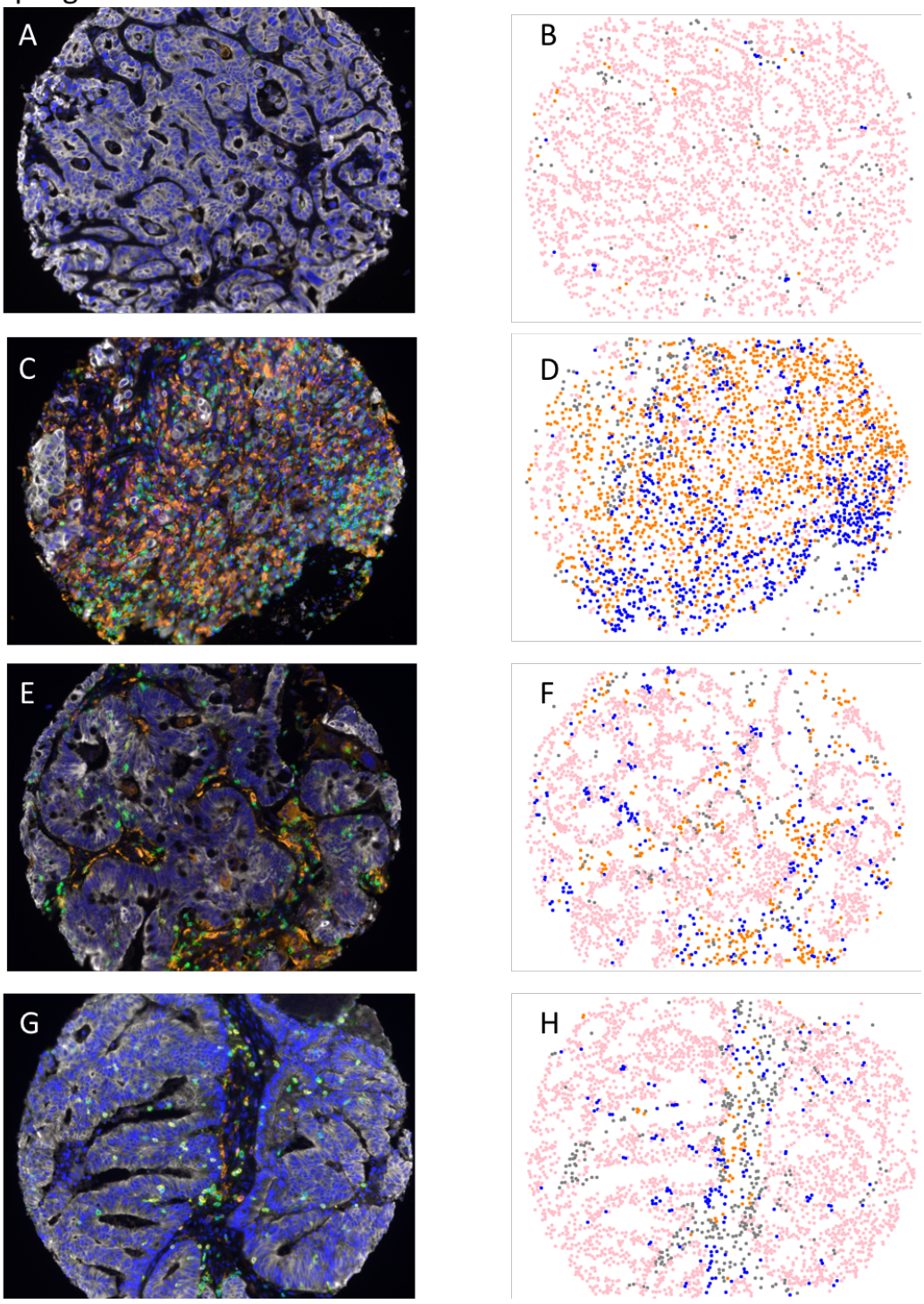
