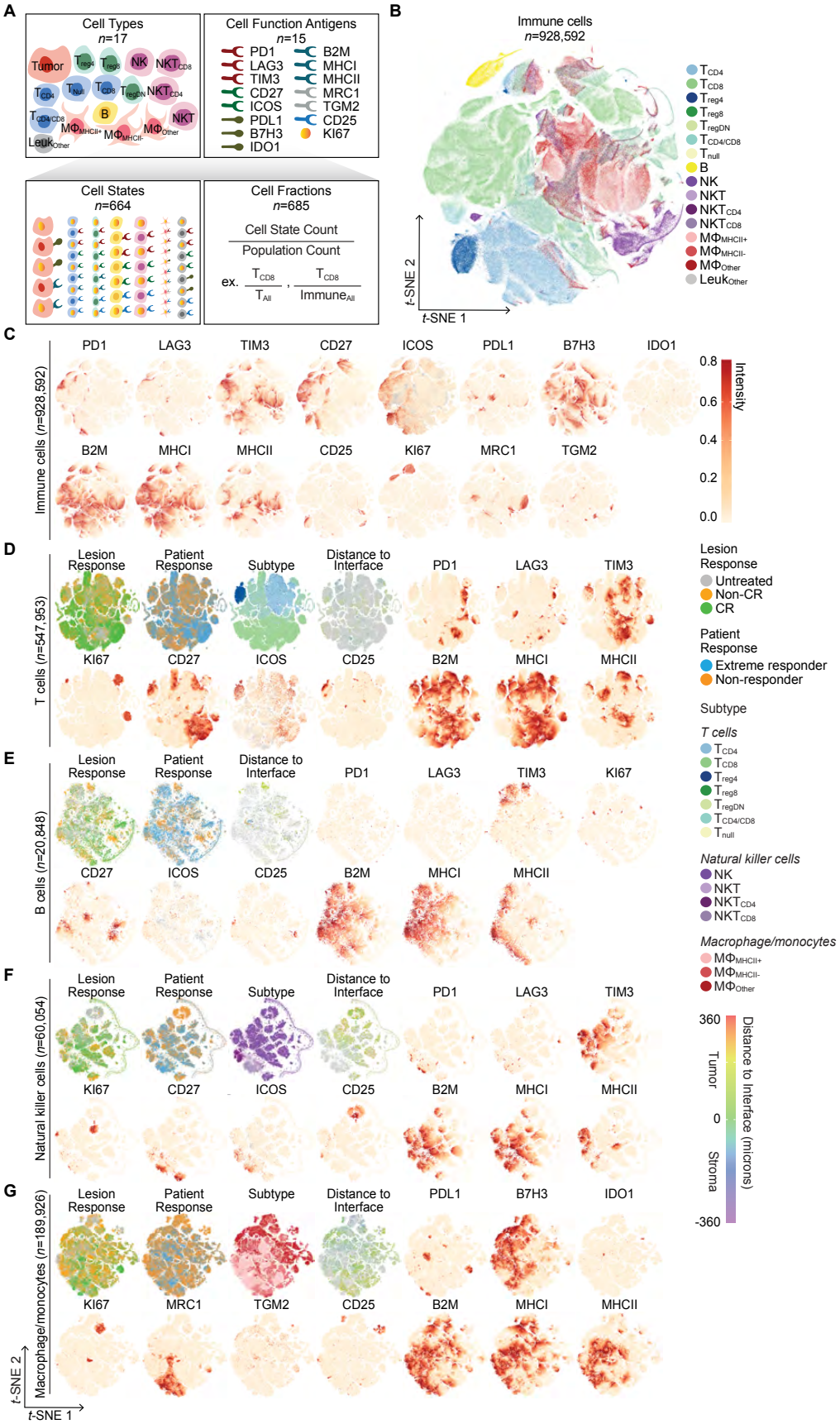


Supplementary Fig. S1. Selection of antibody panel and validation of multiplexed immunofluorescence staining. (**A**) Antibody panel. MHC-II was used as both a cell identity and cell function marker. See also Supplementary Table S2. (**B**) Example of validation process

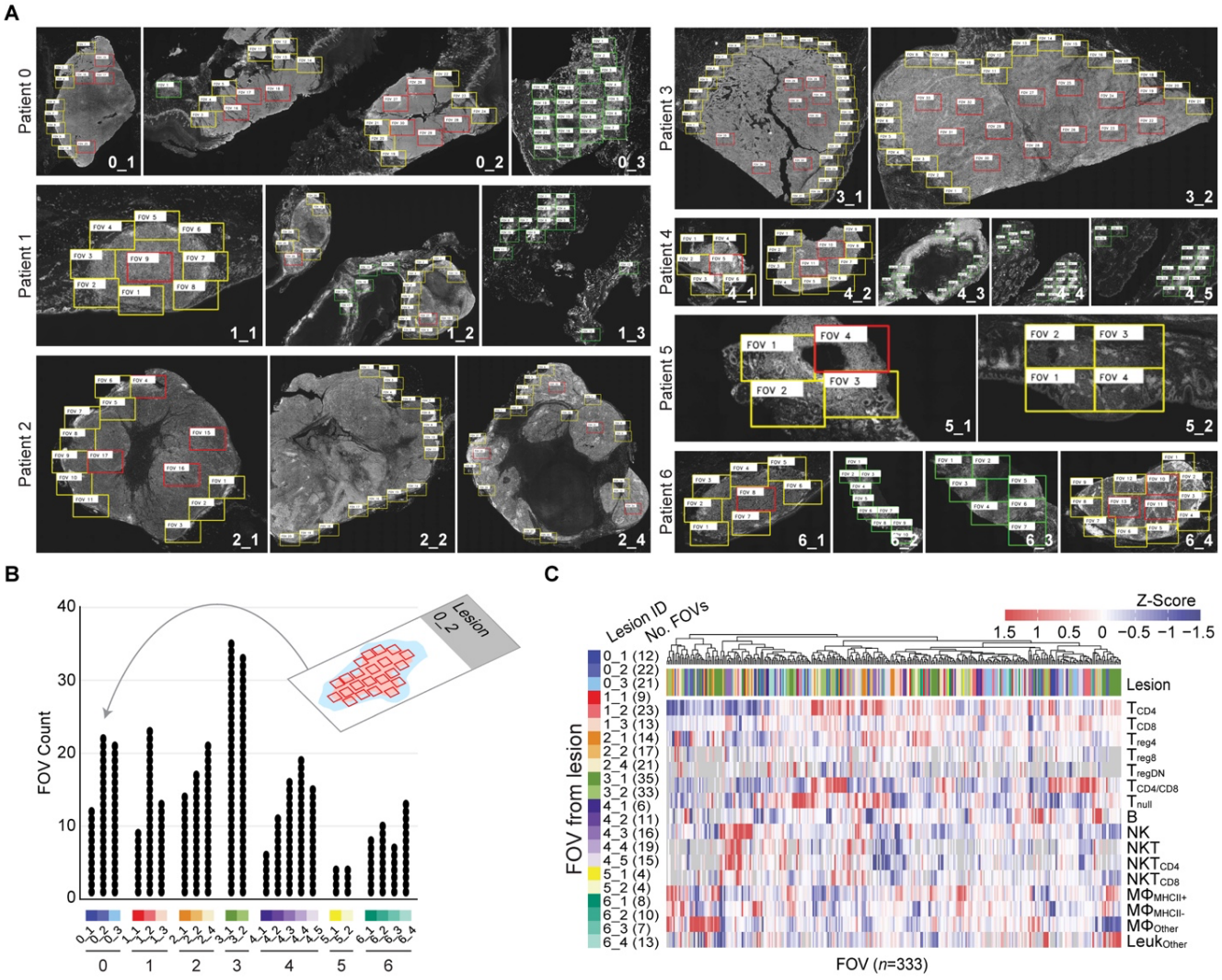
shown for PD-L1 on normal human placenta. Representative images showing 3 dilutions of PD-L1 Cy5 direct conjugate, PD-L1 primary with an anti-rabbit IgG Alexa Fluor 647 secondary, PD-L1 immunohistochemistry (IHC), and hematoxylin and eosin (H&E) stained on adjacent tissue sections. (**C**) Representative images showing the optimal staining concentration for each direct conjugate on appropriate normal human controls. Skin: B2M, CD68, CD163, MRC1, PCK26 (panCK), SOX-10. Lymph node: CD3, CD4, CD8, CD20, CD27, CD45, KI-67. Tonsil: B7-H3, CD14, CD25, FOXP3, ICOS, IDO-1 LAG-3, MHC-I, MHC-II, PD-1, TGM2, TIM-3. Colon: CD56, S100B. Placenta: PD-L1. (**D**) Representative images showing the validation process for epitope stability to dye inactivation for CD27, PD-1, KI-67, and MHC-I on normal human lymph node. (**E**) Representative images showing images acquired using multiplexed immunofluorescence (Cell Dive) and IHC for lesion 3_2. All scale bars are 100-microns.



