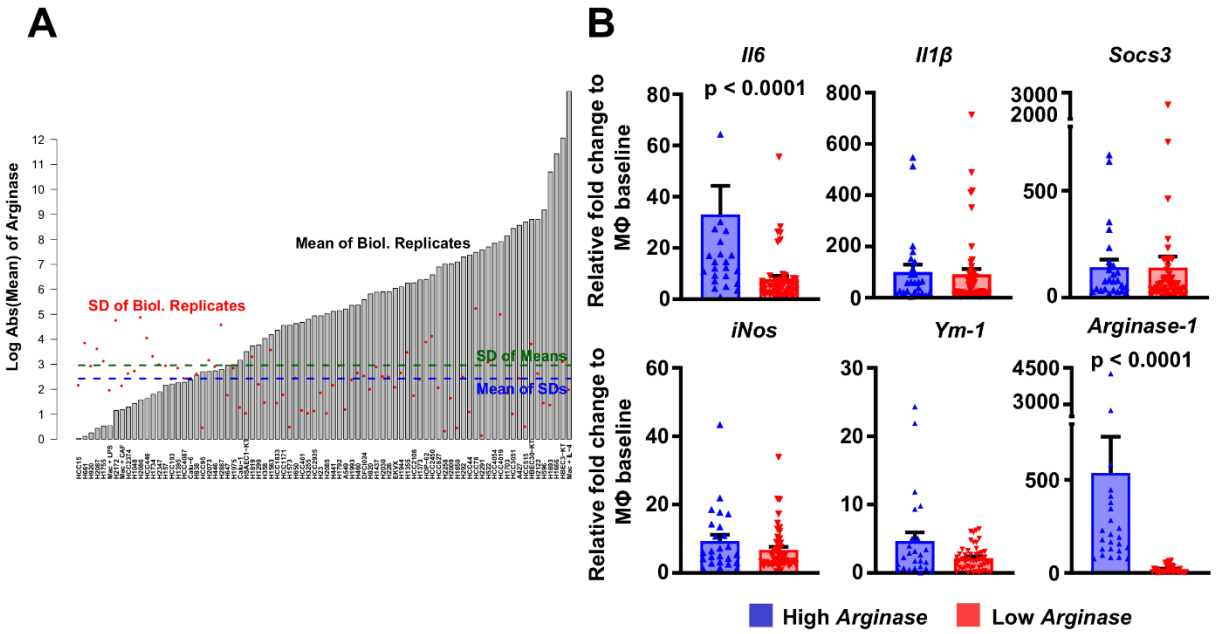
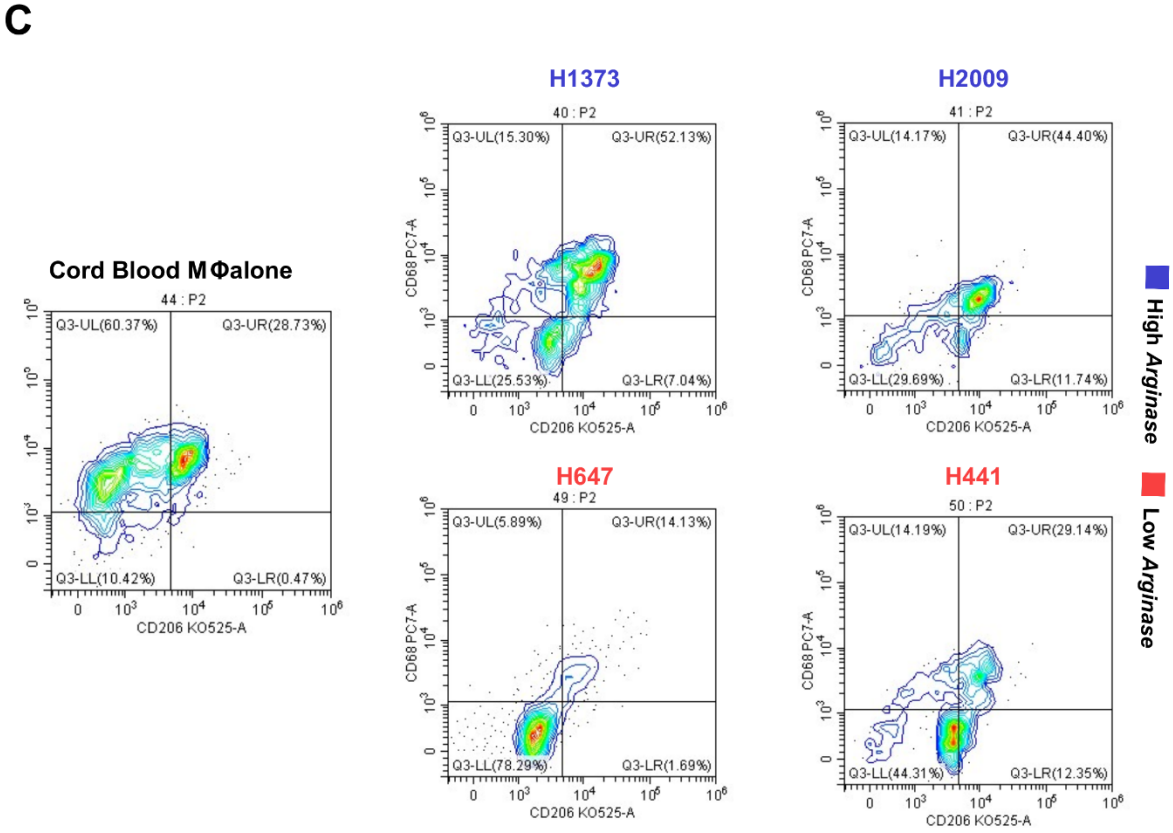