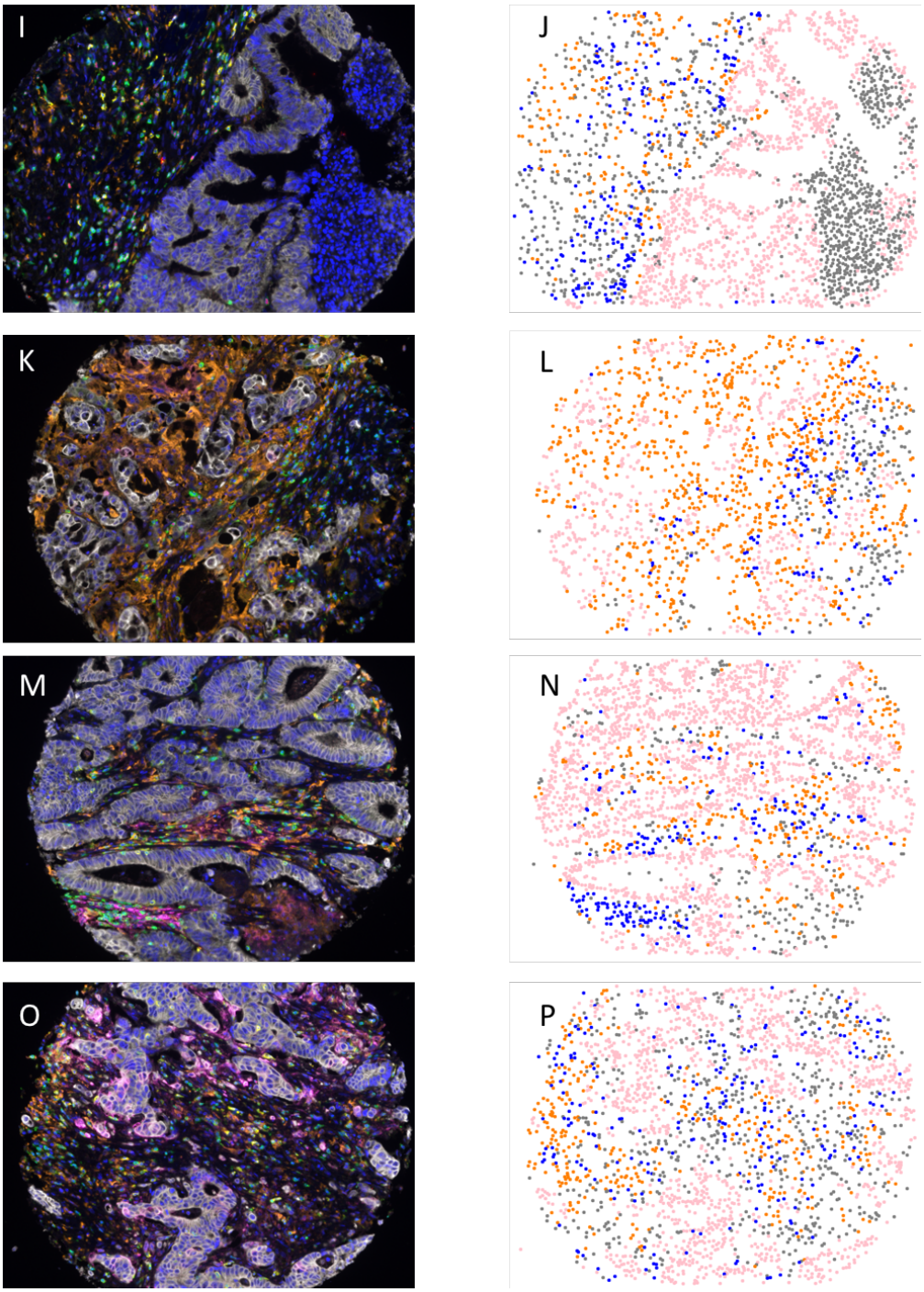