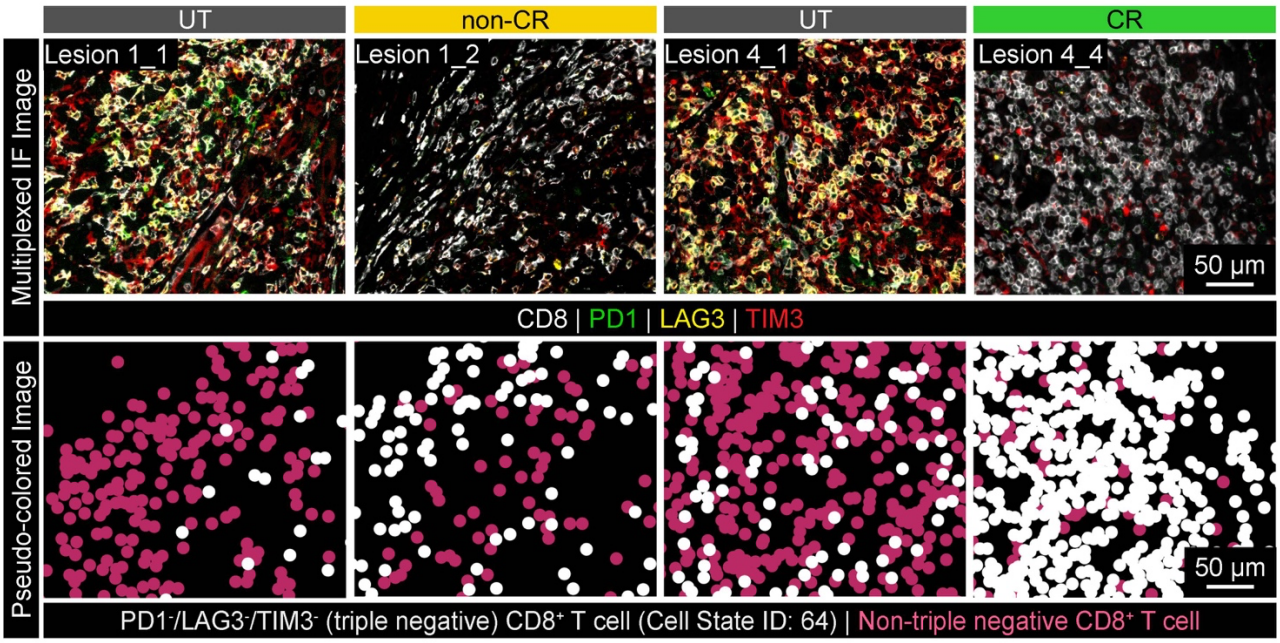
Supplementary Fig. S2. Genomic profile of in-transit melanoma metastases. (**A**) Genome-wide view of gene copy number alterations for the indicated untreated (UT) and non-complete response (non-CR) lesions ordered by lesion ID. Regions of copy gains and loss are indicated in red and blue, respectively. Numbers refer to chromosomal location. (**B**) Genetic alterations in the untreated and non-CR lesions in the cohort ordered by temporality and (**C**) in the MSK-IMPACT cutaneous melanoma cohort. Fraction of lesions altered for each gene in the MSK-IMPACT cohort is noted. N/P, not profiled.


A
Cell Types
n=17
Tumor
Treg4
Treg8
NK
NKTCD8
TCD4
TNull
TCD8
TregDN
NKTCD4
B
TCD4/CD8
NKT
MΦMHCII+
MΦMHCII-
MΦOther
LeukOther
Cell Function Antigens
n=15
PD1
LAG3
TIM3
CD27
ICOS
PDL1
B7H3
IDO1
B2M
MHCI
MHCII
MRC1
TGM2
CD25
KI67
Cell States
n=664
Cell Fractions
n=685
Cell State Count
Population Count
ex. TCD8 / TAll , TCD8 / ImmuneAll
B
Immune cells
n=928,592
t-SNE 2
t-SNE 1
TCD4
TCD8
Treg4
Treg8
TregDN
TCD4/CD8
TNull
B
NK
NKT
NKTCD4
NKTCD8
MΦMHCII+
MΦMHCII-
MΦOther
LeukOther
C
Immune cells (n=928,592)
PD1
LAG3
TIM3
CD27
ICOS
PDL1
B7H3
IDO1
B2M
MHCI
MHCII
CD25
KI67
MRC1
TGM2
Intensity
0.8
0.6
0.4
0.2
0.0
D
T cells (n=547,953)
Lesion Response
Patient Response
Subtype
Distance to Interface
PD1
LAG3
TIM3
KI67
CD27
ICOS
CD25
B2M
MHCI
MHCII
Lesion Response
Untreated
Non-CR
CR
Patient Response
Extreme responder
Non-responder
Subtype
T cells
TCD4
TCD8
Treg4
Treg8
TregDN
TCD4/CD8
TNull
Natural killer cells
NK
NKT
NKTCD4
NKTCD8
Macrophage/monocytes
MΦMHCII+
MΦMHCII-
MΦOther
E
B cells (n=20,848)
Lesion Response
Patient Response
Distance to Interface
PD1
LAG3
TIM3
KI67
CD27
ICOS
CD25
B2M
MHCI
MHCII
F
Natural killer cells (n=60,054)
Lesion Response
Patient Response
Subtype
Distance to Interface
PD1
LAG3
TIM3
KI67
CD27
ICOS
CD25
B2M
MHCI
MHCII
360
Tumor
0
Stroma
-360
Distance to Interface (microns)
G
Macrophage/monocytes (n=189,926)
Lesion Response
Patient Response
Subtype
Distance to Interface
PDL1
B7H3
IDO1
KI67
MRC1
TGM2
CD25
B2M
MHCI
MHCII
t-SNE 2
t-SNE 1

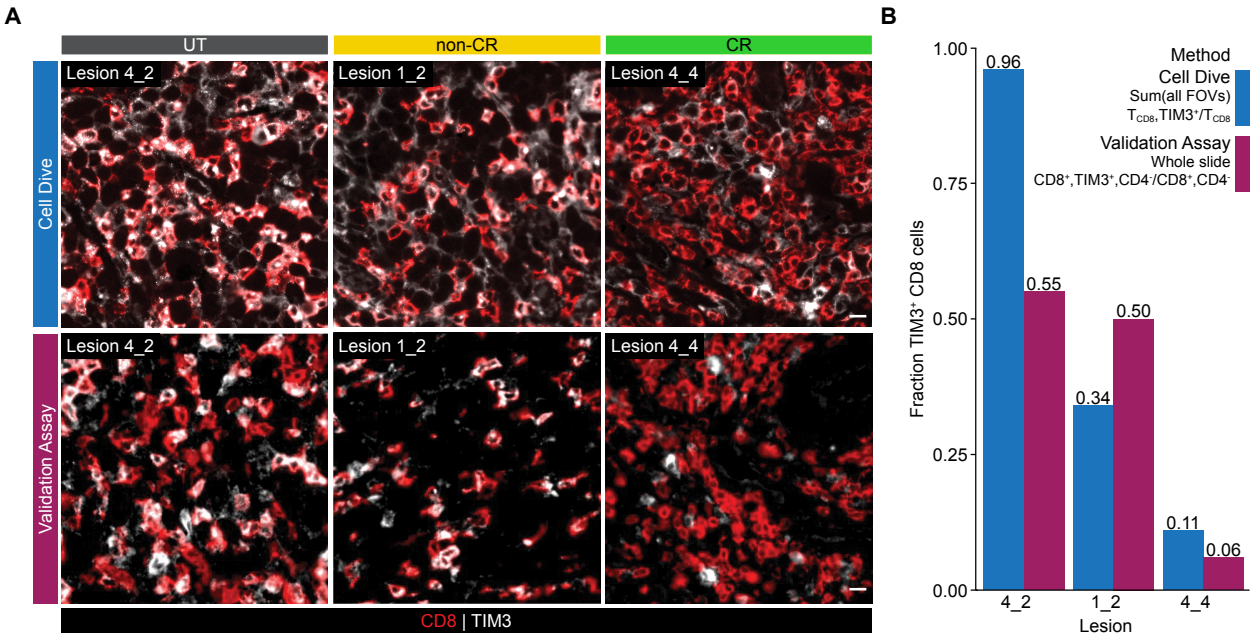
Supplementary Fig. S3. Phenotypic data for T cells, B cells, NK cells, and myeloid cells. (**A**) Single-cell proteomic analysis approach. Derivation of 664 distinct cell states quantified as 685 distinct cell fractions using combinations of cell types and cell function markers. See Supplementary Table S3 for full cell type names. (**B**) Identification of 16 different immune cell types using single-cell proteomics. *t*-distributed stochastic neighbor embedding (*t*-SNE) of all immune cells from 22 lesions colored by cell type. (**C**) *t*-SNE of immune cells colored by normalized intensity of each cell function marker. (**D**) *t*-SNE of all T cells, (**E**) B cells, (**F**) natural killer cells, and (**G**) macrophage/monocytes from 22 lesions colored by the normalized intensity of each cell function marker, lesion response (non-complete response, non-CR; complete response, CR), patient response, subtype, and distance from the interface. Grey cells indicate cells with missing information (ICOS) and cells that are not within 360-microns of the tumor interface (distance to interface).



