Supplementary Table S1. Analyses of MSI, DNA methylation, *KRAS*, *BRAF*, and *PIK3CA* mutations, and neoantigen load

Characteristic	Method	Reference
Microsatellite instability (MSI)	PCR for 10 microsatellite markers (BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, D18S55, D18S56, D18S67, and D18S487). MSI-high was defined as presence of instability in ≥ 30% of the markers.	Ogino et al.(1)
CpG island methylator phenotype (CIMP)	Methylation analyses for eight promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) using bisulfite-treated DNA and real-time PCR. CIMP-high was defined as ≥ 6 methylated promoters.	Ogino et al.(1)
Long interspersed nucleotide element-1 (LINE-1) methylation level	PCR and pyrosequencing using bisulfite-treated DNA.	Ogino et al.(2)
BRAF (codon 600), *KRAS* (codons 12, 13, 61 and 146), and *PIK3CA* (exons 9 and 20) mutation status	PCR and pyrosequencing.	Nosho et al.(3)
Neoantigen load	Whole exome sequencing to identify peptides predicted to bind to HLA molecules with high affinity.	Giannakis et al.(4)

Supplementary Table S2. List of antibodies and fluorophores used in the immunofluorescence procedure

Order	Marker	Clone	Manufacturer, catalogue number	Antibody dilution	Fluorophore	Fluorophore dilution
1	IRF5	EPR17067	Abcam, ab181553	1:2000	Opal 650	1:300
2	MAF	EPR16484	Abcam, ab199424	1:100	Opal 570	1:150
3	CD68	PG-M1	Dako, M0876	1:40	Opal 520	1:150
4	CD86	E2G8P	Cell Signaling Technology, #91882	1:100	Opal 540	1:250
5	MRC1 (CD206)	E2L9N	Cell Signaling Technology, #91992	1:600	Opal 690	1:150
6	CK	AE1/AE3; C11	Dako, M3515; Cell Signaling Technology, #4545	1:50; 1:500	Opal 620	1:400

Supplementary Table S3. Statistical methods

Statistical test or model	Use	Item
Chi-square test	To assess the relationships between ordinal macrophage density categories or year of diagnosis categories and categorical clinicopathologic features	Table 1, Table S6, Fig. 3
Spearman rank correlation test	To assess the correlation between continuous macrophage densities, or continuous macrophage densities and ordinal lymphocytic reaction scores	Fig. S7, Fig. S12
t-Distributed Stochastic Neighbor Embedding (tSNE)	To project the high dimensional data, *i.e*., the fluorophore intensities in different cells, into two-dimensional space for visual inspection of potential heterogeneity in fluorophore intensities across TMAs	Fig. S6
Kaplan-Meier analysis, log rank test	To visualize cumulative survival probabilities according to macrophage densities and compare the differences between categories	Fig. 2, Fig. S8
Univariable Cox proportional hazards regression [A,B,C]	To estimate hazard ratios for cancer specific survival and overall survival according to macrophage density categories	Table 2, Table S4, Table S5, Table S7, Table S8, Fig. S9, Fig. S10, Fig. S11
Multivariable Cox proportional hazards regression [A,B,C,D]	To estimate hazard ratios and for cancer specific survival and overall survival according to macrophage density categories, adjusting for potential confounders	Table 2, Table 3, Table S4, Table S5, Table S7, Table S8, Fig. S9, Fig. S10, Fig. S11

[A] The Schoenfeld residual plots supported the proportionality of hazards during most of the follow-up period up to 10 years (data not shown), and thus, we used Cox regression models limiting the follow-up period to 10 years.

[B] The inverse probability weighting (IPW) method was applied to reduce the potential bias due to the availability of tumor tissue.(5) Using the multivariable logistic regression model for the entire dataset of colorectal cancer cases (regardless of available tissue), we estimated the probability of the availability of tumor tissue. Each patient with complete data was weighted by the inverse probability. Weights greater than the 95th percentile were truncated and set to the value of the 95th percentile to reduce outlier effects. We confirmed that results without weight truncation did not change substantially (data not shown). The Cox regression analyses without IPW yielded similar results to the IPW-adjusted model (data not shown).

[C] To assess the statistical interaction between macrophage densities/density ratio (low vs. high) and MSI status (high vs. non-high) or year of diagnosis (1995 or before vs. 1996-2000 vs. 2001-2008) in relation to cancer-specific survival, a Wald test for the cross product of the macrophage density or density ratio (high vs. low) and MSI status (high vs. non-high) or year of diagnosis

(1995 or before vs. 1996-2000 vs. 2001-2008) was performed in the IPW-adjusted Cox regression model. We estimated HR for colorectal cancer mortality comparing binarized low and high macrophage density or density ratio in the two strata of MSI status using re-parameterization of the interaction term in a single regression model.(6)

[D] Covariates assessed as potential confounders included sex (female vs. male), age at diagnosis (continuous), year of diagnosis (continuous), family history of colorectal cancer in any first-degree relative (present vs. absent), tumor location (proximal colon vs. distal colon vs. rectum), tumor differentiation (well to moderate vs. poor), disease stage (I/II vs. III/IV), MSI status (MSI-high vs. non-MSI-high), CIMP status (high vs. low/negative), LINE-1 methylation level (continuous), *KRAS* mutation (mutant vs. wild-type), *BRAF* mutation (mutant vs. wild-type), and *PIK3CA* mutation (mutant vs. wild-type). A backward elimination was conducted with a threshold *P* of 0.05 to select variables for the final models. Cases with the following missing data (% missing) were included in the majority category of a given categorical covariate to limit the degrees of freedom: family history of colorectal cancer in a first-degree relative (0.3%), tumor location (0.4%), tumor differentiation (0.1%), disease stage (7.2%), MSI (2.9%), CIMP (7.2%), *KRAS* (3.0%), *BRAF* (2.3%), and *PIK3CA* (8.9%). For the cases with missing data on LINE-1 methylation (3.0%), we assigned a separate indicator variable. We confirmed that excluding the cases with missing information in any of the covariates did not substantially alter results (data not shown).

Supplementary Table S4. Macrophage density, M1-like macrophage density, M2-like macrophage density, and M1:M2 macrophage density ratio in overall tissue regions and patient survival with inverse probability weighting (IPW)

		Colorectal cancer-specific survival			Overall survival		
	No. of cases	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†
Macrophage density							
Q1	232	81	1 (referent)	1 (referent)	153	1 (referent)	1 (referent)
Q2	233	76	1.07 (0.76-1.49)	1.25 (0.88-1.77)	149	1.23 (0.93-1.62)	1.28 (0.95-1.73)
Q3	233	73	0.93 (0.66-1.31)	1.11 (0.77-1.59)	136	0.97 (0.73-1.30)	1.02 (0.75-1.40)
Q4	233	58	0.70 (0.49-1.00)	0.93 (0.63-1.37)	129	0.86 (0.64-1.14)	0.93 (0.67-1.30)
P_{trend}‡			0.041	0.73		0.15	0.47
M1-like macrophage density							
Q1	232	89	1 (referent)	1 (referent)	153	1 (referent)	1 (referent)
Q2	233	75	0.91 (0.66-1.27)	1.10 (0.79-1.54)	154	1.05 (0.79-1.40)	1.22 (0.91-1.64)
Q3	233	73	0.73 (0.52-1.02)	0.89 (0.62-1.28)	138	0.82 (0.61-1.10)	0.92 (0.68-1.25)
Q4	233	51	0.52 (0.36-0.75)	0.75 (0.51-1.11)	122	0.74 (0.55-0.99)	0.89 (0.65-1.23)
P_{trend}‡			<0.001	0.12		0.017	0.27
M2-like macrophage density							
Q1	232	65	1 (referent)	1 (referent)	148	1 (referent)	1 (referent)
Q2	233	69	1.18 (0.82-1.69)	1.33 (0.92-1.93)	135	1.20 (0.90-1.60)	1.30 (0.95-1.78)
Q3	233	77	1.39 (0.98-1.98)	1.67 (1.16-2.40)	143	1.42 (1.07-1.88)	1.60 (1.17-2.18)
Q4	233	77	1.37 (0.96-1.96)	1.51 (1.04-2.21)	141	1.19 (0.89-1.61)	1.21 (0.87-1.69)
P_{trend}‡			0.054	0.016		0.14	0.15
M1:M2 density ratio							
Q1	232	86	1 (referent)	1 (referent)	157	1 (referent)	1 (referent)
Q2	232	74	0.82 (0.59-1.16)	0.97 (0.69-1.36)	137	0.96 (0.72-1.27)	1.03 (0.77-1.37)
Q3	233	77	0.83 (0.59-1.16)	0.83 (0.59-1.17)	146	0.87 (0.65-1.17)	0.85 (0.63-1.15)
Q4	233	51	0.48 (0.33-0.69)	0.61 (0.41-0.90)	127	0.65 (0.48-0.88)	0.67 (0.49-0.92)
P_{trend}‡			<0.001	0.012		0.005	0.008