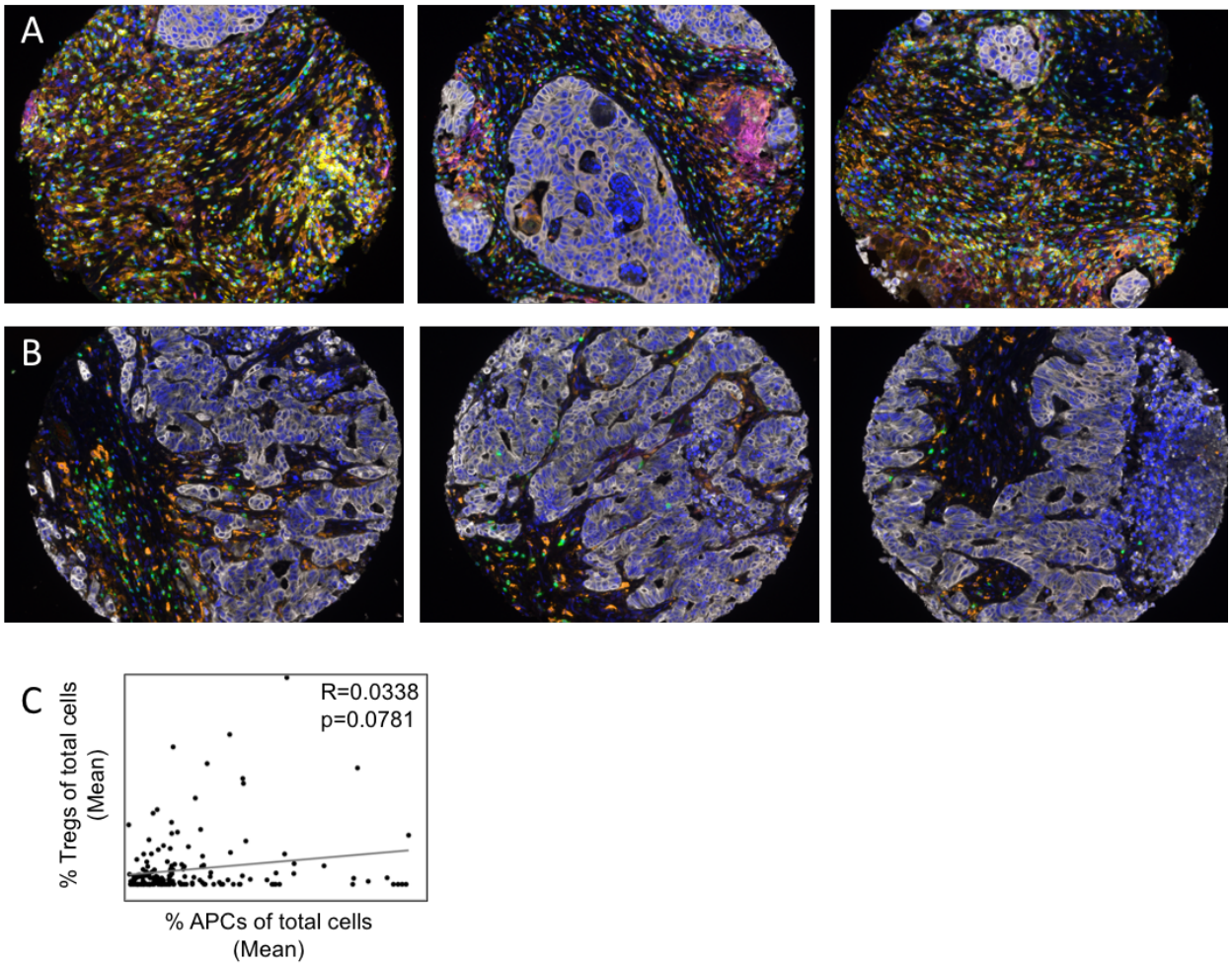
Sup Figure 1