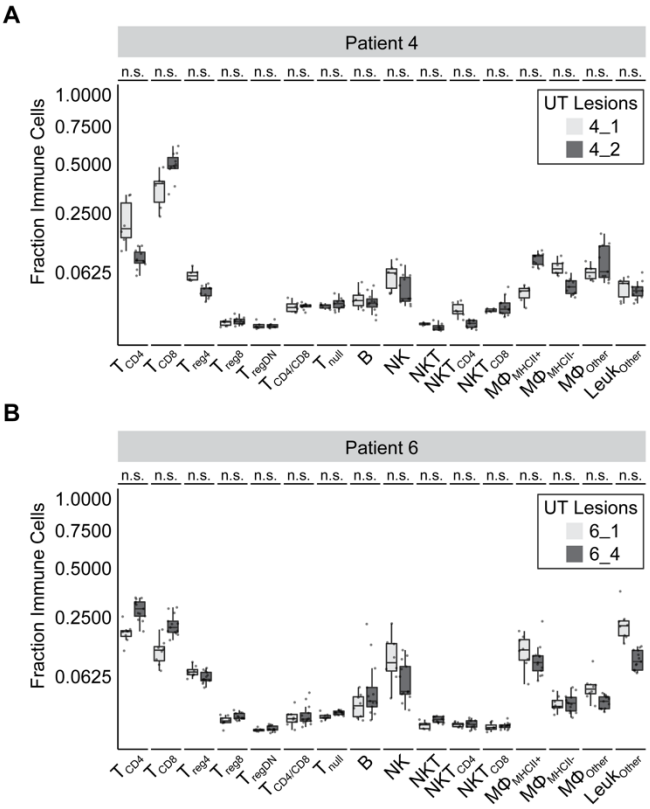
Supplementary Fig. S4. Intra-tumoral heterogeneity in immune cell composition. (**A**) Location of all fields of view (FOVs) in all lesions (*n*=22). Interface FOVs (yellow) cover approximately 50% tumor and 50% stroma, center FOVs (red) cover 100% tumor, and regressed FOVs (green) cover regions of regressed tumor. See also Supplementary Table S5. (**B**) Number of FOVs examined per lesion. (**C**) Intratumoral heterogeneity in immune cell infiltrates. Shown is hierarchical clustering of immune cell fractions over total immune cells in each FOV (*n*=333). FOVs belonging to the same lesion are indicated in the same color (column annotations). For cell type abbreviations, see Supplementary Table S3.



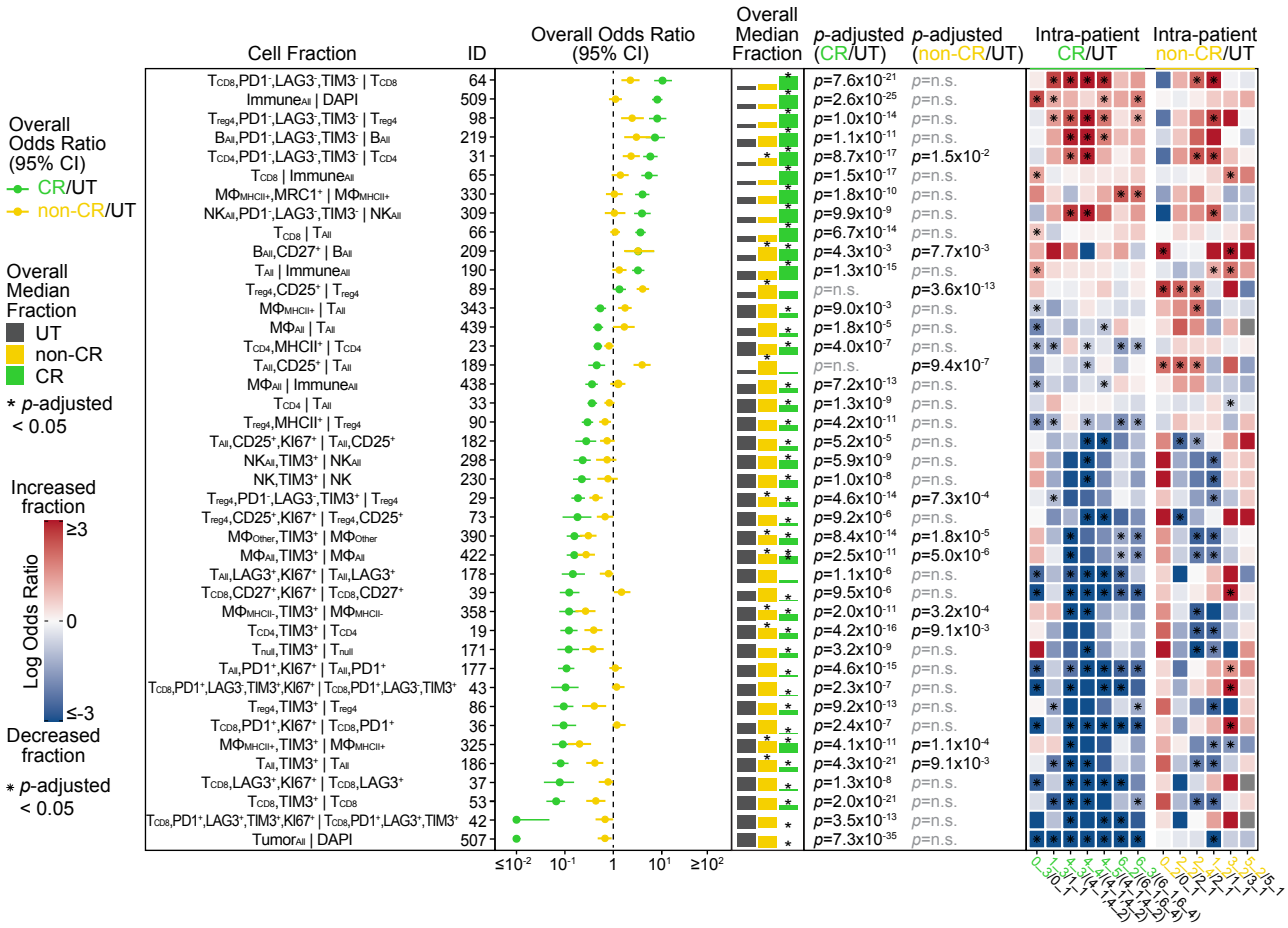
Supplementary Fig. S5. Increase in triple negative PD-1/LAG-3/TIM-3 CD8$^+$ T cells in lesions that completely regress following intralesional interleukin-2 injection. Representative multiplexed immunofluorescence (IF) and pseudo-colored images for untreated (UT) lesion 1_1 field of view (FOV) 7, non-complete response (non-CR) lesion 1_2 FOV 25, UT lesion 4_1 FOV 4, and CR lesion 4_4 FOV 3. The fraction of triple negative PD-1/LAG-3/TIM-3 CD8$^+$ T cells over total CD8$^+$ T cells in each FOV are 0.110, 0.636, 0.249, and 0.777, respectively.



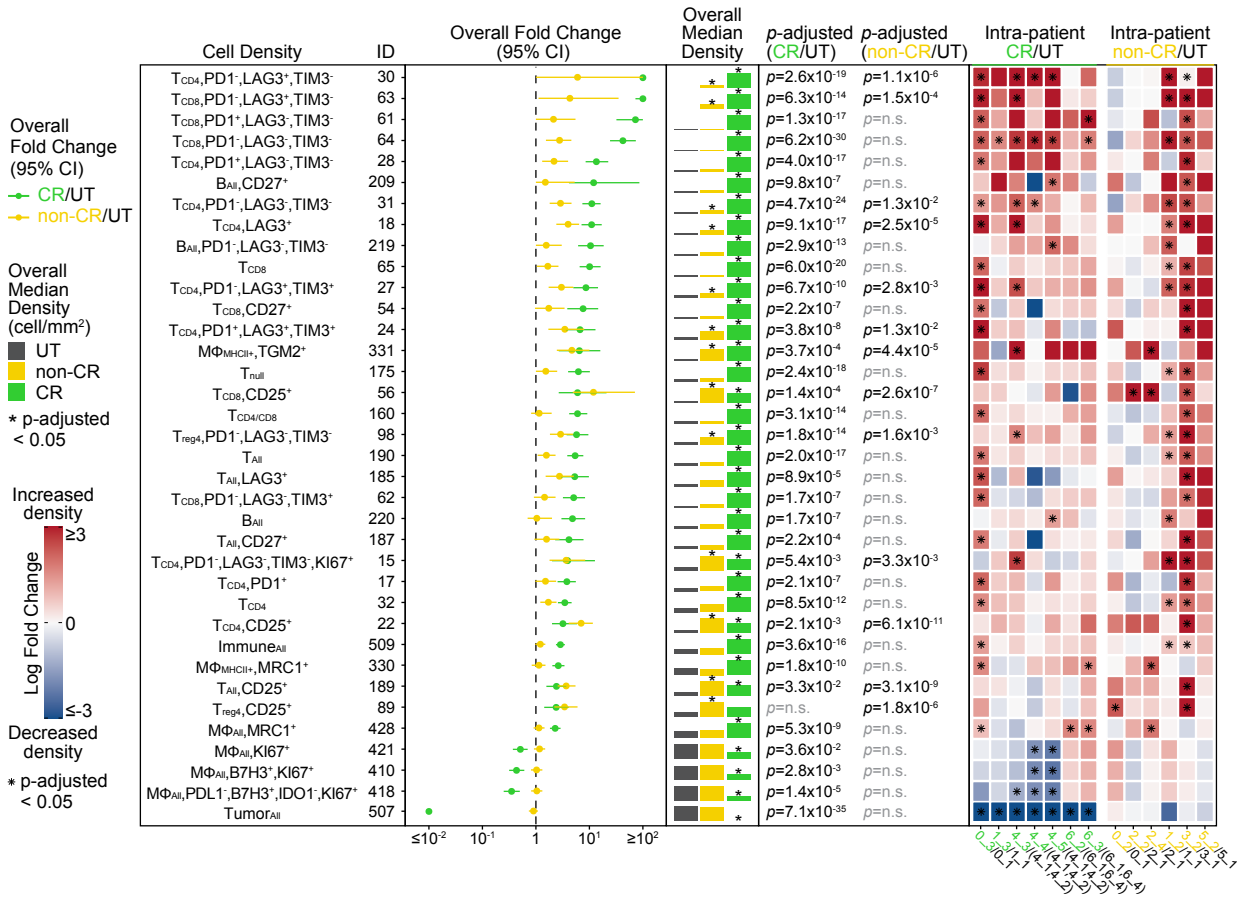
Supplementary Fig. S6. Decrease in TIM-3 positive CD8$^+$ T cells in lesions that completely regress following intralesional interleukin-2 injection. (**A**) Representative images showing CD8 (red) and TIM-3 (white) in untreated (UT) lesion 4_2, non-complete response (non-CR) lesion 1_2, and complete response (CR) lesion 4_4 acquired using Cell Dive and a different multiplexed immunofluorescence method (validation assay – see methods). Scale bar is 10-microns. (**B**) Summary of quantified fractions of TIM-3 positive CD8$^+$ T cells (Cell Dive) and TIM-3 positive CD8$^+$/CD4$^-$ cells (validation assay). Cell Dive fractions are computed over sum of all fields of view. Validation assay fractions are computed over the entire tissue (whole slide).