* IPW was applied to reduce a bias due to the availability of tumor tissue after cancer diagnosis (see “Statistical Analysis” subsection for details).
† The multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer, tumor location, tumor differentiation, disease stage, microsatellite instability, CpG island methylator phenotype, *KRAS, BRAF*, and *PIK3CA* mutations, and long-interspersed nucleotide element-1 methylation level. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models.
‡ P_{trend} value was calculated across the ordinal quartiles of each macrophage density or M1 to M2 density ratio within overall region in the IPW-adjusted Cox regression model.
Abbreviations: CI, confidence interval; HR, hazard ratio; IPW, inverse probability weighting.

Supplementary Table S5. Densities of macrophage populations defined by positivity for single polarization marker in tumor intraepithelial and stromal regions and patient survival with inverse probability weighting (IPW)

		Colorectal cancer-specific survival			Overall survival		
	No. of cases	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†
Tumor intraepithelial region							
$CD86^+$ macrophage density							
Q1	320	106	1 (referent)	1 (referent)	203	1 (referent)	1 (referent)
Q2	203	66	0.99 (0.71-1.38)	1.12 (0.80-1.56)	124	0.94 (0.71-1.25)	0.94 (0.69-1.27)
Q3	204	73	1.14 (0.83-1.57)	1.16 (0.82-1.65)	127	1.19 (0.91-1.56)	1.23 (0.92-1.65)
Q4	204	43	0.56 (0.38-0.82)	0.75 (0.49-1.16)	113	0.81 (0.61-1.07)	0.84 (0.61-1.15)
P_{trend}‡			0.025	0.52		0.46	0.77
$IRF5^+$ macrophage density							
Q1	232	84	1 (referent)	1 (referent)	157	1 (referent)	1 (referent)
Q2	233	69	0.80 (0.57-1.13)	0.89 (0.62-1.28)	137	0.97 (0.72-1.29)	0.98 (0.73-1.33)
Q3	233	66	0.80 (0.56-1.13)	0.97 (0.68-1.38)	135	1.00 (0.74-1.33)	1.05 (0.77-1.43)
Q4	233	69	0.85 (0.61-1.19)	1.03 (0.70-1.50)	138	1.01 (0.76-1.34)	1.09 (0.79-1.50)
P_{trend}‡			0.33	0.86		0.93	0.55
MAF^+ macrophage density							
Q1	394	123	1 (referent)	1 (referent)	246	1 (referent)	1 (referent)
Q2	178	49	0.86 (0.60-1.23)	0.94 (0.66-1.34)	108	1.03 (0.78-1.36)	1.07 (0.80-1.43)
Q3	180	70	1.31 (0.96-1.78)	1.51 (1.09-2.08)	118	1.28 (0.98-1.66)	1.34 (1.01-1.76)
Q4	179	46	0.77 (0.53-1.11)	0.83 (0.55-1.26)	95	0.82 (0.60-1.10)	0.83 (0.58-1.17)
P_{trend}‡			0.64	0.82		0.67	0.81
$MRC1^+$ macrophage density							
Q1	232	79	1 (referent)	1 (referent)	147	1 (referent)	1 (referent)
Q2	233	72	0.97 (0.68-1.37)	1.13 (0.79-1.61)	143	1.10 (0.82-1.48)	1.12 (0.81-1.53)
Q3	233	67	0.92 (0.65-1.30)	1.04 (0.72-1.48)	131	1.07 (0.80-1.44)	1.08 (0.79-1.48)
Q4	233	70	0.96 (0.68-1.35)	1.09 (0.74-1.61)	146	1.18 (0.89-1.57)	1.12 (0.81-1.56)
P_{trend}‡			0.73	0.77		0.30	0.55

Tumor stromal region							
CD86$^+$ macrophage density							
Q1	232	76	1 (referent)	1 (referent)	147	1 (referent)	1 (referent)
Q2	233	83	1.14 (0.81-1.59)	1.33 (0.94-1.89)	161	1.29 (0.97-1.72)	1.39 (1.02-1.90)
Q3	233	79	1.12 (0.80-1.58)	1.37 (0.96-1.96)	128	1.17 (0.87-1.57)	1.28 (0.93-1.76)
Q4	233	50	0.59 (0.40-0.87)	0.92 (0.60-1.40)	131	0.86 (0.64-1.15)	0.99 (0.71-1.39)
P_{trend}‡			0.015	0.87		0.26	0.98
IRF5$^+$ macrophage density							
Q1	232	75	1 (referent)	1 (referent)	156	1 (referent)	1 (referent)
Q2	233	73	1.07 (0.75-1.52)	1.40 (0.97-2.01)	144	1.10 (0.83-1.49)	1.26 (0.92-1.73)
Q3	233	76	1.05 (0.75-1.47)	1.33 (0.93-1.91)	142	1.21 (0.92-1.61)	1.28 (0.93-1.76)
Q4	233	64	0.88 (0.61-1.26)	1.26 (0.84-1.90)	125	0.98 (0.73-1.32)	1.15 (0.82-1.62)
P_{trend}‡			0.52	0.25		0.87	0.38
MAF$^+$ macrophage density							
Q1	280	82	1 (referent)	1 (referent)	177	1 (referent)	1 (referent)
Q2	217	64	1.01 (0.72-1.43)	1.20 (0.86-1.67)	135	1.15 (0.87-1.51)	1.27 (0.96-1.69)
Q3	217	74	1.23 (0.88-1.72)	1.46 (1.01-2.09)	135	1.27 (0.97-1.67)	1.35 (0.99-1.82)
Q4	217	68	1.08 (0.76-1.54)	1.33 (0.90-1.98)	120	0.99 (0.74-1.34)	1.10 (0.79-1.53)
P_{trend}‡			0.44	0.082		0.73	0.41
MRC1$^+$ macrophage density							
Q1	232	72	1 (referent)	1 (referent)	153	1 (referent)	1 (referent)
Q2	233	78	1.21 (0.86-1.71)	1.39 (0.97-1.97)	138	1.17 (0.87-1.56)	1.30 (0.95-1.78)
Q3	233	81	1.40 (1.00-1.98)	1.76 (1.24-2.51)	143	1.36 (1.02-1.81)	1.50 (1.10-2.04)
Q4	233	57	0.80 (0.55-1.17)	1.00 (0.66-1.50)	133	0.97 (0.73-1.29)	1.02 (0.73-1.42)
P_{trend}‡			0.53	0.41		0.81	0.57

* IPW was applied to reduce bias due to the availability of tumor tissue after cancer diagnosis (see “Statistical Analysis” subsection for details).

† The multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer, tumor location, tumor differentiation, disease stage, microsatellite instability, CpG island methylator phenotype, *KRAS, BRAF*, and *PIK3CA* mutations, and long-interspersed nucleotide element-1 methylation level. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models.