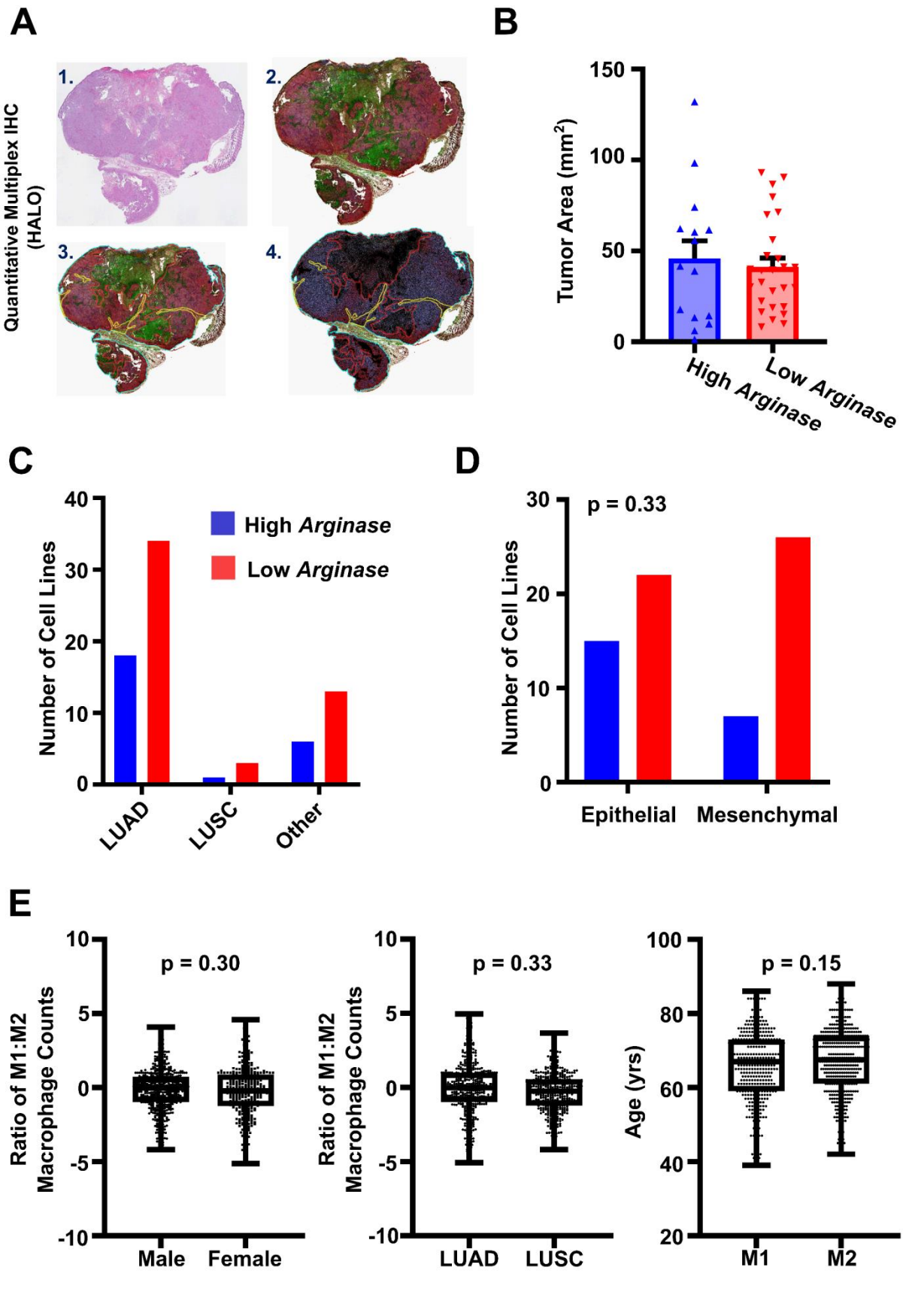
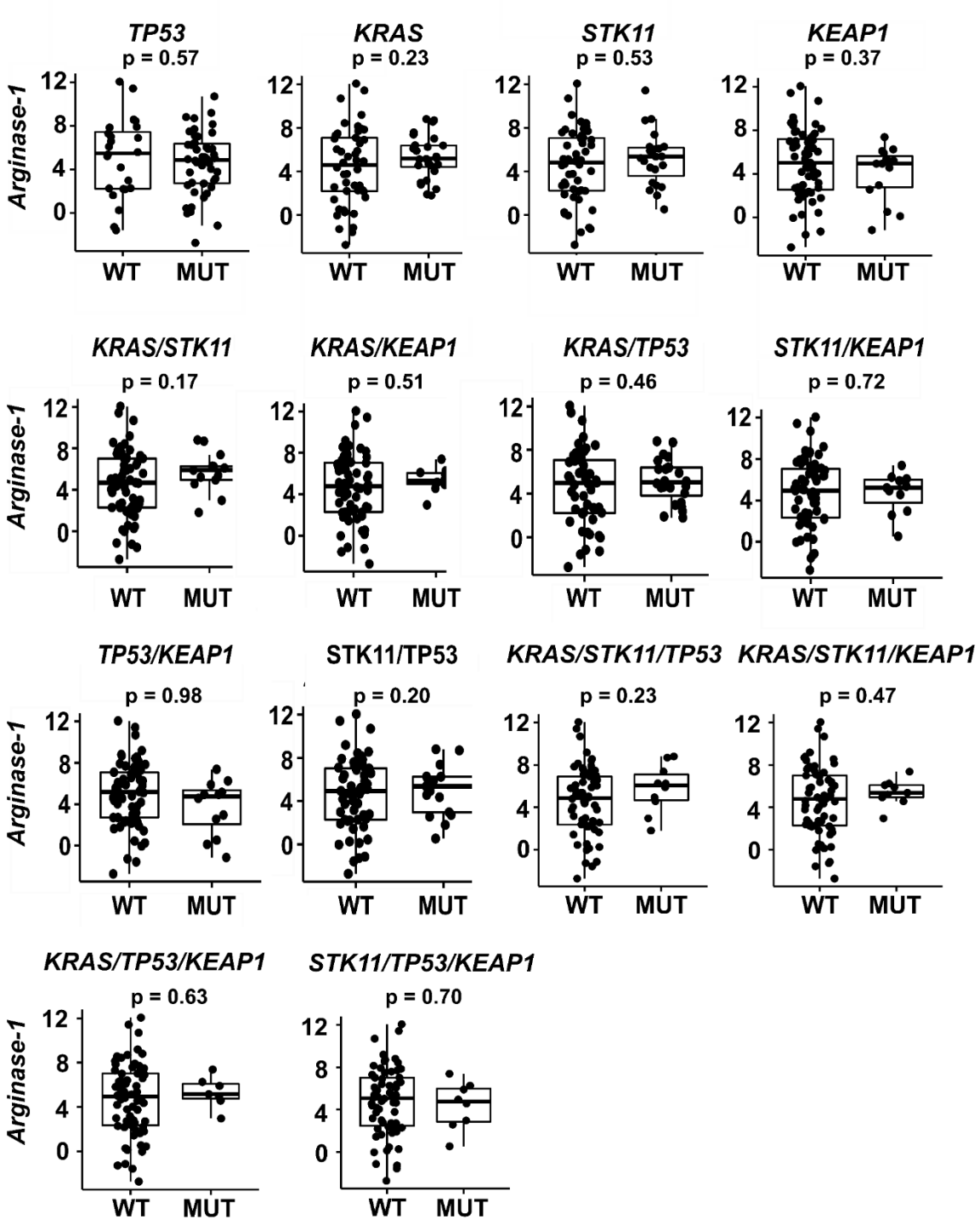