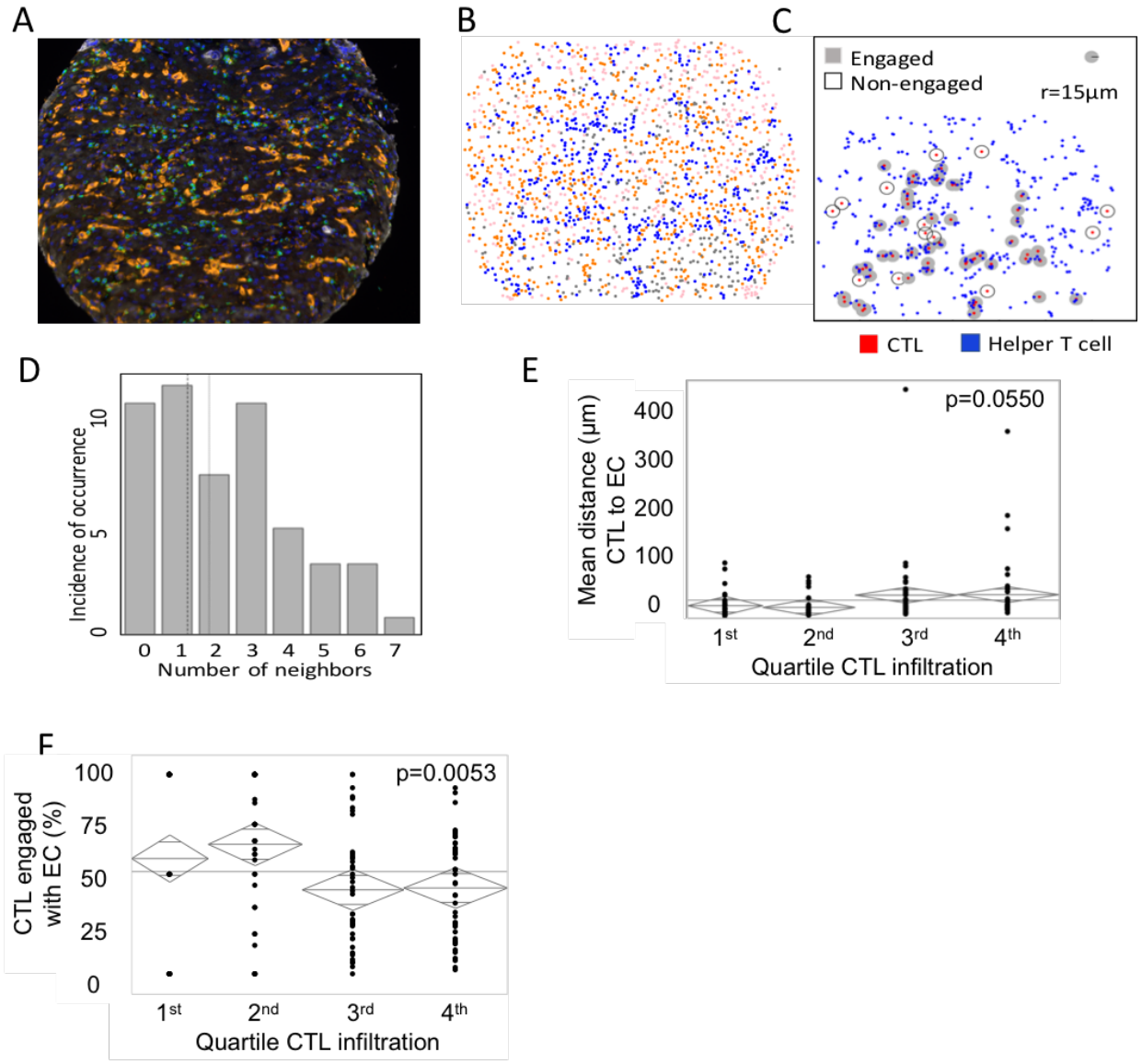
Supplemental Figure 1: *The immune infiltrate of colorectal liver metastases (CRLM) is heterogeneous.* Multiplex fluorescent immunohistochemistry (mfIHC) and immune cell phenotyping was applied to samples from 177 patients with resected CRLM (20x magnification)(white – epithelial tumor cell (EC), green – helper T cell, yellow – cytotoxic T lymphocyte (CTL), red – T_{reg}, orange – antigen presenting cell (APC), magenta – PD-L1). Immune infiltrates varied from (A,B) relatively devoid of immune cells to (C,D) high infiltration. Predominant cell types included (E,F) helper T cells, (G,H) cytotoxic T lymphocytes (CTLs), (I,J) T_{regs}, (K,L) APCs, (M,N) PD-L1^{+}APCs and (O,P) PD-L1^{+} epithelial cells (ECs).