‡ P_{trend} value was calculated across the ordinal quartiles of each macrophage density within tumor intraepithelial and stromal regions in the IPW-adjusted Cox regression model.
Abbreviations: CI, confidence interval; HR, hazard ratio; IPW, inverse probability weighting.

Supplementary Table S6. Clinical, pathological, and molecular characteristics of colorectal cancer cases according to year of diagnosis

Characteristic*	All cases (N = 931)	Year of diagnosis: 1995 or before (N = 304)	1996-2000 (N = 303)	2001-2008 (N = 324)	*P* value†
Sex					0.54
Female (NHS)	517 (56%)	172 (57%)	173 (57%)	172 (53%)	
Male (HPFS)	414 (44%)	132 (43%)	130 (43%)	152 (47%)	
Mean age ± SD (years)	68.9 ± 8.9	63.8 ± 8.1	69.0 ± 7.7	73.7 ± 7.9	<0.001
Family history of colorectal cancer in first-degree relative(s)					0.44
Absent	738 (80%)	232 (77%)	245 (81%)	261 (81%)	
Present	190 (20%)	69 (1239%)	58 (19%)	63 (63%)	
Tumor location					0.018
Cecum	161 (17%)	44 (14%)	53 (17%)	64 (20%)	
Ascending to transverse colon	301 (32%)	85 (28%)	96 (32%)	120 (37%)	
Descending to sigmoid colon	283 (31%)	112 (37%)	93 (31%)	78 (24%)	
Rectum	182 (20%)	63 (21%)	58 (19%)	61 (19%)	
Tumor differentiation					
Well to moderate	843 (91%)	269 (89%)	277 (91%)	297 (92%)	
Poor	87 (9.4%)	34 (11%)	26 (8.6%)	27 (8.3%)	
AJCC disease stage					0.021
I	199 (23%)	58 (21%)	61 (21%)	80 (27%)	
II	282 (33%)	89 (32%)	89 (31%)	104 (35%)	
III	249 (29%)	81 (29%)	85 (30%)	83 (28%)	
IV	134 (16%)	54 (19%)	52 (18%)	28 (9.5%)	
MSI status					
Non-MSI-high	750 (83%)	260 (86%)	253 (84%)	237 (78%)	0.030

MSI-high	154 (17%)	41 (14%)	48 (16%)	65 (22%)	
CIMP status					
Low/negative	705 (82%)	260 (87%)	239 (81%)	206 (76%)	0.004
High	159 (18%)	39 (13%)	56 (19%)	64 (24%)	
Mean LINE-1 methylation level ± SD (%)	62.5 ± 9.5	60.6 ± 9.3	60.8 ± 9.7	66.0 ± 8.6	<0.001
KRAS mutation					0.69
Wild-type	535 (59%)	182 (61%)	173 (58%)	180 (59%)	
Mutant	368 (41%)	116 (39%)	127 (42%)	125 (41%)	
BRAF mutation					0.33
Wild-type	771 (85%)	260 (87%)	252 (84%)	259 (83%)	
Mutant	139 (15%)	38 (13%)	49 (16%)	52 (17%)	
PIK3CA mutation					0.74
Wild-type	712 (84%)	229 (85%)	230 (85%)	253 (83%)	
Mutant	136 (16%)	41 (15%)	42 (15%)	53 (17%)	
Neoantigen load					0.076
Q1 (lowest)	104 (25%)	19 (20%)	33 (26%)	52 (27%)	
Q2	104 (25%)	22 (23%)	25 (20%)	57 (30%)	
Q3	104 (25%)	25 (26%)	41 (32%)	38 (20%)	
Q4 (highest)	104 (25%)	30 (31%)	29 (23%)	45 (23%)	
Macrophage density					<0.001
Q1 (lowest)	232 (25%)	107 (35%)	60 (20%)	65 (20%)	
Q2	233 (25%)	82 (27%)	74 (24%)	77 (24%)	
Q3	233 (25%)	57 (19%)	82 (27%)	94 (29%)	
Q4 (highest)	233 (25%)	58 (19%)	87 (29%)	88 (27%)	
Tumor cell density					0.52
Q1 (lowest)	232 (25%)	87 (29%)	72 (24%)	73 (23%)	
Q2	233 (25%)	72 (24%)	82 (27%)	79 (24%)	
Q3	233 (25%)	76 (25%)	70 (23%)	87 (27%)	

Q4 (highest)	233 (25%)	69 (23%)	79 (26%)	85 (26%)	
Mean cytoplasmic KRT intensity					0.31
Q1 (lowest)	232 (25%)	77 (25%)	73 (24%)	82 (25%)	
Q2	233 (25%)	86 (28%)	64 (21%)	83 (26%)	
Q3	233 (25%)	65 (21%)	83 (27%)	85 (26%)	
Q4 (highest)	233 (25%)	76 (25%)	83 (27%)	74 (23%)	

* Percentage indicates the proportion of patients with a specific clinical, pathologic, or molecular characteristic among all patients or in strata of year of diagnosis

† To compare categorical data between the ordinal categories of macrophage density, the chi-square test was performed. To compare continuous variables, an analysis of variance was performed.

Abbreviations: AJCC, American Joint Committee on Cancer; CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; LINE-1, long-interspersed nucleotide element-1; MSI, microsatellite instability; NHS, Nurses' Health Study; SD, standard deviation.

Supplementary Table S7. Macrophage densities and M1:M2 macrophage density ratio in tumor intraepithelial and stromal regions and patient survival in strata of MSI status with inverse probability weighting (IPW)

	Tumor intraepithelial region				Stromal region			
		Colorectal cancer-specific survival				Colorectal cancer-specific survival		
	No. of cases	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†	No. of cases	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†
Non-MSI-high								
Macrophage density								
Low	415	145	1 (referent)	1 (referent)	400	148	1 (referent)	1 (referent)
High	335	112	1.00 (0.76-1.30)	0.92 (0.69-1.22)	350	109	0.86 (0.66-1.12)	0.93 (0.71-1.24)
MSI-high								
Macrophage density								
Low	37	7	1 (referent)	1 (referent)	52	11	1 (referent)	1 (referent)
High	117	15	0.45 (0.18-1.14)	0.27 (0.11-0.66)	102	11	0.35 (0.14-0.86)	0.25 (0.10-0.63)
$P_{interaction}$‡			0.11	0.018			0.061	0.011
Non-MSI-high								
M1-like macrophage density								
Low	406	145	1 (referent)	1 (referent)	401	160	1 (referent)	1 (referent)
High	344	112	0.87 (0.67-1.13)	0.93 (0.71-1.22)	349	97	0.63 (0.49-0.83)	0.74 (0.56-0.98)
MSI-high								
M1-like macrophage density								
Low	46	10	1 (referent)	1 (referent)	51	14	1 (referent)	1 (referent)
High	108	12	0.36 (0.15-0.88)	0.37 (0.14-0.94)	103	8	0.16 (0.07-0.42)	0.24 (0.09-0.67)
$P_{interaction}$‡			0.062	0.080			0.006	0.057
Non-MSI-high								
M2-like macrophage density								
Low	403	133	1 (referent)	1 (referent)	375	116	1 (referent)	1 (referent)

High	347	124	1.24 (0.95-1.61)	1.22 (0.93-1.61)	375	141	1.40 (1.08-1.83)	1.62 (1.22-2.14)
MSI-high								
M2-like macrophage density								
Low	49	8	1 (referent)	1 (referent)	77	10	1 (referent)	1 (referent)
High	105	14	0.86 (0.33-2.25)	0.64 (0.23-1.75)	77	12	1.29 (0.52-3.21)	1.09 (0.41-2.87)
$P_{interaction}$‡			0.47	0.21			0.86	0.41
Non-MSI-high								
M1:M2 density ratio								
Low	369	134	1 (referent)	1 (referent)	385	143	1 (referent)	1 (referent)
High	358	111	0.79 (0.60-1.03)	0.75 (0.57-0.99)	361	112	0.76 (0.58-0.98)	0.75 (0.57-0.98)
MSI-high								
M1:M2 density ratio								
Low	68	12	1 (referent)	1 (referent)	65	14	1 (referent)	1 (referent)
High	84	10	0.50 (0.20-1.23)	0.77 (0.30-2.01)	88	8	0.25 (0.10-0.65)	0.40 (0.15-1.10)
$P_{interaction}$‡			0.34	0.93			0.028	0.31

* IPW was applied to reduce bias due to the availability of tumor tissue after cancer diagnosis (see “Statistical Analysis” subsection for details).