A
Log Abs(Mean) of Arginase
Mean of Biol. Replicates
SD of Biol. Replicates
SD of Means
Mean of SDs
B
Il6
p < 0.0001
Il1β
Socs3
iNos
Ym-1
Arginase-1
p < 0.0001
Relative fold change to MΦ baseline
Relative fold change to MΦ baseline
High Arginase
Low Arginase


C
Cord Blood MΦalone
44 : P2
Q3-UL(60.37%)
Q3-UR(28.73%)
Q3-LL(10.42%)
Q3-LR(0.47%)
CD206 KO525-A
H1373
40 : P2
Q3-UL(15.30%)
Q3-UR(52.13%)
Q3-LL(25.53%)
Q3-LR(7.04%)
CD68 PC7-A
CD206 KO525-A
H2009
41 : P2
Q3-UL(14.17%)
Q3-UR(44.40%)
Q3-LL(29.69%)
Q3-LR(11.74%)
H647
49 : P2
Q3-UL(5.89%)
Q3-UR(14.13%)
Q3-LL(78.29%)
Q3-LR(1.69%)
H441
50 : P2
Q3-UL(14.19%)
Q3-UR(29.14%)
Q3-LL(44.31%)
Q3-LR(12.35%)
High Arginase
Low Arginase

Supplemental Figure 1: **A) High expression of macrophage *Arginase-1* co-cultured with NSCLC cells is highly reproducible.** For each biologic replicate of macrophages co-cultured with lung cancer and benign immortalized cells, the mean and standard deviation of *Arginase-1* expression were calculated. Across all replicates of individual cell lines, the mean of the standard deviations was less than the standard deviation of the mean expression values, reflecting high reproducibility between replicates when compared to the overall range of expression values. Murine-specific primers were used to assess gene expression changes. Standard deviation values for individual genes across replicates for each lung cancer co-culture system is provided in **Supplemental File 1 ("Co-culture qPCR box plots"). B) Quantitative expression of macrophage genes between *Arg*^{hi} and *Arg*^{low} cluster lines.** qRT-PCR gene expression analysis of differential expression of murine *Arginase-1, Socs3, Il1β, iNos, Il6, Ym-1* was compared between both cohorts. Each dot represents a test of an individual NSCLC line on co-cultured macrophage transcriptional activity. *Arg*^{hi} cluster lines induced significantly higher levels of *Arginase-1* and *Il6*. Median expression levels were compared using Mann-Whitney U tests. NSCLC (72), SCLC (2) and benign immortalized (3) cell lines for panels A and B. **C) Induced macrophage phenotypes identified in co-cultured murine macrophages are recapitulated in co-cultured human umbilical cord blood-derived macrophages.** A panel of 2 *Arg*^{hi} (H1373 and H2009) and 2 *Arg*^{low} (H647 and H441) were co-cultured with cord-blood derived macrophages (2 replicates of patient-derived samples) and subsequently prepared for flow cytometric analysis. CD68 was used as a pan-macrophage marker and CD206 was utilized as an M2-like marker. Those cell lines which induced high macrophage expression of *Arginase-1* in murine macrophages similarly polarized human macrophages towards an M2-like (CD68+/CD206+) phenotype.


A
Quantitative Multiplex IHC (HALO)
1.
2.
3.
4.
B
Tumor Area (mm²)
150
100
50
0
High Arginase
Low Arginase
C
Number of Cell Lines
40
30
20
10
0
High Arginase
Low Arginase
LUAD
LUSC
Other
D
Number of Cell Lines
30
20
10
0
p = 0.33
Epithelial
Mesenchymal
E
Ratio of M1:M2 Macrophage Counts
10
5
0
-5
-10
p = 0.30
Male
Female
Ratio of M1:M2 Macrophage Counts
10
5
0
-5
-10
p = 0.33
LUAD
LUSC
Age (yrs)
100
80
60
40
20
p = 0.15
M1
M2

Supplemental Figure 2: **A) Example of pipeline used for quantitative multiplex immunohistochemical analysis of macrophages in nude mice xenografts.** Hematoxylin and eosin (H and E) stains of individual tumor sections were generated for each tumor to identify tumor borders, stroma, and areas of necrosis **(1)**. Sequentially stained tumor sections were inspected **(2)** and key areas identified in H and E stains were annotated **(3).** Within the annotated tumor border, quantitative analyses of overall macrophage (F4/80+ cells) and ARG+ macrophage (ARG+ / F4/80+ cells) density were performed **(4). B) Comparison of tumor area.** Median tumor area between tumors established in murine xenografts was not significantly different between *Arg*^{hi} and *Arg*^{low} cohorts on Mann-Whitney U analysis. NSCLC xenografts studied in Panels A and B were established from 5 *Arg*^{hi} cluster lines (A427, H1373, H1666, H2009, H522) and 6 *Arg*^{low} cluster lines (H1993, Calu-6, H460, H647, H2073, H441). **C) Distribution of NSCLC subtype between cell line clusters.** Adenocarcinoma (LUAD), squamous cell carcinoma (LUSC), or other histologic subtypes were comprised of a mixed distribution of *Arg*^{hi} and *Arg*^{low} low-cluster cell lines. **D) Distribution of EMT subtype classification between cell line clusters.** No significant differences were noted between the frequency of epithelial or mesenchymal phenotypes of NSCLC cells in either cohort using Fisher-exact tests. **E) Impact of sex, NSCLC subtype, and age on M1:M2 distribution in TCGA samples.** In TCGA-deposited clinical NSCLC tumor samples, sex, histologic subtype, and age were not significantly associated with M1:M2 ratios of macrophages characterized by CIBERSORT analysis. Mann-Whitney U tests were used for comparative analyses. Available data from a total of 980 patient lung cancer samples were used for TCGA analyses.