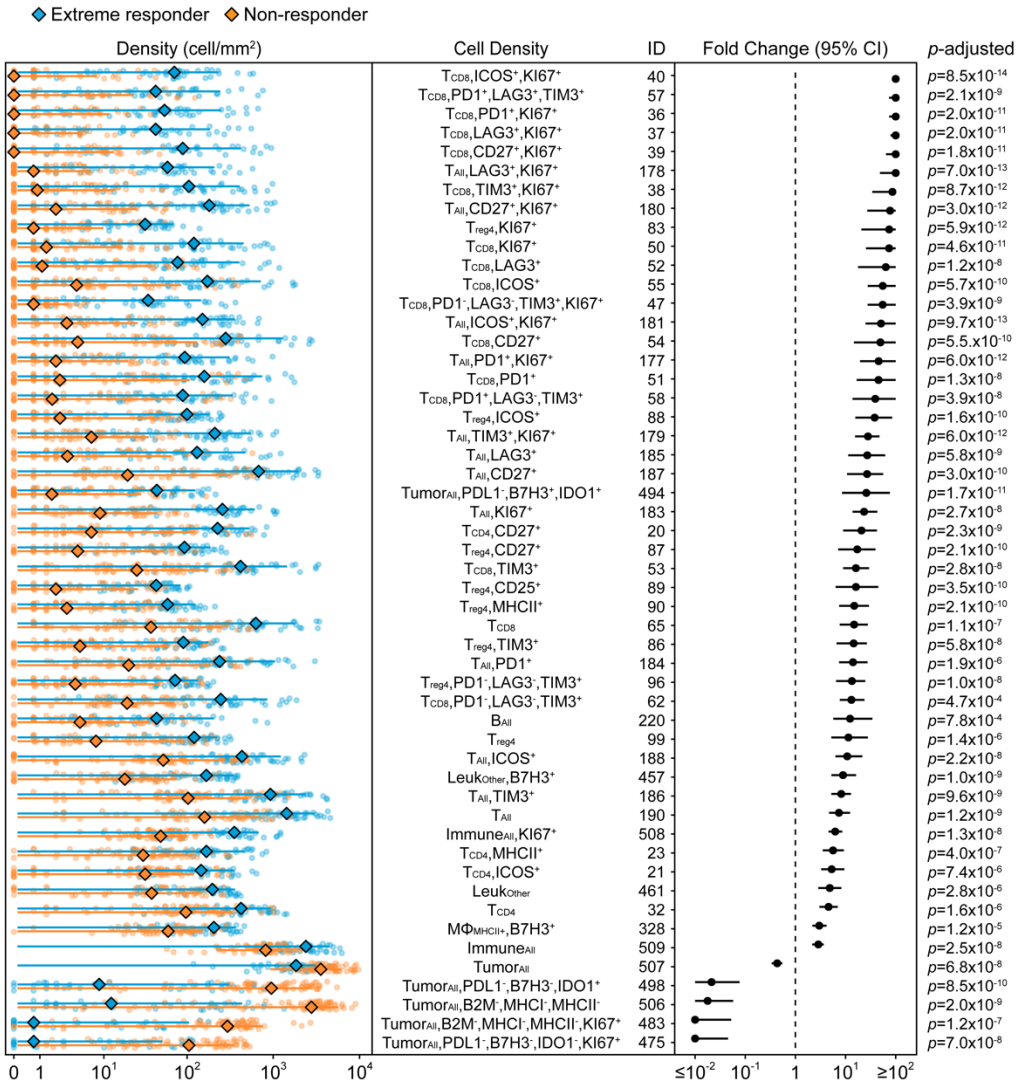
Supplementary Fig. S7. Untreated lesions from the same patient show a similar immune cell composition. (**A**) Box plots (center line: median fraction, box limits: upper and lower quartiles, whiskers: 1.5x interquartile range, points: field of view (FOV) fractions) comparing the fraction of each immune cell type (*n*=16) over total immune cells using a two-sided Wilcoxon test adjusted by Bonferroni correction for untreated (UT) lesions 4_1 (*n*=6 FOVs) and 4_2 (*n*=11 FOVs) from patient 4 and (**B**) for UT lesions 6_1 (*n*=8 FOVs) and 6_4 (*n*=13 FOVs) from patient 6. n.s., not significant (*p*-adjusted>0.001). For exact *p*-values, see Supplementary Table S7. For cell type abbreviations, see Supplementary Table S3.



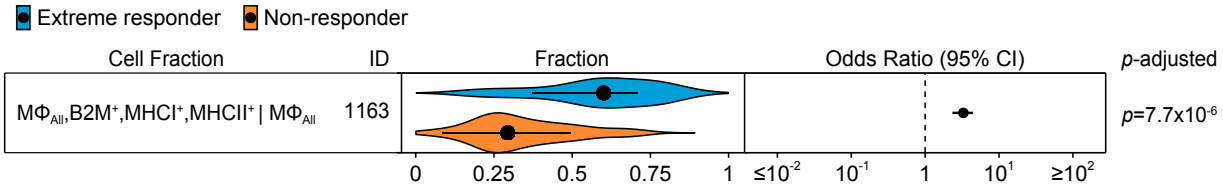
Supplementary Fig. S8. Intralesional interleukin-2 associated changes in immune cell state fractions. Shown are cell fractions (rows) with significant changes following interleukin-2 (IL-2) injection. The forest plot shows the overall effect size and 95% confidence interval (CI) of each cell fraction across all patients for complete response (CR) (*n*=101 fields of view, FOVs) versus untreated (*n*=112 FOVs) and non-complete response (non-CR) (*n*=120 FOVs) versus untreated. The overall median fraction, scaled to 1 for the largest fraction, is shown for untreated, non-CR, and CR lesions. Significant results, determined using a two-sided Wilcoxon test adjusted by Bonferroni correction, are indicated with an asterisk above the median fraction with *p*-adjusted noted (n.s., not significant). The heatmap indicates the effect size of each cell fraction in the IL-2 injected lesion from a single patient versus the untreated lesion(s) from the same patient. Red denotes an increase in cell fraction, blue denotes a decrease. The asterisks in the heatmap indicate significance when effect size is in the same direction as the overall. The FOV counts for intra-patient CR/UT are 21/12, 13/9, 16/17, 19/17, 15/17, 10/21, 7/21 and for intra-patient non-CR/UT are 22/12, 23/9, 17/14, 21/14, 33/35, 4/4 (in order listed above). See also Supplementary Table S6. See Supplementary Table S3 for full cell type names.