† The multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer, tumor location, tumor differentiation, disease stage, microsatellite instability, CpG island methylator phenotype, *KRAS, BRAF*, and *PIK3CA* mutations, and long-interspersed nucleotide element-1 methylation level. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models.

‡ $P_{interaction}$ (two-sided) was calculated using the Wald test for the cross product of the macrophage density or density ratio (high vs. low) and MSI status (high vs. non-high) in the IPW-adjusted Cox regression model.

Abbreviations: CI, confidence interval; HR, hazard ratio; IPW, inverse probability weighting.

Supplementary Table S8. Macrophage densities and M1:M2 macrophage density ratio in tumor intraepithelial and stromal regions and patient survival in strata of year of diagnosis with inverse probability weighting (IPW)

	Tumor intraepithelial region				Stromal region			
		Colorectal cancer-specific survival				Colorectal cancer-specific survival		
	No. of cases	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†	No. of cases	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†
Diagnosed in 1995 or before								
Macrophage density								
Low	172	59	1 (referent)	1 (referent)	187	61	1 (referent)	1 (referent)
High	132	41	0.80 (0.52-1.24)	0.74 (0.47-1.16)	117	39	0.94 (0.61-1.47)	0.94 (0.61-1.46)
Diagnosed in 1996 to 2000								
Macrophage density								
Low	131	52	1 (referent)	1 (referent)	129	58	1 (referent)	1 (referent)
High	172	55	0.91 (0.61-1.36)	0.94 (0.61-1.43)	174	49	0.60 (0.40-0.91)	0.70 (0.46-1.07)
Diagnosed in 2001 to 2008								
Macrophage density								
Low	160	43	1 (referent)	1 (referent)	145	42	1 (referent)	1 (referent)
High	164	38	0.89 (0.57-1.39)	0.96 (0.59-1.58)	179	39	0.77 (0.49-1.20)	0.74 (0.46-1.19)
$P_{interaction}$‡			0.77	0.39			0.38	0.37
Diagnosed in 1995 or before								
M1-like macrophage density								
Low	175	59	1 (referent)	1 (referent)	189	73	1 (referent)	1 (referent)
High	129	41	0.78 (0.51-1.20)	0.78 (0.51-1.20)	115	27	0.46 (0.28-0.74)	0.53 (0.33-0.87)
Diagnosed in 1996 to 2000								
M1-like macrophage density								
Low	129	50	1 (referent)	1 (referent)	131	55	1 (referent)	1 (referent)
High	174	57	0.90 (0.60-1.34)	1.09 (0.71-1.66)	172	52	0.68 (0.45-1.01)	0.90 (0.59-1.39)
Diagnosed in 2001 to 2008								

M1-like macrophage density								
Low	162	49	1 (referent)	1 (referent)	141	49	1 (referent)	1 (referent)
High	162	32	0.62 (0.39-0.98)	0.74 (0.46-1.20)	183	32	0.51 (0.33-0.81)	0.59 (0.37-0.95)
$P_{interaction}$‡			0.51	0.99			0.82	0.77
Diagnosed in 1995 or before								
M2-like macrophage density								
Low	170	56	1 (referent)	1 (referent)	178	55	1 (referent)	1 (referent)
High	134	44	1.11 (0.72-1.70)	0.99 (0.64-1.53)	126	45	1.38 (0.90-2.13)	1.41 (0.91-2.17)
Diagnosed in 1996 to 2000								
M2-like macrophage density								
Low	138	46	1 (referent)	1 (referent)	140	44	1 (referent)	1 (referent)
High	165	61	1.35 (0.90-2.04)	1.48 (0.96-2.27)	163	63	1.39 (0.92-2.09)	1.31 (0.86-2.02)
Diagnosed in 2001 to 2008								
M2-like macrophage density								
Low	154	42	1 (referent)	1 (referent)	141	28	1 (referent)	1 (referent)
High	170	39	0.89 (0.57-1.40)	1.08 (0.67-1.72)	183	53	1.74 (1.09-2.77)	1.74 (1.08-2.81)
$P_{interaction}$‡			0.55	0.68			0.58	0.62
Diagnosed in 1995 or before								
M1:M2 density ratio								
Low	146	48	1 (referent)	1 (referent)	149	53	1 (referent)	1 (referent)
High	143	44	0.78 (0.50-1.22)	0.73 (0.47-1.14)	151	45	0.65 (0.43-1.00)	0.68 (0.45-1.04)
Diagnosed in 1996 to 2000								
M1:M2 density ratio								
Low	143	56	1 (referent)	1 (referent)	150	62	1 (referent)	1 (referent)
High	158	50	0.73 (0.48-1.09)	0.77 (0.51-1.16)	152	45	0.66 (0.44-0.99)	0.74 (0.48-1.12)
Diagnosed in 2001 to 2008								

M1:M2 density ratio								
Low	163	48	1 (referent)	1 (referent)	170	50	1 (referent)	1 (referent)
High	152	30	0.64 (0.40-1.02)	0.70 (0.43-1.13)	154	31	0.64 (0.41-1.01)	0.75 (0.47-1.20)
$P_{interaction}$‡			0.60	0.94			0.97	0.74

* IPW was applied to reduce a bias due to the availability of tumor tissue after cancer diagnosis (see "Statistical Analysis" subsection for details).

† The multivariable Cox regression model initially included sex, age, family history of colorectal cancer, tumor location, tumor differentiation, disease stage, microsatellite instability, CpG island methylator phenotype, *KRAS, BRAF*, and *PIK3CA* mutations, and long-interspersed nucleotide element-1 methylation level. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models.

‡ $P_{interaction}$ (two-sided) was calculated using the Wald test for the cross product of the macrophage density or density ratio of (high vs. low) and year of diagnosis (1995 or before, 1996-2000, and 2001-2008) in the IPW-adjusted Cox regression model.

Abbreviations: CI, confidence interval; HR, hazard ratio; IPW, inverse probability weighting.