TP53
p = 0.57
KRAS
p = 0.23
STK11
p = 0.53
KEAP1
p = 0.37
KRAS/STK11
p = 0.17
KRAS/KEAP1
p = 0.51
KRAS/TP53
p = 0.46
STK11/KEAP1
p = 0.72
TP53/KEAP1
p = 0.98
STK11/TP53
p = 0.20
KRAS/STK11/TP53
p = 0.23
KRAS/STK11/KEAP1
p = 0.47
KRAS/TP53/KEAP1
p = 0.63
STK11/TP53/KEAP1
p = 0.70
Arginase-1
12
8
4
0
WT
MUT

663

Supplemental Figure 3: Impact of key driver mutations in NSCLC on quantitative expression of *Arginase-1* in co-cultured macrophages. Across the most frequently mutated single or combination of oncogenes, relative expression of *Arginase-1* compared to macrophages alone was compared between wild-type and mutant status using Wilcoxon signed-rank tests. No significant associations with induced macrophage *Arginase-1* expression were observed across oncogenotypes. 72 individual NSCLC cell lines were studied.

Supplemental Table S1: Clinical characteristics and oncogenotypes of the 74 lung cancer lines and 3 benign immortalized lines used in this study.

Cell Line	*Arg* Status	Age	Race	Gender	Subtype	EMT Status	Smoking	TMB	*TP53*	*KRAS*	*LKB1*	*EGFR*	*KEAP1*	Tumor Source	Stage	Chemotherapy	Radiation	Response
A427	High	52	C	M	NSCLC			548	WT	Codon 12	HD	WT	WT	Primary				
H1355	High	53	C	M	ADN	Mes	Y	589	MUT	Codon 13	FS	Other	MUT	Distant Metastasis	4	N	N	
H1650	High	27	C	M	ADN	Epi	Y	258	MUT	WT	WT	Exon 19	WT	Distant Metastasis	3	N	N	PD
H1666	High	50	C	F	ADN	Epi		515	WT	WT	FS	WT	WT	Distant Metastasis	3	N	Y	
H1693	High	55	C	F	ADN	Epi	Y	280	MUT	WT	WT	WT	WT	Lymph Node	3	N	Y	PR
H1703	High	56	C	M	ADSQ	Mes	Y	674	MUT	WT	WT	WT	WT	Primary	1	N	N	
H2009	High	68	C	F	ADN	Epi	Y	1196	MUT	Codon 12	WT	WT	WT	Lymph Node	4	Y	N	PD
H2122	High	46	C	F	ADN	Epi	Y	233	MUT	Codon 12	FS	WT	WT	Distant Metastasis	4	N	N	PD
H2258	High	46	C	M	ADN	Epi	Y	97	MUT	WT	WT	WT	WT	Distant Metastasis	3	Y	N	PD
H2291	High			M	ADN	Epi	N	701	MUT	Codon 12	WT	Other	WT	Lymph Node		N	N	
H292	High	32	B	F	MEC	Epi	N	520	WT	Codon 12	WT	WT	WT	Lymph Node	4	N	N	PD
H522	High	60	C	M	ADN	Mes	Y	439	MUT	WT	WT	WT	WT	Primary	2	N	N	ND
H596	High	73	C	M	ADSQ	Epi		620	MUT	WT	WT	WT	WT	Primary	3	N	Y	
HBEC3-KT	High	65		F	Immortalized Benign		Y		WT	WT	WT	WT	WT					
HBEC30-KT	High	62	C	F	Immortalized Benign		Y		WT	WT	WT	WT	WT					
HCC3051	High	63	B	M	LCC	Mes		306	MUT	WT	WT	WT	WT					
HCC4019	High	40	C	M	ADN	Epi	Y	502	MUT	Codon 12	WT	WT	MUT		4			
HCC4054	High	73	C	M	ADN			280	MUT	Codon 12	WT	WT	WT	Primary	1			
HCC44	High	54	C	F	ADN	Mes		408	MUT	Codon 12	FS	WT	MUT	Distant Metastasis				
HCC515	High	39	C	F	ADN	Epi		980	MUT	Codon 13	SS	WT	MUT	Lymph Node				
HCC78	High	55	C	M	ADN	Epi		260	MUT	Codon 12	WT	Other	WT	Distant Metastasis				
HCC827	High	38	C	F	ADN	Epi		251	MUT	WT	WT	Exon 19	WT	Primary				
HOP-62	High			F	ADN	Mes		708	MUT	Codon 12	WT	WT	WT					
H1373	High	56	B	M	ADN	Epi	Y	981	MUT	Codon 12	WT	WT	WT	Primary	3	Y	Y	ND
HCC2108	High			M	ADN	Mes		178	MUT	Other	MS	WT	WT	Lymph Node				
HCC2450	High	52	C	M	SCC	Epi		1926	WT	WT	WT	Other	WT		3			
A549	Low	58	C	M	ADN	Mes		462	WT	Codon 12	NS	WT	MUT	Primary				
H1299	Low	43	C	M	LCC	Mes	Y	524	MUT	WT	WT	WT	WT	Lymph Node	3	N	Y	PD
H1437	Low	60	C	M	ADN	Epi	Y	366	MUT	WT	HD	WT	WT	Distant Metastasis	1	N	N	
H1563	Low			M	ADN	Mes		763	WT	WT	NS	WT	WT	Primary		N	N	
H1792	Low	50	C	M	ADN	Mes	Y	518	MUT	Codon 12	WT	WT	MUT	Distant Metastasis	4	N	N	PD
H1819	Low	55	C	F	ADN	Epi	Y	295	MUT	WT	WT	WT	WT	Lymph Node	3	Y	N	PR
H1944	Low	62	C	F	ADN	Mes	Y	643	WT	Codon 13	MS	WT	MUT	Distant Metastasis	3	N	Y	
H1993	Low	47	C	F	ADN	Epi	Y	525	MUT	WT	NS	WT	MUT	Lymph Node	3	N	N	
H2030	Low			M	ADN	Mes	N	493	MUT	Codon 12	NS	WT	MUT	Lymph Node		N	N	
H2085	Low	45		M	ADN	Epi		474	MUT	WT	WT	WT	WT	Primary		N	N	