Sup Figure 2



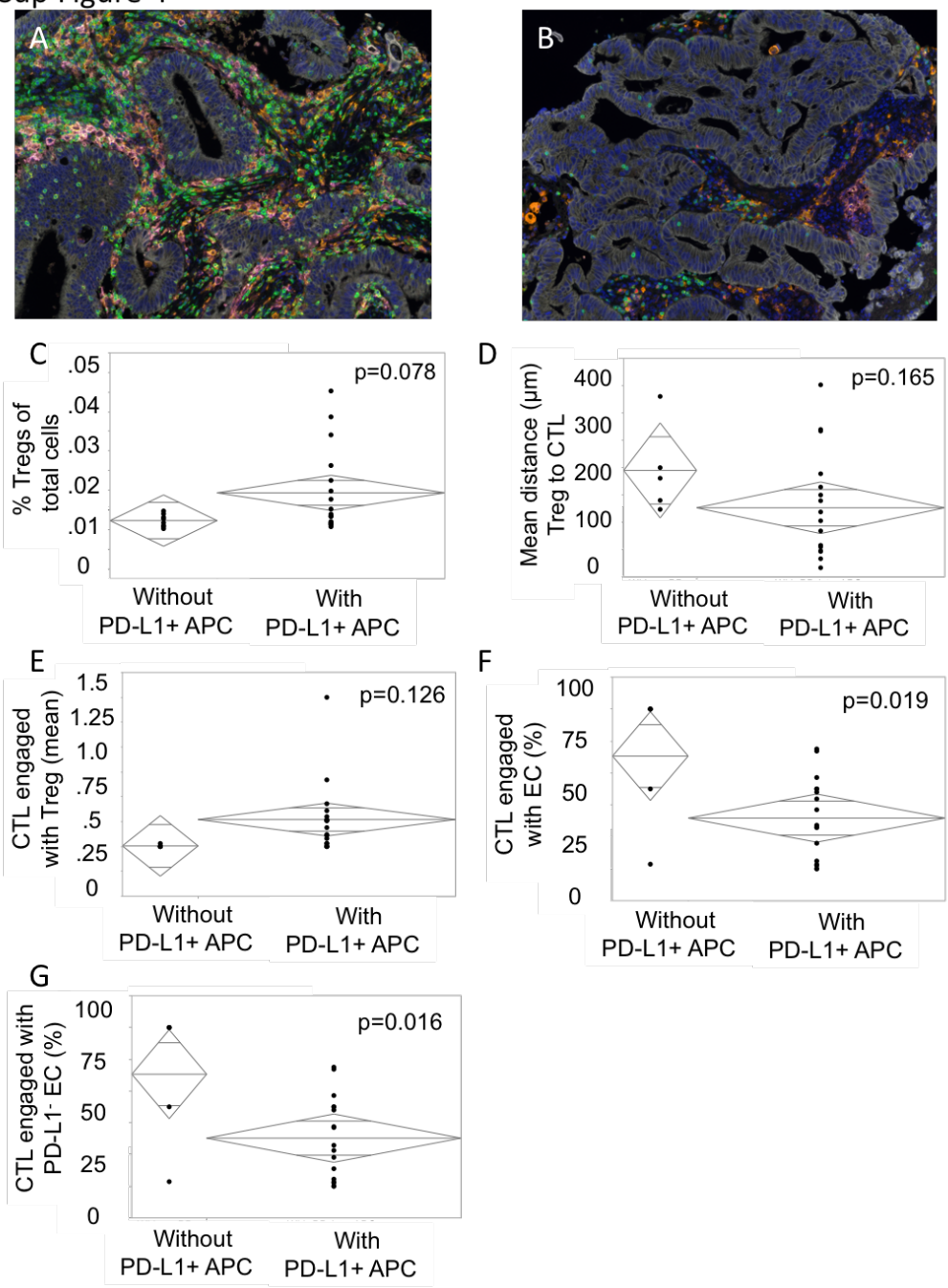
Supplemental Figure 2: *Intra-tumoral heterogeneity varied amongst patients.* Intra-tumoral heterogeneity varied with some patients displaying great differences between cores (A) while other remained relatively consistent (B) (20x magnification)(white – epithelial cell (EC), green – helper T cell, yellow – cytotoxic T lymphocyte (CTL), red – T_{reg}, orange – antigen presenting cell (APC), magenta – PD-L1). (C) Lack of correlation between antigen presenting cells (APC) and T_{reg} infiltration confirms that associations with cytotoxic T lymphocytes (CTLs) are not due to non-specific increase in immune cell infiltration.