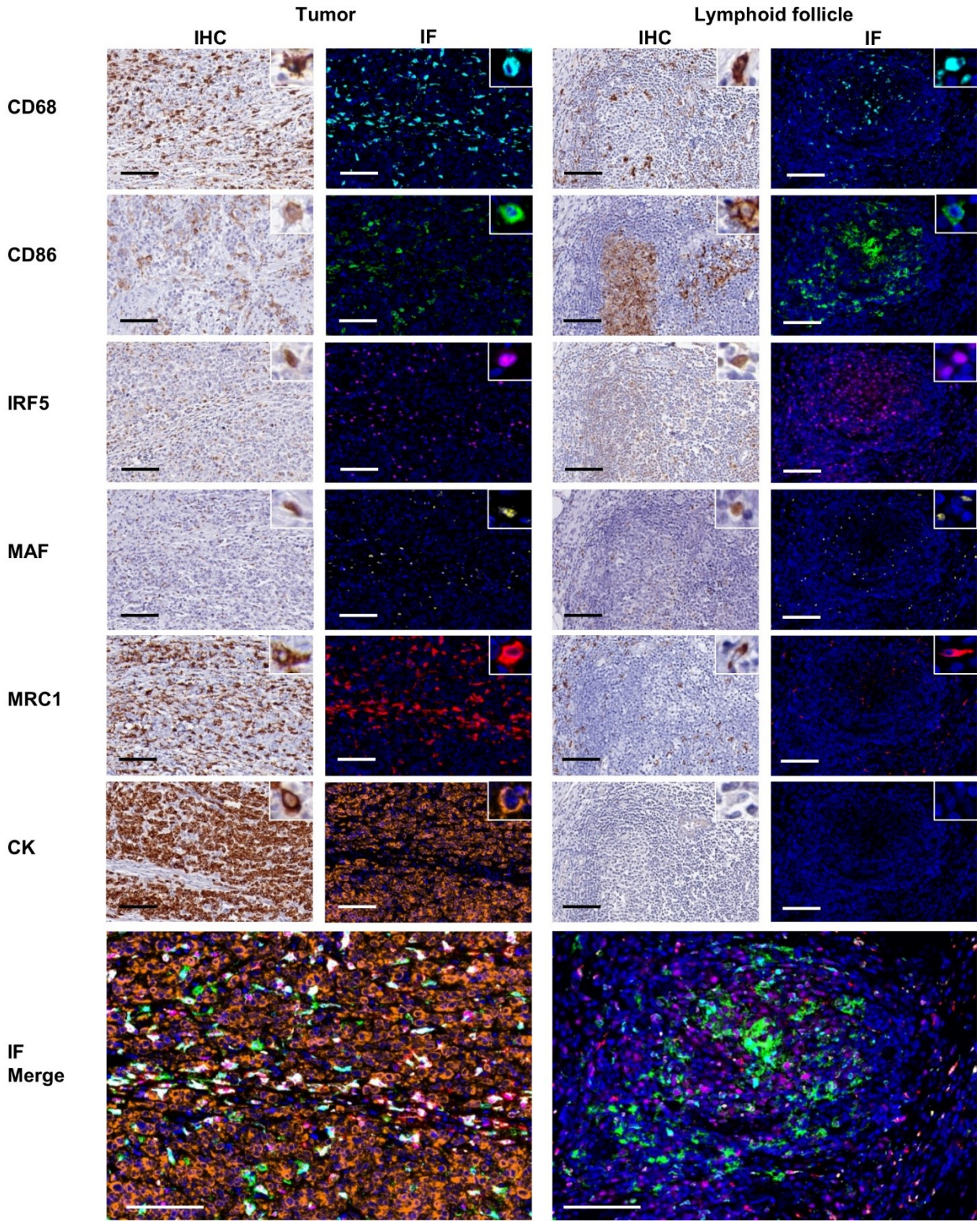


Figure S1. Comparison of staining patterns between multiplex immunofluorescence and standard immunohistochemistry. Scale bar is 100 μm.

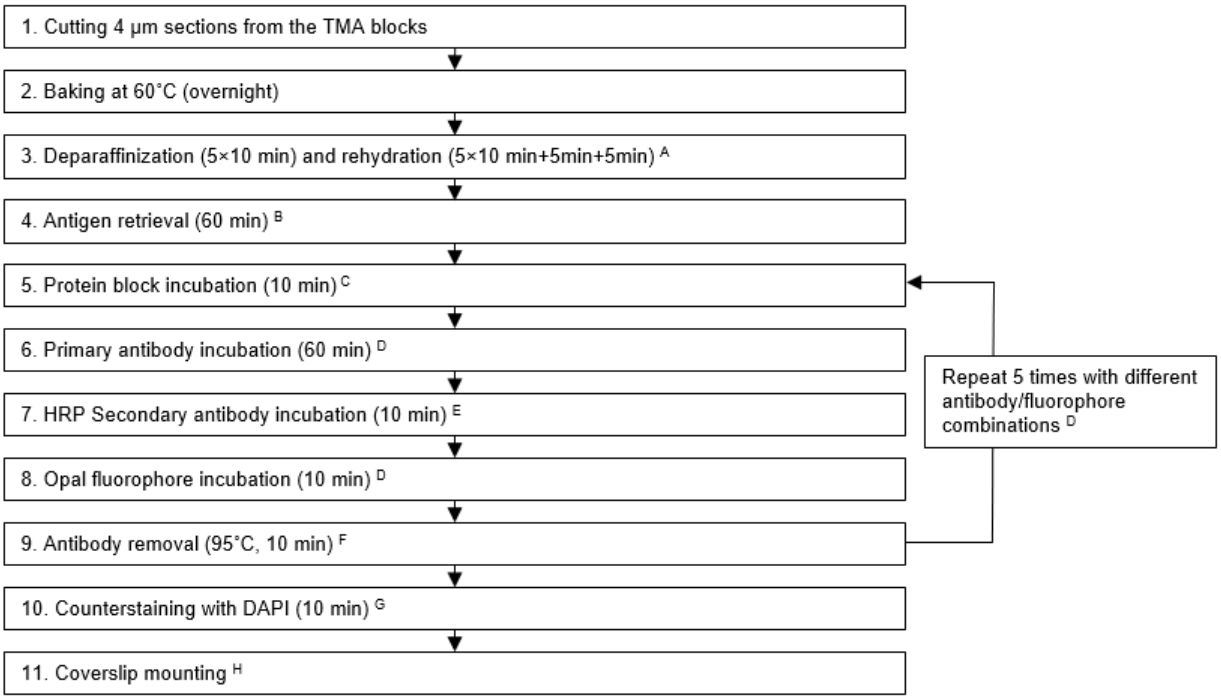


Figure S2. Flowchart of the cyclic immunofluorescence procedure. Steps 5-10 were performed with a Leica Bond RX Research Stainer (Leica Biosystems, Buffalo, IL, USA).
[A] Deparaffinization in Xylene (X3P1GAL, Fisher Scientific, Pittsburgh, PA, USA) and rehydration through graded alcohol series (100%×3+95%+80%) (HC-800-1GAL, Fisher Scientific).
[B] With 2100-Retriever (62700-10, Electron Microscopy Sciences, Hatfiled, PA, USA) in Citrate buffer pH 6.0 (S1699, Dako, Cophenhagen, Denmark)
[C] Protein Block, Serum-Free (X0909, Dako)
[D] See Table S1 for details about antibodies and fluorophores
[E] Opal Polymer HRP Ms + Rb (ARH1001EA, Akoya Biosciences, Hopkinton, MA, USA)
[F] BOND Epitope Retrieval Solution 1 (AR9961, Leica Biosystems)
[G] Spectral DAPI (FP1490, Akoya Biosciences)
[H] FF Cover Glass (125485M, Fisher Scientific); ProLong Diamond Antifade Mountant (P36970, Thermo Fisher Scientific, Waltham, MA, USA)