H226	Low			M	SCC M	Mes		374	WT	WT	WT	WT	WT	Distant Metastasis		N	N	
H23	Low	51	B	M	ADN	Mes	Y	1182	MUT	Codon 12	NS	WT	MUT	Primary		N	N	PD
H3255	Low	47	C	F	ADN	Epi	N	322	MUT	WT	WT	Exon 21	WT		3			
H358	Low		C	M	ADN	Epi		1194	MUT	Codon 12	WT	WT	WT	Primary		N	N	
H460	Low			M	LCC	Mes		588	WT	Other	NS	WT	MUT	Distant Metastasis		N	N	
H820	Low	53	C	M	ADN	Epi		358	MUT	WT	WT	Exon 19	WT	Lymph Node	4	Y	N	PD
HCC1171	Low	58	C	M	NSCLC	Epi		373	MUT	Codon 12	WT	WT	WT	Primary				
HCC2935	Low	39	C	M	ADN	Epi	N	339	MUT	WT	WT	Exon 19	MUT	Primary				
HCC461	Low	69	C	M	ADN	Mes		164	WT	Codon 12	WT	Other	WT	Primary				
Calu-6	Low	61	C	F	ADN	Mes		314	MUT	Other	WT	WT	WT	Primary				
EKVX	Low			M	ADN	Mes		617	MUT	WT	MS	WT	WT					
H1048	Low	53		F	SCLC	Epi		995	MUT	WT	WT	WT	WT	Distant Metastasis		N	N	
H1395	Low	55	C	F	ADN	Mes	Y	208	WT	WT	FS	WT	WT	Primary	2	N	N	
H157	Low	59	C	M	SCC	Mes	Y	1608	MUT	Codon 12	WT	WT	MUT	Distant Metastasis	3	N	N	PD
H1734	Low	56	C	F	ADN	Mes		715	MUT	Codon 13	FS	WT	WT	Primary		N	N	
H2073	Low	47	C	F	ADN	Mes	Y	487	MUT	WT	NS	WT	WT	Primary	3	Y	N	
H2086	Low	45		M	ADN	Epi		460	MUT	WT	WT	WT	WT	Lymph Node		N	N	
H441	Low	33		M	ADN	Epi		768	MUT	Codon 12	WT	WT	WT	Distant Metastasis	3	N	N	
H446	Low	61	C	M	SCLC	Mes		866	MUT	WT	WT	WT	WT	Distant Metastasis				
H838	Low	59	C	M	ADN	Mes	Y	680	MUT	WT	FS	WT	MUT	Lymph Node	3	N	N	
HCC15	Low	55	B	M	SCC	Mes		674	MUT	WT	WT	WT	WT	Lymph Node				
HCC1833	Low	69	C	F	ADN	Epi		394	MUT	WT	MS	WT	WT	Primary				
HCC193	Low	71	C	F	ADN	Epi		263	MUT	WT	WT	WT	WT	Distant Metastasis				
HCC4087	Low	82	C	F	ADSQ		Y	235	MUT	Codon 13	WT	WT	MUT					
HCC446	Low	49		F	ADN			161	MUT	WT	WT	WT	WT	Primary				
Calu-1	Low	47	C	M	MEC	Mes		383	MUT	Codon 12	WT	WT	WT	Distant Metastasis				
DFCI024	Low			F	ADN	Mes	N	477	WT	Codon 12	WT	WT	MUT					
H1573	Low	35	C	F	ADN	Epi	Y	1526	MUT	Codon 12	MS	WT	MUT	Distant Metastasis	4	N	Y	
H1755	Low	65	C	F	ADN	Epi	Y	491	MUT	WT	MS	WT	MUT	Distant Metastasis	4	N	N	
H1975	Low			F	ADN	Epi	N	450	MUT	WT	WT	Exon 20	WT	Primary		N	N	
H2087	Low	69	C	M	ADN	Epi	Y	602	MUT	WT	WT	WT	WT	Lymph Node	1	N	N	PD
H2172	Low			F	NSCLC	Mes		1623	MUT	WT	HD	Other	MUT	Primary		N	N	
H2347	Low	54	C	F	ADN	Epi		919	MUT	Codon 13	WT	WT	WT	Primary	1	N	N	
H2887	Low	31		M	NSCLC	Mes		152	MUT	Codon 12	WT	WT	WT		4			
H647	Low	56	C	M	ADSQ	Epi	N	407	MUT	Codon 13	SS	WT	MUT	Distant Metastasis	3	N	Y	
H650	Low			M	ADN	Mes	N	2077	MUT	Other	WT	WT	WT	Lymph Node		N	N	
H661	Low	43	C	M	LCC	Mes		791	MUT	WT	WT	WT	MUT	Lymph Node	3	Y	Y	MR
H920	Low	44	C	M	ADN	Mes	Y	1288	MUT	WT	WT	WT	MUT	Lymph Node	4	N	Y	PD

HCC2374	Low	69	B	F	LCN	Epi		806	MUT	Codon 12	WT	WT	WT	Primary				
HCC95	Low	65	C	M	SCC	Epi		288	MUT	WT	WT	WT	WT	Distant Metastasis	4			
HSAEC1-KT	Low	22	B	M	Immortalized Benign		Y		WT	WT	WT	WT	WT					