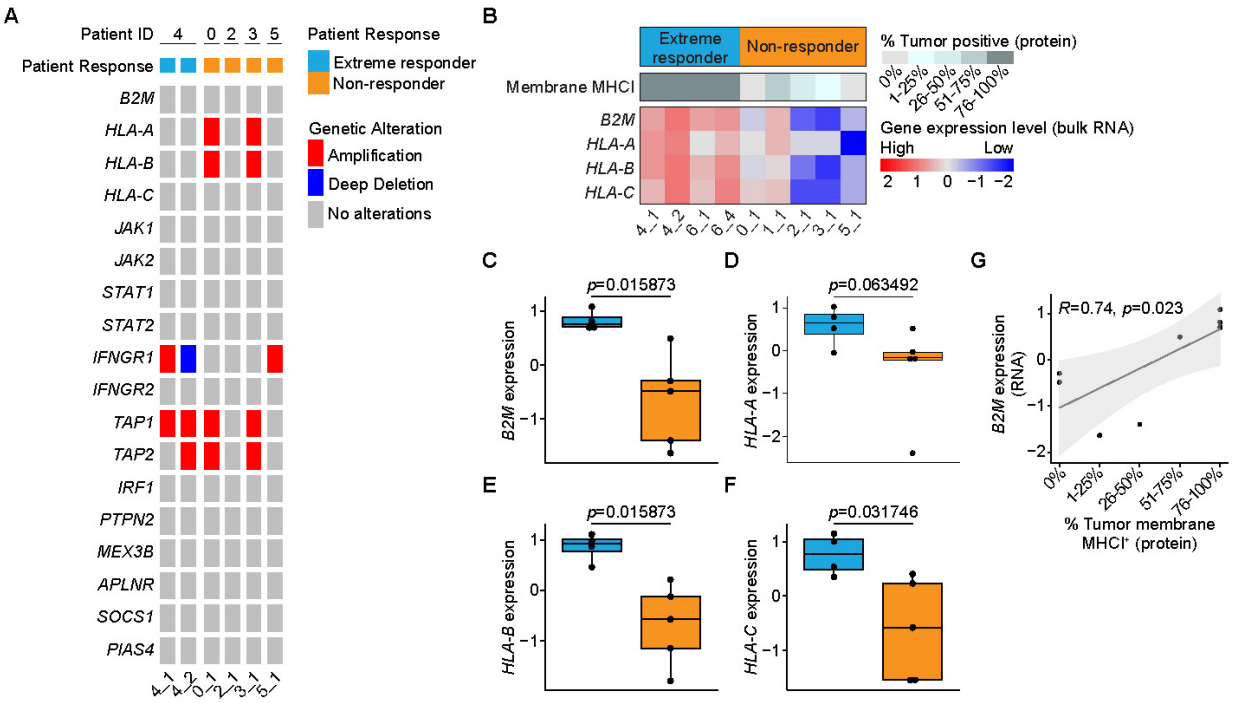
Supplementary Fig. S9. Intralesional interleukin-2 associated changes in immune cell state densities. Shown are cell densities (rows) with significant changes following interleukin-2 (IL-2) injection. The forest plot shows the overall effect size and 95% confidence interval (CI) of each cell density across all patients for complete response (CR) (*n*=101 fields of view, FOVs) versus untreated (UT) (*n*=112 FOVs) and non-complete response (non-CR) (*n*=120 FOVs) versus untreated. The overall median density, scaled to 1 for the largest density, is shown for untreated, non-CR, and CR lesions. Significant results, determined using a two-sided Wilcoxon test adjusted by Bonferroni correction, are indicated with an asterisk above the median density with *p*-adjusted noted (n.s., not significant). The heatmap indicates the effect size of each cell density in the IL-2 injected lesion from a single patient versus the untreated lesion(s) from the same patient. Red denotes an increase in cell density, blue denotes a decrease. The asterisks in the heatmap indicate significance when effect size is in the same direction as the overall. The FOV counts for intra-patient CR/UT are 21/12, 13/9, 16/17, 19/17, 15/17, 10/21, 7/21 and for intra-patient non-CR/UT are 22/12, 23/9, 17/14, 21/14, 33/35, 4/4 (in order listed above). See also Supplementary Table S8. For cell type abbreviations, see Supplementary Table S3.



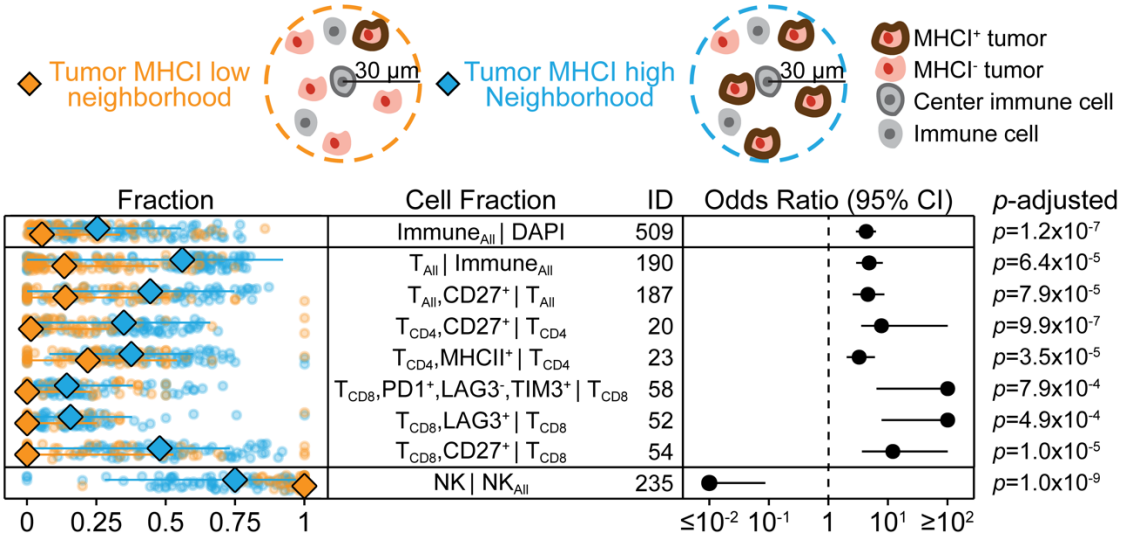
Supplementary Fig. S10. Determinants of response to intralesional interleukin-2 (cell densities). Shown are cell densities (rows) with significant differences in untreated lesions from extreme responders (*n*=38 fields of view, FOVs) versus non-/mixed responders (labeled "non-responder" in figure) (*n*=74 FOVs). The left panel shows densities in each FOV, with overall median, minimum, and maximum. The forest plot shows effect size and 95% confidence interval (CI) of each cell density with *p*-adjusted, determined using a two-sided Wilcoxon test adjusted by Bonferroni correction, noted. See also Supplementary Table S11. For cell type abbreviations, see Supplementary Table S3.



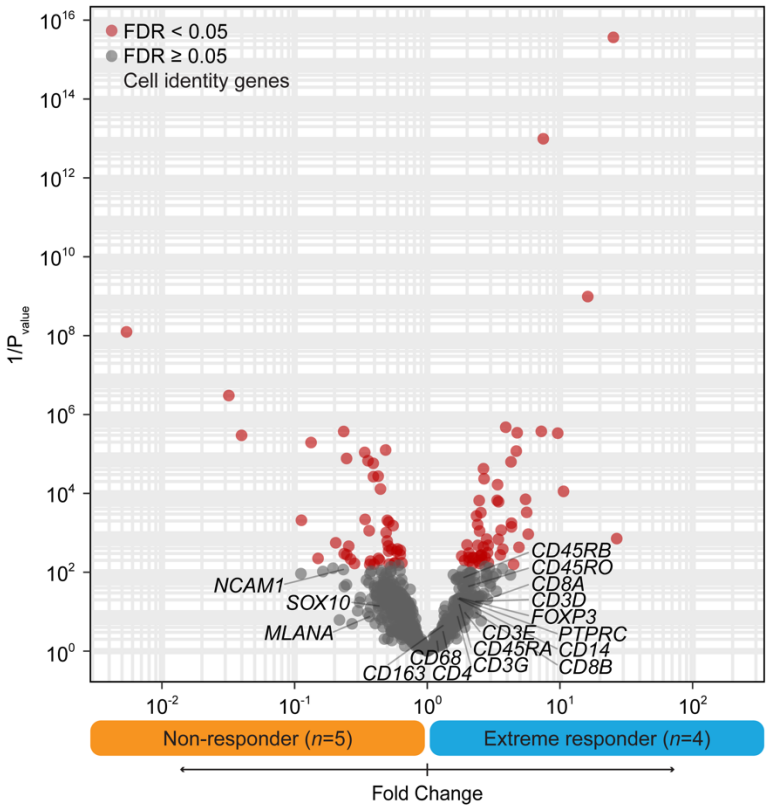
Supplementary Fig. S11. Expression of B2M/MHC-I/MHC-II in macrophage/monocytes in untreated lesions is associated with IL-2 response. Violin plot showing fraction of triple-positive B2M/MHC-I/MHC-II macrophage/monocytes in untreated lesions from extreme responders (*n*=38 fields of view, FOVs) versus non-/mixed responders (labeled "non-responder" in figure) (*n*=74 FOVs) (with overall median, minimum, and maximum shown). The forest plot shows effect size and 95% confidence interval (CI) with *p*-adjusted noted. See also Supplementary Table S10. See Supplementary Table S3 for full cell type names.