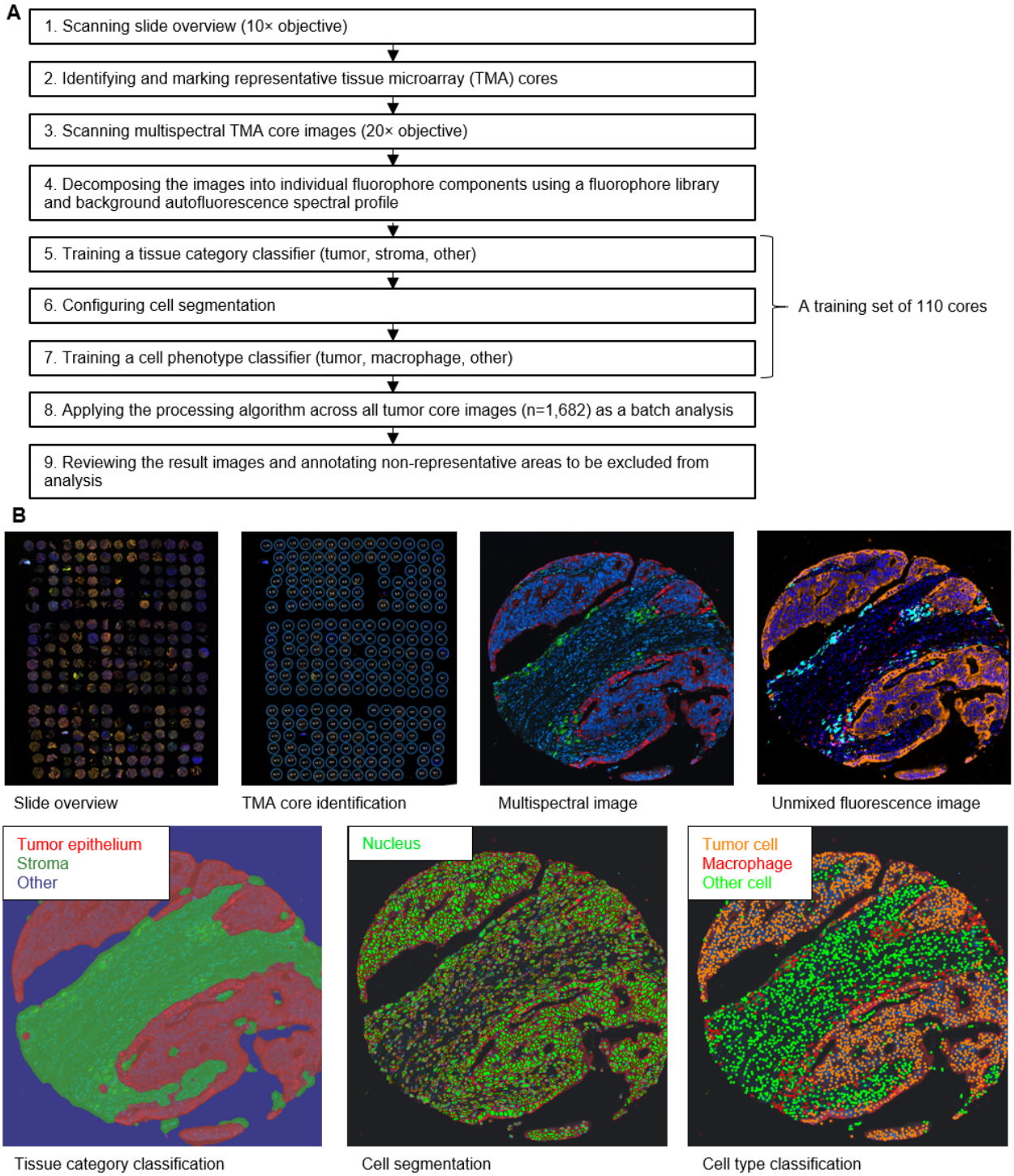


Figure S3. Analysis of immunofluorescence slides to quantify tumor intraepithelial and stromal macrophage densities. A. Flowchart of the scanning of the immunofluorescence slides with Vectra 3.0 Automated Quantitative Pathology Imaging System (steps 1 and 3) (Akoya Biosciences, Hopkinton, MA, USA) and processing of the images with the Phenochart (step 2) (Akoya Biosciences) and inForm software packages (steps 4-9) (Akoya Biosciences) to perform tissue category segmentation (tumor epithelium, stroma, other; using KRT expression to delineate epithelial areas), cell segmentation (using the DAPI signal to identify nuclei), and cell type classification [macrophage, tumor cell, other; using a combination of cellular morphology,

CD68 expression (macrophages), and KRT expression (tumor cells) to distinguish these phenotypes]. The cell phenotype classification implemented in the inForm software package was based on multinomial logistic regression utilizing image features derived from texture analysis and cell segmentation. B. Example images in various steps of analysis.

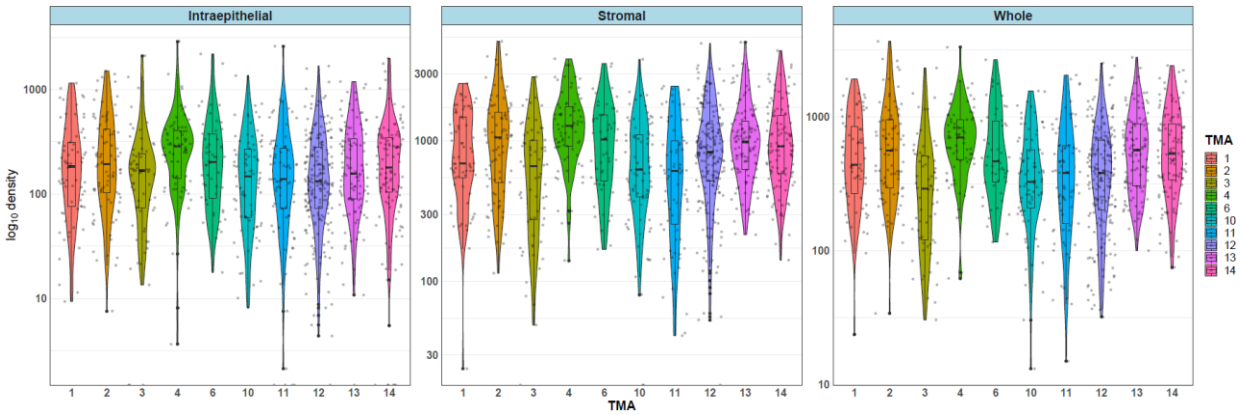


Figure S4. Macrophage densities across 10 TMAs (per mm^2).

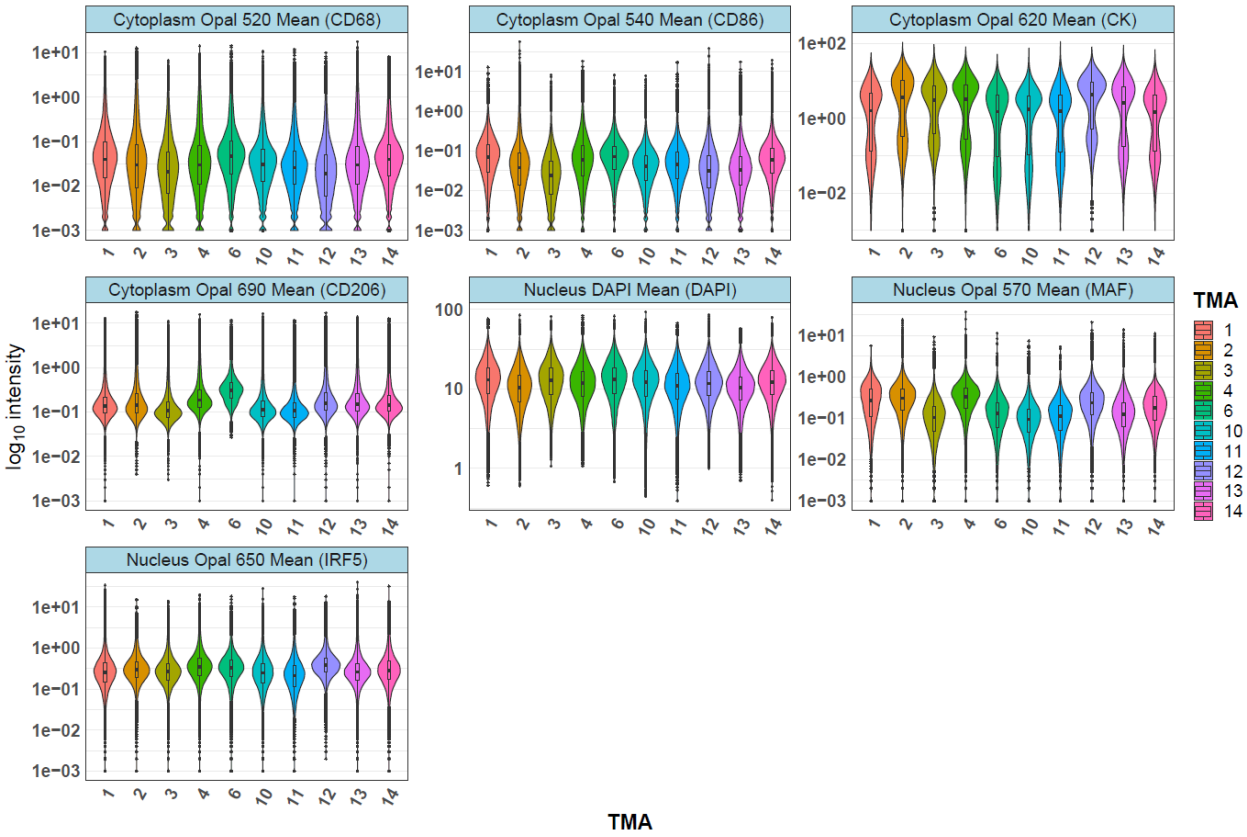


Figure S5. Fluorescence signal intensities across 10 TMAs.