Legend: C = Caucasian, B = Black, M = Male, F = Female, ADN = adenocarcinoma, MEC = Muco-epidermoid carcinoma, LCN = Large cell neuroendocrine, ADSQ = adenosquamous, SCC = Squamous cell carcinoma, LCC = Large cell, M = Mesothelioma, NSCLC = Non-small cell lung cancer, SCLC = Small cell lung cancer, P = Primary site, L = Lymph node metastasis, M = Metastasis/Malignant pleural/pericardial effusion, Mes = Mesenchymal, Epi = Epithelial, TMB = Total mutational burden, Y = Yes, N = No, WT = Wild Type, MUT = Mutant, Codon 12 = KRAS Codon 12 mutant, Codon 13 = KRAS Codon 13 mutant, HD = Homozygous deletion, NS = Nonsense substitution, FS = Frameshift substitution, MS = Missense substitution, SS = Splice site mutation, Exon 19 = *EGFR* Exon 19 mutant, Exon 20 = *EGFR* Exon 20 mutant, Exon 21 = *EGFR* Exon 21 mutant, Other = alterative mutation sites; PR = Partial response; PD = Progressive disease; ND = No disease; MR = Metabolic response; Blank cells indicate unavailable data

Supplemental Table S2: Transcriptome and DNA mutation profile-matched TCGA-deposited NSCLC clinical samples to patient-derived NSCLC samples studied in the co-culture model. Lung cancer cell lines were matched to patient-derived lung tumor specimens from the TCGA NSCLC datasets using RNAseq expression and somatic mutation data (**Supplemental Methods**). Specifically, the best tumor specimen match for each cell line was chosen based on the Pearson correlation of 2000 genes together with a mutation similarity score for 700 cancer-related genes.

Cell line	TCGA-Matched Patient Sample
H596	TCGA-66-2759-01
HCC827	TCGA-75-6203-01
HOP-62	TCGA-64-5775-01
H1373	TCGA-38-4629-01
H1693	TCGA-J2-A4AD-01
HCC515	TCGA-05-4395-01
HCC3051	TCGA-22-0944-01
HCC44	TCGA-05-4395-01
H1650	TCGA-75-6207-01
H1355	TCGA-05-4415-01
H2122	TCGA-05-4415-01
H2009	TCGA-55-A493-01
HCC2108	TCGA-05-4415-01
H2258	TCGA-62-A46Y-01
H2030	TCGA-05-4415-01
H1437	TCGA-50-5939-01
H820	TCGA-95-8494-01
EKVX	TCGA-MP-A4TA-01
H1792	TCGA-05-4395-01
HCC461	TCGA-55-8090-01
HCC1171	TCGA-MP-A4T4-01
H1993	TCGA-55-7913-01
H23	TCGA-05-4415-01
H441	TCGA-95-7567-01
H2085	TCGA-97-8175-01
H1819	TCGA-60-2711-01
H647	TCGA-05-4395-01
H2887	TCGA-05-4433-01
H2073	TCGA-05-4415-01
HCC95	TCGA-43-6770-01
Calu-6	TCGA-95-8494-01

H2347	TCGA-95-7567-01
H1734	TCGA-05-4415-01
H1755	TCGA-66-2754-01
H2087	TCGA-95-8494-01
HCC15	TCGA-05-4415-01

Supplemental Materials and Methods

Fingerprinting ID

All cell lines were verified by DNA fingerprinting with the Promega Fusion system (Cat# DC2408) which consists of 24 short tandem repeat (STR) markers. These loci collectively provide a genetic profile with a random match probability of 10^{-28}. Fingerprints were compared against our database of more than 10,000 reference fingerprints that were collected from ATCC (www.atcc.org), DSMZ (www.dsmz.de), JCRB (cellbank.nibiohn.go.jp), RIKEN (en.brc.riken.jp), Cellosaurus (web.expasy.org/cellosaurus), and from our own resources. [42] A match is called between two fingerprints when at least 80% of the alleles are identical according to the shared allele match algorithm defined by the International Cell Line Authentication Committee.

qPCR

RNA was extracted with the RNeasy Mini Kit (QIAGEN) and QIAcube robot (QIAGEN) following the manufacturer's recommended protocol. 1 µg of total RNA was mixed with qScript cDNA SuperMix for cDNA synthesis (BioRad) per the manufacturer's protocol. After reverse transcription, qRT-PCR was performed with SYBR Green (BioRad) following the manufacturer's recommended protocol for marker (*Actin, iNos, Il6, Arg1, Ym-1, Il-1b, Socs3*). Mouse specific primers were used to ensure only macrophage transcripts would be detected. All primers were

cross-examined for no activity on human RNA. qRT-PCR was performed on a CFX384 Touch Real-Time PCR Detection System (BioRad). The cycling program was 95°C for 10 min, 95°C for 15 s, and 60°C for 40 cycles. Each sample was run in quadruplicate, normalized to the actin probe, and then normalized to macrophage baseline, and analyzed by the comparative CT method.

Supplemental Table S3: Primers

Gene Name	Forward Primer	Reverse Primer
Actin	CTGAGAGGGAAATCGTGCGT	AGGGTGTAAAACGCAGCTCAG
Arginase-1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
iNOS	GTTCAGCTACGCCTTCAACAC	CAAGGCCAAACACAGCATACC
Il-6	CGTGGAAATGAGAAAAGAGTTGTGC	TGGTACTCCAGAAGACCAGAGG
Ym-1	TCTGGGTACAAGATCCCTGAA	TTTCTCCAGTGTAGCCATCCTT
Il-1β	TGCCACCTTTTGACAGTGATG	TTCTTGTGACCCTGAGCGAC
Socs3	CAAAAATCCAGCCCCAACGG	GGCTGGCTCCACTTGAAAGA

qPCR data processing and analysis

All qPCR processing was completed in R (see **Supplemental File 2: "Co-culture qPCR R code.R").** All PCR results were first combined in a single table containing 7 columns (**Supplemental File 3: "Co-culture qPCR raw data"-Note: available on request**): Group (which refers to different PCR plate results analyzed together as a biological replicate group), Plate (name of the PCR plate), Plate_Sort (for sorting plates by date), Well, Target, Sample, and Cq. Technical replicates for each assay within each plate were averaged, excluding Cq values = 40, unless all such values for a given assay were 40. The resulting average was called