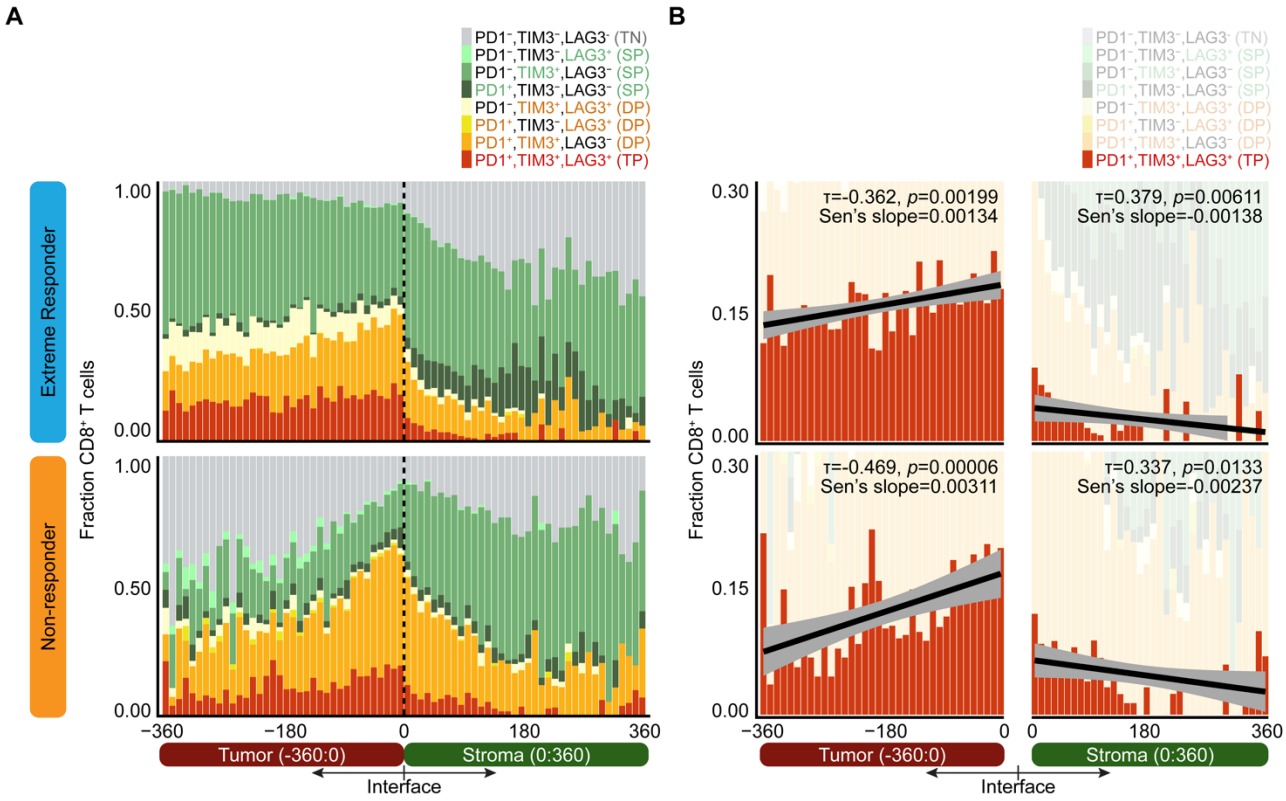
Supplementary Fig. S12. Increased RNA expression of *B2M* and *HLA* genes in untreated lesions of extreme responders. (**A**) Oncoplot showing genetic alterations in antigen presentation pathway genes. "Non-responder" refers to all non-/mixed responder patients. (**B**) Heatmap showing MHC-I scores and scaled RNA expression values of *B2M*, *HLA-A*, *HLA-B*, and *HLA-C* in untreated lesions from extreme responders and non-/mixed responders. (**C**) Boxplots showing expression of *B2M*, (**D**) *HLA-A*, (**E**) *HLA-B*, and (**F**) *HLA-C* genes in untreated lesions of extreme responders (n=4) and non-/mixed responders (n=5). p-values are derived from a two-sided Wilcoxon test. (**G**) Correlation (Pearson) of untreated lesion tumor cell MHC-I membrane positivity (%) with *B2M* RNA expression value.



Supplementary Fig. S13. Activated tumor microenvironment in tumor MHC-I high neighborhoods. Analysis approach to defining tumor MHC-I high neighborhoods (minimum 75% MHC-I positive tumor cells) and tumor MHC-I low neighborhoods (maximum 25% MHC-I positive tumor cells). Shown are cell fractions (rows) with significant differences in tumor MHC-I high versus tumor MHC-I low neighborhoods from untreated lesions. See also Supplementary Table S14.



Supplementary Fig. S14. Untreated lesions of extreme responders and non-/mixed responders exhibit similar expression of cell identity genes. Volcano plot showing differential expression of genes (*n*=770) in untreated lesions of extreme responders (*n*=4) versus non-/mixed responders (labeled "non-responder" in figure) (*n*=5). Cell identity genes (false discovery rate, FDR≥0.05) are labeled in black. See also Supplementary Table S15.



Supplementary Fig. S15. Fraction of triple positive PD-1/LAG-3/TIM-3 CD8⁺ T cells increases as CD8⁺ T cells approach the tumor-stroma interface. (**A**) Fractional distribution of CD8⁺ T cells expressing all combinations of PD-1/TIM-3/LAG-3 in untreated lesions of extreme responders (*n*=31 fields of view, FOVs) and non-/mixed responders (labeled "non-responder" in figure) (*n*=54 FOVs) in 10-micron intervals from -360:360-microns. TN, triple negative. SP, single positive. DP, double positive. TP, triple positive. See also Supplementary Table S18. (**B**) Fraction of CD8⁺ T cells triple positive for PD-1/TIM-3/LAG-3 in untreated lesions of extreme responders and non-/mixed responders in 10-micron intervals from -360:0 (tumor) and 0:360 (stroma). Kendall's tau (τ) and *p*-value, determined using a two-sided Mann-Kendall test, and Sen's slope are noted for each side of the tumor interface (tumor and stroma).

Patient ID	Age	Gender	Patient Response	Lesion ID	Lesion Site*	No. IL-2 Injections	Interval IL-2 Injection/Lesion Excision (days)	Lesion Response
0	56	Male	Non-Responder	0_1	Left groin	0	NA	UT
				0_2	Left foot	7	21	non-CR
				0_3	Left groin	3	28	CR
1	81	Female	Non-Responder	1_1	Right leg	0	NA	UT
				1_2	Right leg	12	134	non-CR
				1_3	Right leg	2	288	CR
2	67	Female	Non-Responder	2_1	Right leg	0	NA	UT
				2_2	Right leg	3	23	non-CR
				2_3	Right leg	3	23	non-CR
				2_4	Right leg	3	23	non-CR
3	86	Female	Non-Responder	3_1	Left thigh	0	NA	UT
				3_2	Left thigh	2	126	non-CR
4	42	Male	Extreme responder	4_1	Right chest	0	NA	UT
				4_2	Right chest	0	NA	UT
				4_3	Right chest	5	7	CR
				4_4	Right axilla	4	14	CR
				4_5	Right arm	3	14	CR
				4_6	Right chest	3	21	CR
				4_7	Right axilla	4	14	CR
5	69	Male	Non-Responder	5_1	Left ear	0	NA	UT
				5_2	Left ear	4	78	non-CR
				5_3	Left ear	4	78	non-CR
				5_4	Left ear	4	78	non-CR
6	74	Female	Extreme responder	6_1	Right leg	0	NA	UT
				6_2	Right leg	3	29	CR
				6_3	Right leg	3	29	CR
				6_4	Right leg	0	NA	UT

*Each lesion represents a discrete metastasis.

Supplementary Table S1. Patient characteristics. Clinical information for 7 patients and 27 in-transit melanoma metastases. Abbreviations: No., number; Non-Responder, Non-/Mixed Responder; IL-2, interleukin-2; NA, not applicable; UT, untreated; non-CR, non-complete response; CR, complete response.