Sup Figure 3



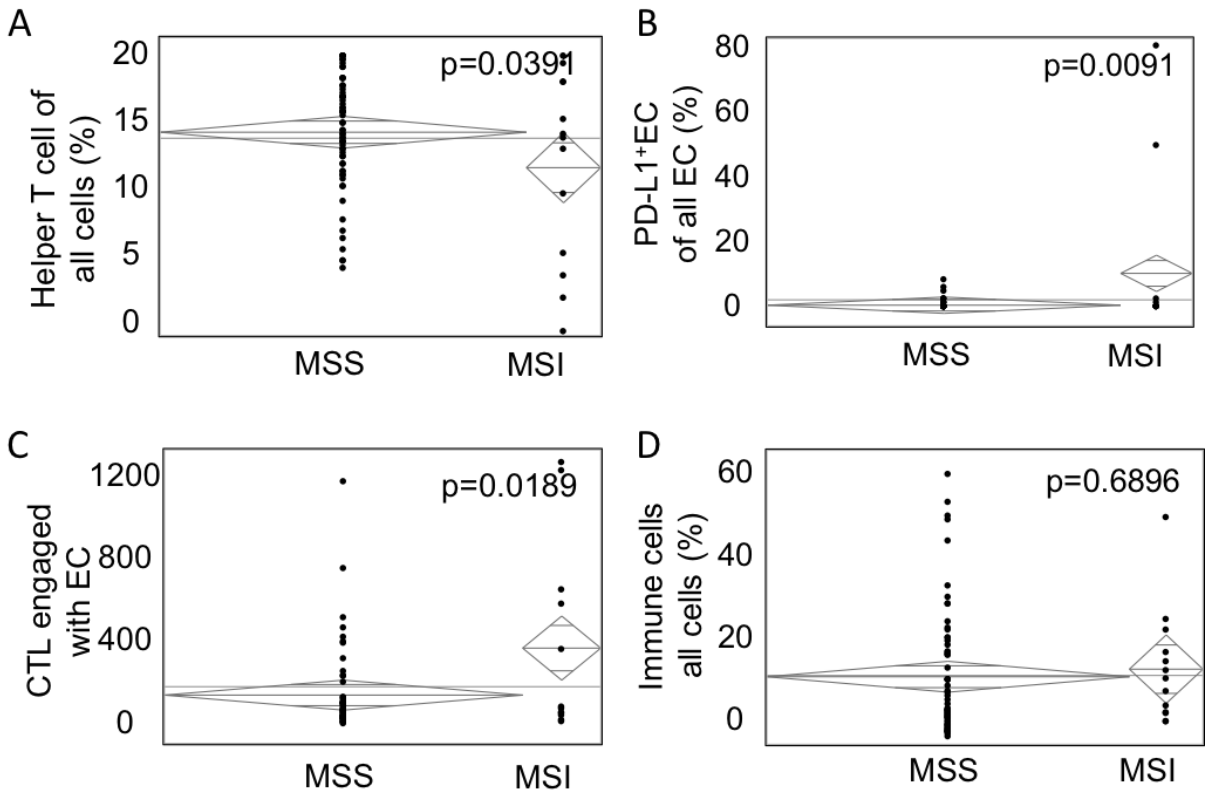
Supplemental Figure 3: *Degree of infiltration had a paradoxical effect on engagement and intercellular distance.* After multiplex fluorescent immunohistochemistry (mfIHC) (A), cells were phenotyped (B) and assigned coordinates. (C) Frequency of engagement of cytotoxic T lymphocytes (CTLs) and helper T cells determined for each core. (D) Mean number of neighbors per engaged cell is calculated. (E,F) To confirm that differences in CTL/epithelial cell (EC) engagement and intercellular distances was not simply a marker for increased infiltration, patients were divided into quartiles from lowest (1st quartile) to highest (4th quartile) CTL infiltration. Paradoxically, samples with the greatest concentration of CTLs tended to have greater intercellular distances (E) and less EC engagement (F).