Cq_mean. Next, for each sample within each plate, the Cq_mean values (there is one for each target) were normalized to the Cq_mean of *Actin* for that sample. Specifically, for sample "x" and target "y", the *Actin* normalized value equaled Cq_mean(sample x, target y) - Cq_mean(sample x, *Actin*). These normalized values were generally positive since *Actin* is highly expressed (lower Cq value). However, these values were discarded when *Actin* Cq > 25 as these indicated a problem with the PCR assay. We then normalized each sample to the macrophage sample control, of which there was one per group (one group may comprise multiple plates). If an assay was done on more than one plate within a group, the median of its normalized values was used (called act_norm_Cq_median). Thus, for sample x and target y, the macrophage normalized values = act_norm_Cq_median(sample x, target y) - act_norm_Cq_median(macrophage control, target y). These values (called mac_norm Cq) may be either positive (higher expression in the macrophage control), or negative (higher expression in the test sample). The final results were calculated as 2^(-mac_norm_Cq) and represent expression fold change compared to the macrophage control (**Supplemental File 4: "Co-culture qPCR processed data"- Note: available on request**). Box plots of negative mac_norm_Cq values were generated for each sample and target, with the datapoints representing the biological replicates (**Supplemental File 1: "Co-culture qPCR box plots ").** The standard deviation for *Arginase-1* expression was calculated for each sample, to show reproducibility for this gene (**Supplemental File 5: "Co-culture qPCR box plots - arginase summary" and Supplemental Figure 1A**). A heatmap shows the group mean values for each sample-target pair (same as the mean of each box plot) (**Supplemental File 6: "Co-culture qPCR processed data for heatmap"** and **Figure 2.C**).

DNA/RNA extraction for sequencing

DNA for exome or genome sequencing was purified from frozen cell line pellets using DNeasy reagents and protocols with QIAcube robot (QIAGEN). DNA spectra were quantitated using spectrophotometer (Nanodrop) and samples diluted with nuclease free water (Ambion). Cell lines were grown to approximately 70%–80% confluence, washed 2X with PBS and directly lysed from culture flasks using RLT buffer (QIAGEN). Lysates were snap frozen and stored at -80°C. RNA was purified from lysates using RNeasy kit and QIAcube robot (QIAGEN).

RNA sequencing

RNA samples isolated from multicellular co-cultures experiments were submitted to Novogene genome sequencing company (Sacramento, CA) or UT Southwestern Next Generation Sequencing Core. The processing centers performed quantitative and qualitative assessment of the RNA samples including the RNA integrity and contamination. Novogene prepared the libraries using poly-Y oligoattached magnetic beads. UTSW NGSC prepared the libraries with Illumina's TruSeq Stranded mRNA library prep kit (Illumina, catalog # RS-122-2101) following manufacturer protocols. Novogene sequenced the libraries on the their HiSeq/MiSeq Illumina machines with 150 nucleotide paired-end reads for an average of 60 million total reads. UTSW NGSC sequenced the libraries with the Illumina NextSeq 500 using V2 reagents and 75 nucleotide single-end reads for an average of 50 million reads. Reads were aligned to the human reference genome GRCh38 and/or mouse reference genome GRCm38 using STAR-2.7 (https://github.com/alexdobin/STAR) followed by read duplicate removal with MarkDuplicates (gatk-4.1.2.0; https://gatk.broadinstitute.org). For co-culture samples, human reads were removed by comparing alignment scores (AS flags in bam files) in the human and mouse alignments: reads for which the human AS value was higher than the mouse AS value were removed (gatk-4.1.2.0 FilterSamReads). FPKM values were generated with cufflinks-2.2.1 (http://cole-trapnell-lab.github.io/cufflinks/). These were then normalized (upper-quartile

normalization: Bullard et al, Bioinformatics 2010, 11:94), and log-transformed. The bulk RNAseq data will be available GEO accession number GSE200627.

TCGA Matchup

We compared each lung cancer cell line with each lung tumor from the TCGA NSCLC datasets using RNAseq expression and somatic mutation data. The expression similarity measure was the Pearson correlation (between the tumor lines and the TCGA samples) on the 2,000 most variably expressed genes (derived from the combined cell line and TCGA RNAseq datasets) while the mutation similarity measure used a concordance value on 700 cancer genes (Cancer Gene Census, COSMIC, https://cancer.sanger.ac.uk/census) defined as the number of genes mutated in both cell line and TCGA samples divided by the number of genes mutated in either sample (with genes weighted by their mutation frequency in the tumor sets). Note: the R code for this matchup algorithm is available upon request (luc.girard@utsouthwestern.edu) to allow anyone to repeat this analyses. A combined score showed the degree of similarity between these cell lines and TCGA specimen is available Bulk RNA sequencing from TCGA matched samples were processed using CIBERSORT to deconvolute relative immune cell populations within the TME. [22]

Human umbilical cord blood sample processing

Human umbilical cord blood samples were obtained from UT Southwestern-Parkland Memorial Hospital, in compliance with all associated regulations and approvals of use regarding human cord blood (STU: 112010-047) at UT Southwestern Medical Center. Sterile blood was obtained at the time of cesarean section from de-identified human umbilical cords that are normally discarded. The procedure is approved through a protocol exempt from informed consent as

approved by the UT Southwestern Medical Center Institutional Review Board and the Office for Human Research Protections (OHRP) supported by the U.S. Department of Health and Human Services. To ensure anonymity, links between the donor's medical and social histories including fetal sex are not maintained.