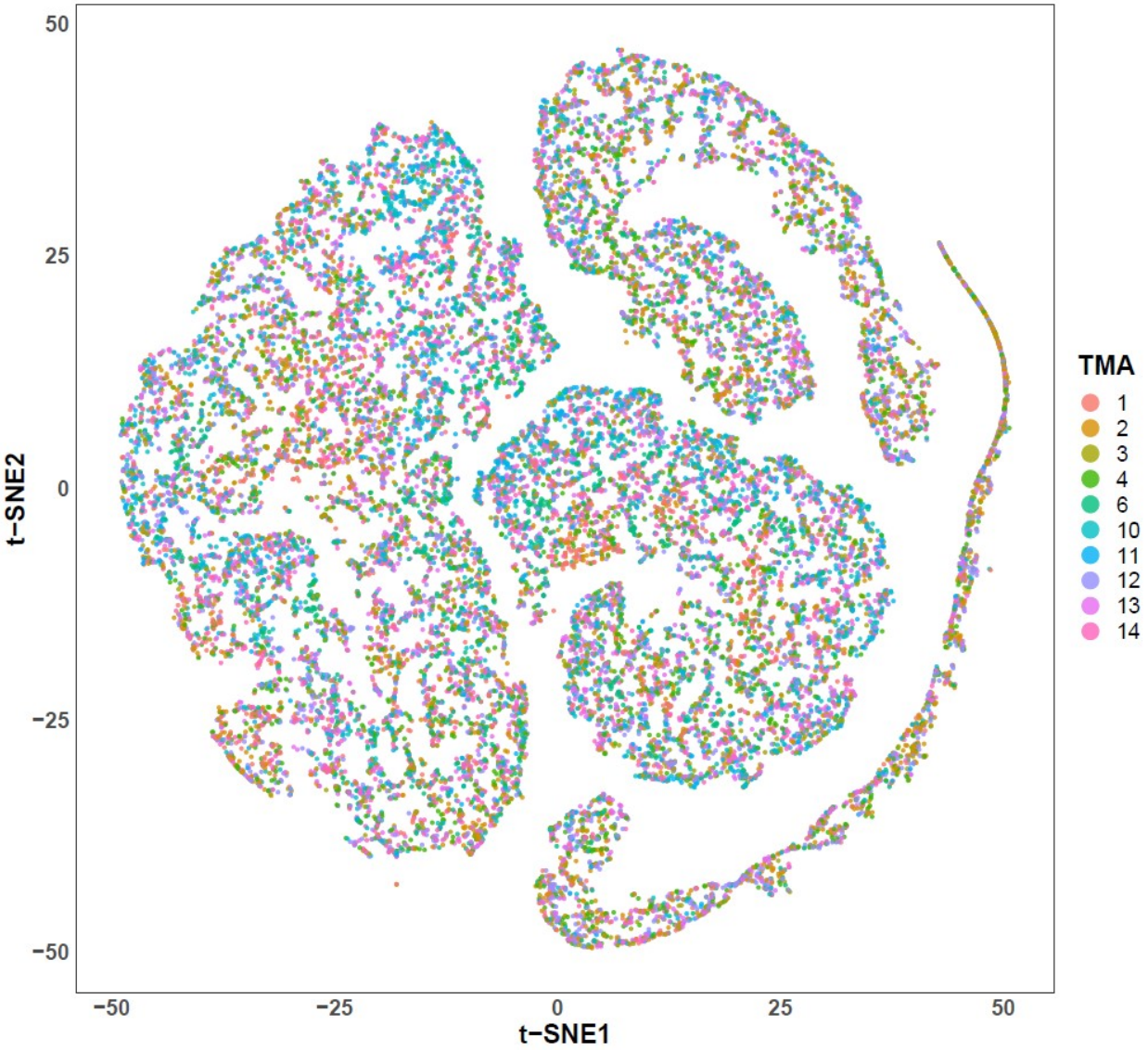


Figure S6. t-SNE analysis of a random sample of 0.5% of all cells based on fluorophore signal intensities shows no clear clustering according to the TMAs.

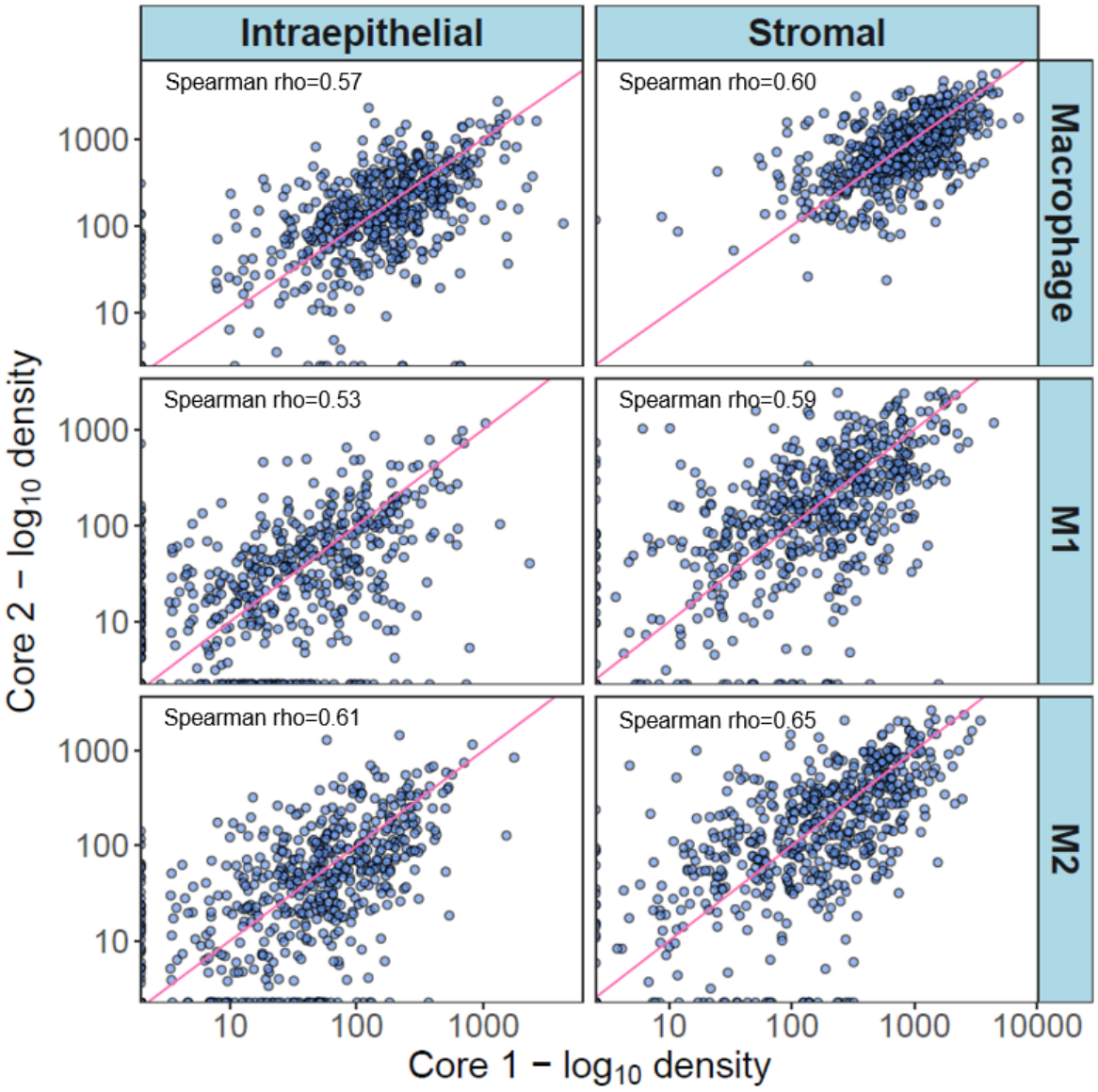
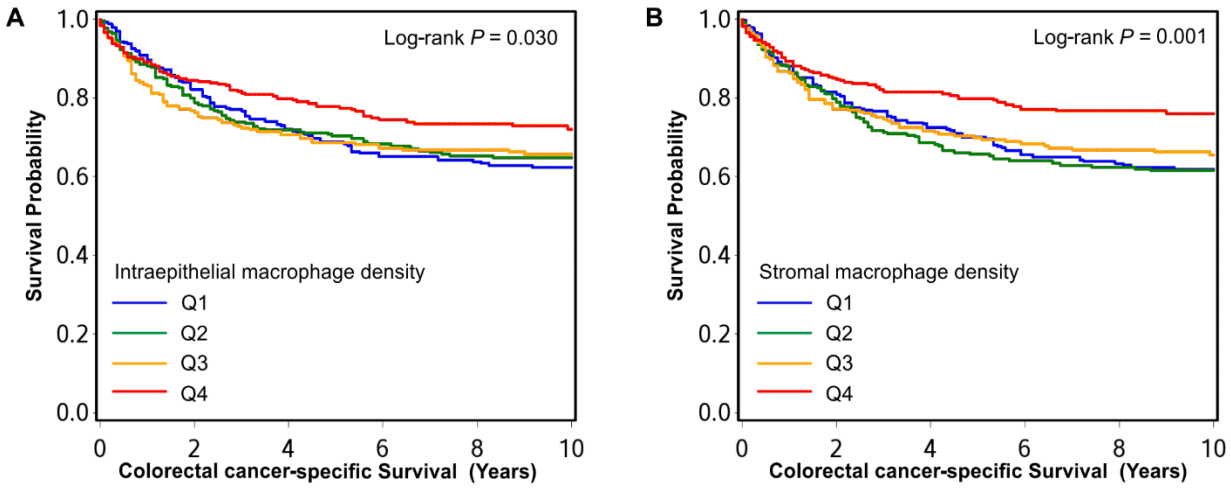