Antigen	Full/Alternative Name	Clone	Vendor, Catalog	Peroxide Stability	Dye	Dye/Protein Ratio	Multiplexed IF Concentration (µg/mL)	IHC Concentration (µg/mL)
B2M	Beta-2-Microglobulin	Polyclonal	Dako, A007202	>10	Cy5	2.07	5.0	0.38
B7H3	CD276	D9M2L	Cell Signaling, 14058	≤5	Cy3	4.73	5.0	0.13
CD14		EPR3653	Abcam, ab196169	>10	Cy5	Commerical conjugate	5.0	0.58
CD163		EDHu-1	Bio-Rad, MCA1853	>10	Cy3	2.57	5.0	0.04
CD20	Pan-B cell antigen	EP459Y	Abcam, ab78237	>10	Cy2	4.34	5.0	0.38
CD25	Interleukin-2 receptor alpha chain	4C9	LSBio, LS-C311984	>10	Cy3	Primary antibody	2.0	1.00
CD27		EPR8569	Abcam, ab192336	≤5	Cy3	3.66	10.0	0.10
CD3		F7.2.38	Dako, M7254	>10	Cy5	2.57	5.0	1.00
CD4		EPR6855	Abcam, ab133616	>10	Cy3	3.60	5.0	0.04
CD45	Leukocyte common antigen	D9M8I	Cell Signaling, 13917	>10	Cy3	4.25	2.5	1.00
CD56	Neural cell adhesion molecule	MRQ-42	Cell Marque, 156R-96	>10	Cy3	4.96	7.5	0.11
CD68		KP1	Thermo, MS-397-PABX	>10	Cy5	3.89	5.0	2.00
CD8		C8/144B	Dako, M7103	>10	Cy5	1.20	5.0	1.67
FOXP3	Forkhead box P3	150D	BioLegend, 320014	<3	Cy5	Commerical conjugate	10.0	0.08
ICOS	CD278/Inducible T Cell Costimulator	D1K2T	Cell Signaling, 89601	>10	Cy5	3.45	10.0	0.31
IDO1	Indoleamine 2,3-Dioxygenase 1	SP260	Spring Bioscience, M5604	>10	Cy3	2.42	10.0	0.70
KI67		Polyclonal	Thermo, RB-1510-PABX	>10	Cy5	3.32	10.0	2.00
LAG3	CD223/Lymphocyte Activating 3	17B4	LSBio, LS-C18692	>10	Cy3	4.75	5.0	0.10
MHCI	Major histocompatibility complex class I	EMR8-5	Abcam, ab70328	>10	Cy5	3.43	5.0	0.67
MHCII	Major histocompatibility complex class II	TAL 1B5	Thermo, MA1-46109	>10	Cy3	5.15	5.0	0.04
MRC1	Mannose receptor C-type 1	Polyclonal	Abcam, ab64693	>10	Cy5	1.33	2.5	2.02
panCK	pan-Cytokeratin	PCK-26	Sigma, C5992	>10	Cy5	1.82	5.0	2.50
PD1	CD279/Programmed cell death protein 1	NAT105	Abcam, ab52587	≤5	Cy5	Commerical conjugate	10.0	2.50
PDL1	CD274/Programmed cell death-ligand 1	E1L3N	Cell Signaling, 13684	>10	Cy5	2.27	10.0	2.02
S100B	S100 calcium-binding protein B	EP1576Y	Abcam, ab215989	>10	Cy3	3.06/2.96 (combined)	10.0	4.00
SOX10	SRY-Box Transcription Factor 10	Polyclonal	Cell Marque, 383A-75	>10	Cy5	3.37	5.0	0.05
TGM2	Transglutaminase 2	CUB 7482	Thermo, MA5-12739	>10	Cy3	3.98	5.0	1.50
TIM3	Hepatitis A virus cellular receptor 2	D5D5R	Cell Signaling, 78226	≤5	Cy5	Commerical conjugate	5.0	0.15

Supplementary Table S2. Antibody information. Abbreviations: IF, immunofluorescence; IHC, immunohistochemistry.

Cell Type	Cell Subtype	Abbreviation	Marker Positives	Marker Negatives
$Tumor_{All}$	Tumor	$Tumor_{All}$	SOX10	CD3,CD4,CD8,FOXP3,CD20
T_{All}	$CD4^+$ T cell	T_{CD4}	CD3,CD4	CD8,FOXP3,CD20,CD56,SOX10
	$CD8^+$ T cell	T_{CD8}	CD3,CD8	CD4,FOXP3,CD20,CD56,SOX10
	$CD4^+$ regulatory T cell	T_{reg4}	CD3,CD4,FOXP3	CD8,CD20,CD56,SOX10
	$CD8^+$ regulatory T cell	T_{reg8}	CD3,CD8,FOXP3	CD4,CD20,CD56,SOX10
	Double negative regulatory T cell	T_{regDN}	CD3,FOXP3	CD4,CD8,CD20,CD56,SOX10
	$CD4^+/CD8^+$ T cell	$T_{CD4/CD8}$	CD3,CD4,CD8	FOXP3,CD20,CD56,SOX10
	T cell/null phenotype	T_{null}	CD3	CD4,CD8,FOXP3,CD20,CD56,SOX10
B_{All}	B cell	B_{All}	CD20	CD3,CD4,CD8,FOXP3,CD56,SOX10
NK_{All}	Natural killer cell	NK	CD56	CD3,CD4,CD8,FOXP3,CD20,SOX10,S100B
	Natural killer T cell	NKT	CD3,CD56	CD4,CD8,FOXP3,CD20,SOX10
	Natural killer T cell/$CD8^+$	NKT_{CD8}	CD3,CD8,CD56	CD4,FOXP3,CD20,SOX10
	Natural killer T cell/$CD4^+$	NKT_{CD4}	CD3,CD4,CD56	CD8,FOXP3,CD20,SOX10
$M\Phi_{All}$	$MHCII^+$ macrophage	$M\Phi_{MHCII+}$	MHCII, CD68 and/or CD163	CD3,CD8,FOXP3,CD20,CD56,SOX10
	$MHCII^-$ macrophage	$M\Phi_{MHCII-}$	CD68 and/or CD163	CD3,CD8,FOXP3,CD20,CD56,SOX10,MHCII
	Other macrophage/monocyte	$M\Phi_{Other}$	CD14 and/or CD4	CD3,CD8,FOXP3,CD20,CD56,CD163,CD68,SOX10
$Leukocyte_{Other}$	Other leukocyte	$Leukocyte_{Other}$	CD45	CD3,CD4,CD8,FOXP3,CD20,CD56,CD14,CD163,CD68,SOX10,S100B
Other	Adipocyte or Langerhans cell	NA	S100B	CD45,CD3,CD4,CD8,FOXP3,CD20,CD56,CD14,CD163,CD68,SOX10,PCK26
	Epithelial cell	NA	panCK	CD45,CD3,CD4,CD8,FOXP3,CD20,CD56,CD14,CD163,CD68,SOX10,S100B
	Nerve	NA	CD56,S100B	CD3,CD4,CD8,FOXP3,CD20,CD14,CD163,CD68,SOX10

Supplementary Table S3. Use of cell identity markers. Each cell type is defined by a combination of positive and negative staining results for specific cell identity markers.

Lesion ID	Cell Count: Cycle 1	Cell Count: Cycle 32	Percent Loss	No. Total FOVs	No. Interface FOVs	No. Center FOVs	No. Regressed FOVs	Area Analyzed (mm^2)
0_1	100,339	99,168	1.17	12	8	4	0	17.44
0_2	188,746	186,198	1.35	22	13	8	1	27.88
0_3	127,427	127,210	0.17	21	0	0	21	29.58
1_1	64,208	63,658	0.86	9	8	1	0	12.52
1_2	208,388	204,483	1.87	23	16	2	5	31.20
1_3	60,467	52,065	13.90	13	0	0	13	18.67
2_1	100,323	91,814	8.48	14	10	4	0	18.21
2_2	123,956	122,231	1.39	17	17	0	0	24.09
2_4	156,872	150,570	4.02	21	17	4	0	29.38
3_1	344,604	342,483	0.62	35	27	8	0	49.94
3_2	354,521	353,809	0.20	33	20	13	0	47.44
4_1	39,045	38,514	1.36	6	5	1	0	7.06
4_2	85,832	84,263	1.83	11	9	2	0	15.21
4_3	147,179	146,997	0.12	16	0	0	16	23.25
4_4	127,819	127,725	0.07	19	0	0	19	27.42
4_5	113,717	113,554	0.14	15	0	0	15	21.80
5_1	22,625	22,472	0.68	4	3	1	0	3.88
5_2	26,635	26,629	0.02	4	4	0	0	5.08
6_1	42,208	40,601	3.81	8	7	1	0	10.92
6_2	23,104	22,383	3.12	10	0	0	10	10.61
6_3	48,366	48,210	0.32	7	0	0	7	8.53
6_4	111,372	107,592	3.39	13	10	3	0	17.80

Supplementary Table S5. Summary of cell loss and fields of view for multiplexed immunofluorescence data. Abbreviations: No., number; FOVs, fields of view.

Cohort	Institution	Patient ID	IL-2 Treatment	Prior Therapies	Patient Response	No. Systemic IL-2 Cycles	No. Intralesional IL-2 Injections
Validation	Calgary	v_01	Intralesional	None	CR	NA	4
Validation	Calgary	v_02	Intralesional	None	CR	NA	8
Validation	Calgary	v_03	Intralesional	None	CR	NA	4
Validation	NIH	v_04	Systemic	None	CR	4	NA
Validation	NIH	v_05	Systemic	None	CR	5	NA
Validation	NIH	v_06	Systemic	None	CR	6	NA
Validation	NIH	v_07	Systemic	None	non-CR	1	NA
Validation	NIH	v_08	Systemic	None	non-CR	2	NA
Validation	NIH	v_09	Systemic	None	non-CR	1	NA
Validation	NIH	v_10	Systemic	None	non-CR	2	NA
Validation	NIH	v_11	Systemic	None	non-CR	2	NA
Validation	NIH	v_12	Systemic	None	non-CR	2	NA
Validation	NIH	v_13	Systemic	None	non-CR	2	NA
Validation	MDACC	v_14	Systemic	None	non-CR	2	NA
Validation	MDACC	v_15	Systemic	None	non-CR	2	NA
Validation	MDACC	v_16	Systemic	None	non-CR	2	NA
Validation	MDACC	v_17	Systemic	None	non-CR	2	NA
Validation	JHMI	v_18	Systemic	none	non-CR	6	NA
Validation	JHMI	v_19	Systemic	none	non-CR	4	NA

Supplementary Table S12. Validation cohort patient characteristics. Clinical information for metastatic melanoma patients in the validation cohort (*n*=19) treated with either intralesional interleukin-2 (IL-2) or high-dose systemic IL-2. Patient response was classified as complete responder (CR) or non-complete responder (non-CR). Abbreviations: Calgary, University of Calgary; NIH, National Institutes of Health; MDACC, MD Anderson Cancer Center; JHMI, Johns Hopkins Medical Institute.