Sup Figure 4



Supplemental Figure 4: *Validation of the impact of PD-L1^{+} antigen presenting cells (APCs) on the immune microenvironment using an independent cohort.* To validate our findings, an independent cohort was created consisting of 19 patient samples representing colorectal metastases to liver (n=9), lung (n=7) and bone (n=3). Variability of PD-L1^{+}APC infiltration was again seen with abundance in some (A; n=11) and absence in others (B; n=8). Similar to our findings in the larger cohort, presence of PD-L1^{+}APCs was associated with a trend towards increased T_{reg} infiltration (C) which tended to closer (D) and more engaged (E) with cytotoxic T lymphocytes (CTLs). They further shaped the microenvironment by decreasing CTL engagement with epithelial cells (ECs) and in particular PD-L1^{-}ECs.

Sup Figure 5



Supplemental Figure 5: *Characteristics of patient with microsatellite instability (MSI) and microsatellite stability (MSS)* A subset of 77 patient samples were subjected to immunohistochemistry (IHC) analysis and 13 determined to be deficient in one or more mismatch repair proteins. ANOVA was used to compare frequency of (A) helper T cells of total cells and (B) PD-L1^{+} epithelial cells (ECs) of total ECs between MSS and MSI patients. (C) Difference in engagement of cytotoxic T lymphocyte (CTLs) with ECs and (D) total immune infiltrate was also examined by ANOVA.

Phenotype	Markers	Color
PD-L1^{-} Epithelial cell	PD-L1^{-} Pancytokeratin^{+}	White
PD-L1^{+} Epithelial cell	PD-L1^{+} Pancytokeratin^{+}	Light purple
Helper T cell	CD3^{+}CD8^{-} FoxP3^{-}	Green
Cytotoxic T cell	CD3^{+}CD8^{+}	Yellow
Regulatory T cell	CD3^{+}CD8^{-}FoxP3^{+}	Red
PD-L1^{-} Antigen Presenting Cell	CD163^{+}PD-L1^{-}	Orange
PD-L1^{+} Antigen Presenting Cell	CD163^{+}PD-L1^{+}	Magenta