Primary human umbilical cord blood-derived monocyte isolation and macrophage differentiation

Mononuclear cells were isolated through Ficoll (Cytiva, Cat# 17544203) differential centrifugation. Harvested mononuclear cells were then subjected to washing and red blood cell lysis. Human primary monocytes were then negatively enriched through immunomagnetic selection (Stem Cell Technologies, Cat# 19359). Isolated human primary monocytes were then seeded at 1 x 10^6 cells/ml in a 10-cm petri dish cultured with RPMI1640 (Millipore Sigma, R8758) supplemented with 10% heat deactivated FBS (ThermoFisher Scientific, Cat# 26140079) to avoid potential immunogenicity. Human M-CSF (50 ng/ml) was supplemented to the medium to allow macrophage induction every 2 days for 7 days. Macrophages alone were seeded at 1.0 x 10^5 cells per well, similar to the mouse-macrophage co-culture protocol. Positive controls for M1 and M2 polarization were established with LPS (20 ng/mL, 4-hour stimulation) and IL-4 (20 ng/mL, 18-hour stimulation), respectively. Co-culture replicates were established with identical proportions of patient-derived NSCLC cells, CAFs, and macrophages as the mouse-macrophage co-culture model.

Flow Cytometry Analysis

Co-cultured cells were scraped and collected from media. Samples were incubated with human anti-Fc blocker (BD Biosciences, Cat# 564220) for 15 min at 4˚C to block potential non-specific

binding of conjugated antibodies. Anti-human CD45, Pacific Blue (Biolegend, Cat# 304021), anti-human CD68 (pan-macrophage marker), PE-Cyanine 7 (BD Biosciences, Cat# 565595), and anti-human CD206 (M2-like phenotype marker), APC (BD Bioscience, Cat# 321138) were incubated with harvested co-culturing cells for 30 min at 4˚C. Fixable viability Dye eFluor 780 (eBioscience, Cat# 65-0865-18) was used to exclude dead cells. Data were collected and analyzed on CytoFLEX flow cytometer (Beckman Coulter Inc.) and associated software. Unstained samples and tumor cell only samples were used for gating out negative populations.

Immunohistochemistry (IHC) and quantification

For IHC, tumors were fixed in 10% Formalin (VWR, catalog # 89370-094) for 72 hours with slight agitation at RT, then embedded in paraffin, sectioned into 5-micron slices and placed onto positively charged slides. Immunohistochemistry (IHC) was performed as previously described. [20] Slides were heated at 60°C for 10 minutes, deparaffinized and rehydrated. Antigen retrieval was performed with a Biocare Medical Decloaking Chamber at 110°C for 17 minutes using Antigen Unmasking Solution, TRIS-BASED (Fisher Scientific, catalog # NC9800748) then allowed to cool to room temperature for 30 minutes. Slides were washed for 5 minutes with PBS, incubated in ice-cold 10% methanol for 10 minutes at room temperature, washed with de-ionized water for 5 minutes, then tissue was blocked for 30 minutes using appropriate blocking solution. Blocking buffer was removed, then primary antibody diluted in Renaissance Buffer (BioCare Catalog # PD905 L) and added to samples at 4°C overnight with agitation. Samples were washed 3 times with 0.5% PBST for 5 minutes, once with PBS for 5 minutes, then secondary antibody solution was added for 30 minutes with agitation at room temperature. Samples were washed once with 0.2% PBST for 5 minutes, twice with 0.5% PBST for 5 minutes, then a solution of 1:250 with the appropriate opal dye in 1X Plus Amplification Diluent (PerkinElmer, catalog # FP1498) was made and added to samples for 3 minutes. After 3

minutes, the solution was quickly aspirated and washed with .05% PBST 3 times, then washed with PBS + 2 mM EDTA for 10 minutes. Sub-sequential staining rounds follow the same protocol for each stained marker, apart from Antigen Unmasking Solution, citrate-based (Fisher Scientific, catalog # H-3300) is used instead of Antigen Unmasking Solution, TRIS-BASED (Fisher Scientific, catalog # NC9800748). The final staining round DAPI was added during the secondary incubation at 1 ug/mL concentration. Slides were then mounted with Vectamount AQ Aqueous Mounting Medium (Fisher Scientific catalog # H550160) and cover slipped (VWR catalog # 48404-133). Images were captured at 40X magnification using Vectra Polaris Slide Scanner (AKOYA Biosciences, Delaware, USA). Images were then deconvoluted and re-stitched using Phenochart and inForm software (Akoya Biosciences). The reconstituted images underwent multiplex quantitative analysis using HALO software (Akoya Biosciences). Tumor borders, regions of necrosis, and visible stroma were annotated and cross-checked to each tissue's associated H&E stain. For individual macrophage subtype characterization, Arginase-1(+) macrophages were characterized as F4/80+/ARG (+). Primary tumor cells were marked as pan-cytokeratin (+). All nuclei were identified with DAPI staining. Cell quantity, distribution in the tumor area, and density of each subtype of macrophage were analyzed for the overall tumor area, areas of necrosis, and stromal regions. Spatial infiltrative analyses into regions of necrosis or stroma were also conducted. Quantitative data were abstracted from analyses from each tumor section and analyzed on GraphPad Prism statistical analysis software (GraphPad Software, CA, USA). Quantitative IHC cell count and density analyses were conducted using Mann-Whitney U non-parametric Y-tests.

Tissue was stained with multiple rounds of antigen retrieval.

1st: Blocking buffer = Rodent Block (Biocare Medical catalog # RBM961H), Primary = Pan-Cytokeratin (Fisher Scientific # NC0581968, 1:300), Secondary = anti-Mouse HRP (Fisher Scientific # NC0141382), Opal – Opal 520 (Perkin Elmer # FP1487001KT)

2nd: Blocking Buffer = 2.5% Goat Serum (Fisher Scientific # NC0533036), Primary = Arginase1 (Cell Signaling # 936685, 1:500), Secondary = anti-Rabbit HRP (Fisher Scientific # MP-7451), Opal = Opal 570 (Perkin Elmer # FP1488001KT)

3rd: Blocking Buffer = 2.5% Goat Serum (Fisher Scientific # NC0533036), Primary = F4/80 (Fisher Scientific # NC1397643, 1:500), Secondary = anti-Rabbit HRP (Fisher Scientific # MP-7451), Opal = Opal 620 (Fisher Scientific # NC1612059)

4th: DAPI (Sigma Aldrich # D9542-10MG) added to secondary block solution for the final round of staining for nuclear identification