Lesion ID	Patient Response	Lesion Response	Mutation Count	Tumor Mutation Burden
4_1	Extreme responder	UT	8	7.08
4_2	Extreme responder	UT	8	7.08
0_1	Non-responder	UT	4	3.54
2_1	Non-responder	UT	5	4.42
3_1	Non-responder	UT	6	5.31
5_1	Non-responder	UT	16	14.16

Supplementary Table S16. High tumor mutation burden in untreated lesions is not associated with response to intralesional IL-2. Tumor mutation burden is measured in mutations per megabase. Abbreviations: Non-responder, Non-/mixed responder; UT, untreated.

Captions for Supplementary Table Files:

Supplementary Table S4. List of cell states, cell fractions, and cell densities. First tab "Summary" includes a summary of cell states. Second tab "Cell_states" includes the list of 664 cell states. Third tab "Cell_fractions" includes the list of 685 cell fractions with cell state (numerator) and population (denominator) noted. Fourth tab "Cell_densities" includes the list of 664 cell densities. Abbreviations: No, number. For cell type abbreviations, see Supplementary Table S3.

Supplementary Table S6. Interleukin-2 associated changes in cell fractions. First tab "all_fractions (*n*=685)" includes statistics for all cell fractions in both the overall and intra-patient comparisons of complete response (CR) versus untreated (UT) and non-complete response (non-CR) versus untreated. Second tab "sig_fractions (*n*=62)" includes statistics for statistically significant cell fractions. Third tab "sig_fractions_Fig2A_S8 (*n*=41)" includes statistics for statistically significant cell fractions shown in Fig. 2A and Supplementary Fig. S8. "NumSame" refers to the number of intra-patient comparisons with effect size in the same direction as the overall effect size. The number of fields of view (FOVs) included in the analysis: untreated, *n*=112; CR, *n*=101; non-CR, *n*=120. All significant cell fractions have a minimum cell state cell count 300, minimum population cell count 1,000, minimum median fraction difference of 10% between comparison groups, and FDR<0.05 (for at least one of two comparison groups). For cell type abbreviations, see Supplementary Table S3. Abbreviations: OR, odds ratio; CI, 95% confidence interval; FDR, false discovery rate; HMP, harmonic *p*-value.

Supplementary Table S7. Comparison of multiple untreated lesions from the same patient. Statistics for immune cell fractions from patient 4 are included in the first tab "patient_4" and from patient 6 are included in the second tab "patient_6". For cell type abbreviations, see Supplementary Table S3. Abbreviations: CI, 95% confidence interval.

Supplementary Table S8. Interleukin-2 associated changes in cell densities. First tab "all_densities (*n*=664)" includes statistics for all cell densities in both the overall and intra-patient comparisons of complete response (CR) versus untreated (UT) and non-complete response (non-CR) versus untreated. Second tab "sig_densities (*n*=74)" includes statistics for statistically significant cell densities. Third tab "sig_densities_FigS9 (*n*=36)" includes statistics for statistically significant cell densities shown in Supplementary Fig. S9. "NumSame" refers to the number of intra-patient comparisons with effect size in the same direction as the overall effect size. The number of fields of view (FOVs) included in the analysis: untreated, *n*=112; CR, *n*=101; non-CR, *n*=120. All significant cell densities have a minimum cell state cell count 1000, minimum median density difference of 10 between comparison groups, and FDR<0.05 (for at least one of two comparison groups). For cell type abbreviations, see Supplementary Table S3. Abbreviations: FC, fold change; CI, 95% confidence interval; FDR, false discovery rate; HMP, harmonic *p*-value.

Supplementary Table S9. Differential expression of genes in interleukin-2 injected lesions versus untreated lesions. First tab "CRvUT_all_genes (*n*=770)" includes results from differential expression analysis of complete response (CR) lesions versus untreated (UT) lesions for 770 genes in the NanoString IO 360 panel. Second tab "nonCRvUT_all_genes (*n*=770)"

includes results from differential expression analysis of non-CR lesions versus untreated lesions. Third tab "gene_annotations" includes different pathways each gene is associated with. Significant differentially expressed genes are defined by *p*-adjusted (padj)<0.0001.

Supplementary Table S10. Determinants of response to intralesional interleukin-2 (cell fractions). First tab "all_fractions (*n*=685)" includes statistics for all cell fractions in comparison of untreated lesions from extreme responders versus non-/mixed responders (labeled "non-responder throughout table). Second tab "sig_fractions (*n*=77)" includes statistics for statistically significant cell fractions. Third tab "sig_fractions_Fig3B (*n*=33)" includes statistics for statistically significant cell fractions shown in Fig. 3B. The number of fields of view (FOVs) included in the analysis: extreme responder, *n*=38; non-/mixed responder, *n*=74. All significant cell fractions have a minimum cell state cell count 300, minimum population cell count 1,000, minimum median fraction difference of 7% between comparison groups, and *p*-adjusted<0.05. For cell type abbreviations, see Supplementary Table S3. Abbreviations: CI, 95% confidence interval.

Supplementary Table S11. Determinants of response to intralesional interleukin-2 (cell densities). First tab "all_densities (*n*=664)" includes statistics for all cell densities in comparison of untreated lesions from extreme responders versus non-/mixed responders (labeled "non-responder throughout table). Second tab "sig_densities (*n*=239)" includes statistics for statistically significant cell densities. Third tab "sig_densities_FigS10 (*n*=52)" includes statistics for statistically significant cell densities shown in Supplementary Fig. S10. The number of fields of view (FOVs) included in the analysis: extreme responder, *n*=38; non-/mixed responder, *n*=74. All significant cell densities have a minimum cell state cell count 1000, minimum median density difference of 10 between comparison groups, and *p*-adjusted<0.05. For cell type abbreviations, see Supplementary Table S3. Abbreviations: CI, 95% confidence interval.

Supplementary Table S13. Summary of MHC-I scores in initial and validation cohorts. First tab "MHCI_scores" includes the score (fraction of tumor cells with membranous MHC-I positivity) for all patients. Second tab "MHCI_scores_summary" includes a summary of MHC-I scores grouped by over 75% tumor cell positive and under 75% tumor cells positive for the initial and validation cohorts. Abbreviations: MSKCC, Memorial Sloan Kettering Cancer Center; Calgary, University of Calgary; NIH, National Institutes of Health; MDACC, MD Anderson Cancer Center; JHMI, Johns Hopkins Medical Institute; CR, complete responder; non-CR, non-complete responder.

Supplementary Table S14. Comparison of tumor MHC-I high *versus* tumor MHC-I low cellular neighborhoods. First tab "all_fractions (*n*=685)" includes statistics for all cell fractions in comparison of tumor MHC-I high versus tumor MHC-I low neighborhoods. Second tab "sig_fractions (*n*=54)" includes statistics for statistically significant cell fractions. Third tab "sig_fractions_FigS13 (*n*=9)" includes statistics for statistically significant cell fractions shown in Supplementary Fig. S13. All significant cell densities have a minimum cell state cell count 1000, minimum median density difference of 10 between comparison groups, and *p*-adjusted<0.05. For cell type abbreviations, see Supplementary Table S3. Abbreviations: CI, 95% confidence interval.

Supplementary Table S15. Differential expression of genes in untreated lesions from extreme responders versus non-/mixed responders. First tab "all_genes (*n*=770)" includes results from differential expression analysis of untreated lesions from extreme responders versus non-/mixed responders (labeled "non-responder throughout table) for 770 genes in the NanoString IO 360 panel. Second tab "pathways" includes list of all genes in the annotated pathways in Fig. 4A.

Supplementary Table S17. Quantification of B cell density and aggregates. First tab "Fig4C" includes raw data for Fig. 4C. Second tab "Fig4D-E" includes raw data for Fig. 4, D and E. Density is measured in cells per millimeter squared. Abbreviations: FOV, field of view; FDR, false discovery rate.

Supplementary Table S18. Tumor-stroma interface analysis of $CD8^+$ T cells expressing all combinations of PD-1/LAG-3/TIM-3. First tab "Fig4H_FigS15" includes raw data for Fig. 4H and Supplementary Fig. S15. Second tab "Fig4I" includes raw data for Fig. 4I. Density is measured in cells per millimeter squared. Band is measured in microns. Abbreviations: FOV, field of view; UT, untreated.

Supplementary Table S19. Cellular neighborhoods of $CD8^+$ T cells with an exhausted phenotype. Includes raw data for Fig. 4J. For cell type abbreviations, see Supplementary Table S3. Abbreviations: LO, log odds; LOR, log odds ratio.