Supplemental Table 1: *Multiplex fluorescent immunohistochemistry (mfIHC) allows for phenotyping and visualization of multiple different cell types in a single tissue core.*

	%CTL of total	%Th of total	% APC of total	%PD-L1^{+}APC of total	%EC of total
Sample A	27.7	6.7	46.9	10.9	6.3
Core 1	4.9	6.9	18.5	6.5	47.3
Core 2	15.3	10.9	53.3	9.9	8.2
Core 3					
Sample B	0.2	5.6	11.4	0	67.0
Core 1	0.7	1.1	3.3	0.1	86.7
Core 2	0	1.1	2.2	0	85.4
Core 3					

Supplemental Table 2: *Variability of the immune microenvironment in cores taken from the same patient.*

Phenotype	Distance (µm)	CI
PD-L1^{+}EC	181.9	158.5 - 205.4
PD-L1^{-}EC	31.1	24.0 - 38.3
Helper T cell	30.0	25.0 - 35.1
Regulatory T cell	166.7	140.7 - 180.7
PD-L1^{-}APC	32.1	27.2 - 37.0
PD-L1^{+}APC	117.4	98 - 136.7

Supplemental Table 3: *Mean intercellular distance from cytotoxic T lymphocytes (CTLs) and neighboring cells.* After imaging and phenotyping, cells were assigned locations on x and y axes. Weighted mean distances between CTLs, Helper T cells, T_{regs}, antigen presenting cells (APCs) and epithelial cells (ECs) was determined.

	MSI n=13	MSS n=64	p value
Mean tumor size	3.82cm	4.88cm	0.3120
Numerous tumors	7.69%	29.7%	0.0903
DFI	18.7mos	16.6mos	0.6615

Supplemental Table 4: *Tumor characteristics of patients with and without microsatellite instability (MSI)*. There were no significant differences in largest tumor size or disease-free interval but a trend towards more numerous tumors

PRIMARY ANTIBODY/ANTIGEN	COMPANY-CATALOGUE NUMBER	DILUTION	SECONDARY ANTIBODY	COMPANY-CATALOGUE NUMBER	OPAL TSA™ FLUOROPHORE
CD8	SpringBio-M5390	1:400	Opal Polymer®	ARH1A01EA	570
CD3	Dako-A0452	1:400	Opal Polymer®	ARH1A01EA	520
CD163	Leica-NCL-L-CD163	1:400	Opal Polymer®	ARH1A01EA	650
PDL1	CST-13684	1:200	Opal Polymer®	ARH1A01EA	540
Pancytokeratin	Dako-M3515	1:500	Opal Polymer®	ARH1A01EA	690
FoxP3	CST-12653	1:400	Opal Polymer®	ARH1A01EA	620

FINAL PHENOTYPE	PRIMARY PHENOTYPE	SCORE for DATA ANALYSIS IN R		SCORE for DATA ANALYSIS IN R	
	InForm TRAINING ALGORITHM	ANTIBODY AND CELL SEGMENT	SIGNAL INTENSITY	CELL SEGMENT	MEAN SIGNAL INTENSITY
$PDL1^+$ tumor epithelial cells	Epithelial cells	PDL1 cytoplasm	2-35		
$PDL1^-$ tumor epithelial cells	Epithelial cells			PDL1 cytoplasm	2-35
$PDL1^+$ APCs	APCs	PDL1 cytoplasm	2-35		
$PDL1^-$ APCs	APCs			PDL1 cytoplasm	2-35
Regulatory T cells (T_{reg}s)	T cells	FoxP3 nucleus	3-50	CD8 cytoplasm	
Cytotoxic T cells (CTLs)	T cells	CD8 cytoplasm	3-50		
Helper T cells (Th)	T cells			CD8 cytoplasm FoxP3 nucleus	3-50 3-50

Supplemental Table 5: *Antibody data* Data on antibodies used in multiplexed staining including primary, dilution, secondary and fluorophore. Description of cellular phenotypes is provided.