Figure S7. Core-to-core correlation of macrophage densities (per mm^2) in two randomly chosen cores of tumors with two or more cores. Macrophages present within the 30% tails of the M1:M2 index distribution were classified as M1-like or M2-like.



	Years, Number at risk					
	0	2	4	6	8	10
Q1	232	196	170	142	123	102
Q2	233	187	166	144	116	86
Q3	233	182	163	137	117	93
Q4	233	193	173	140	116	94

	Years, Number at risk					
	0	2	4	6	8	10
Q1	232	193	168	141	118	95
Q2	233	180	157	130	110	81
Q3	233	185	163	139	118	91
Q4	233	200	184	153	126	108

Figure S8. Inverse probability weighting-adjusted Kaplan-Meier survival curves of colorectal cancer survival according to the ordinal quartile categories (Q1-Q4) of intraepithelial (A) and stromal (B) macrophage densities.

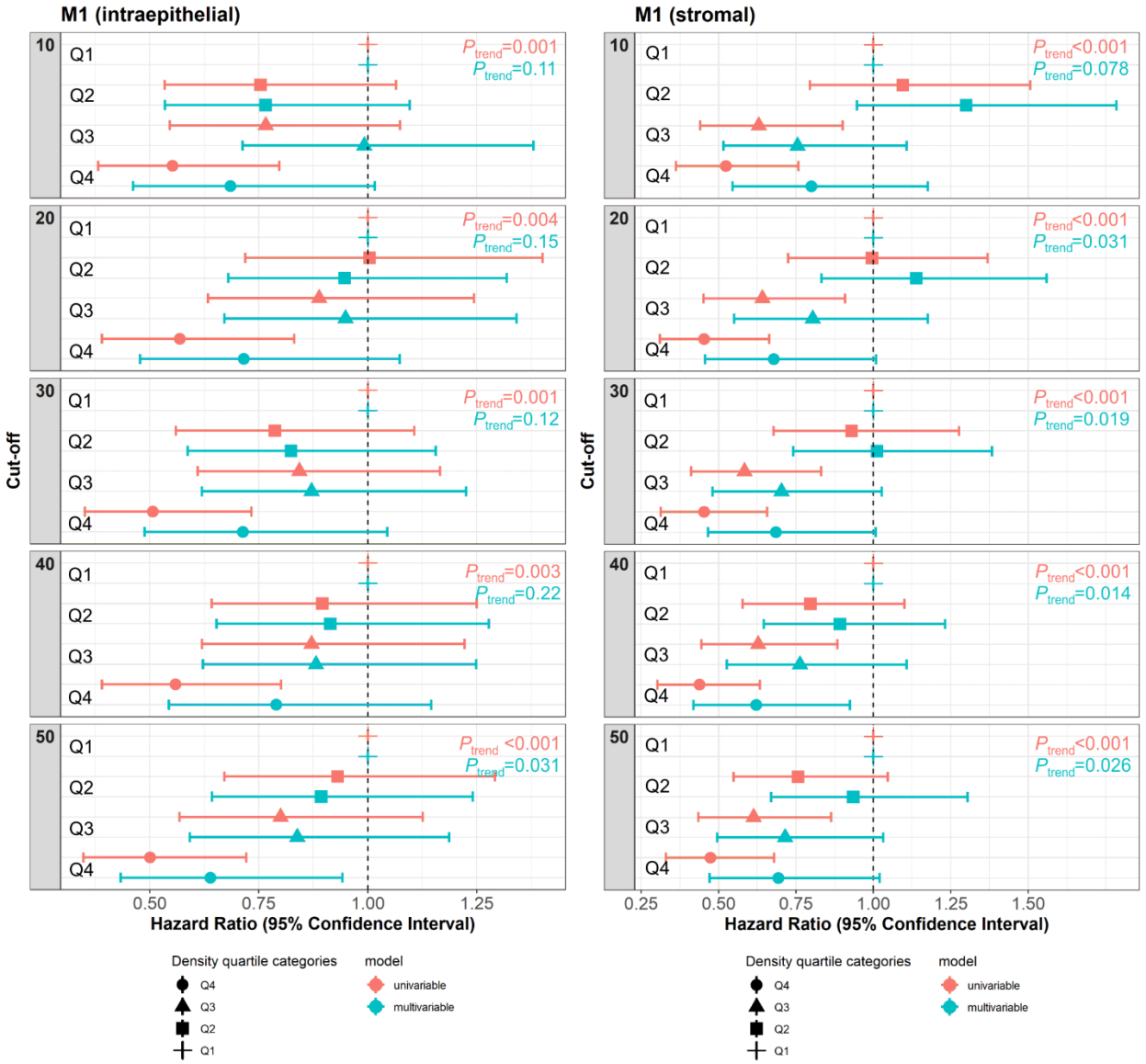


Figure S9. Forest plots of inverse probability weighting-adjusted Cox regression models of colorectal cancer specific survival according to the densities of intraepithelial and stromal M1-like polarized macrophages, with M1-like macrophages defined using different cut-offs (10-50%) of the M1-end of the macrophage polarization (M1:M2) index distribution. The multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer, tumor location, tumor differentiation, disease stage, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations, and long-interspersed nucleotide element-1 methylation level. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models.

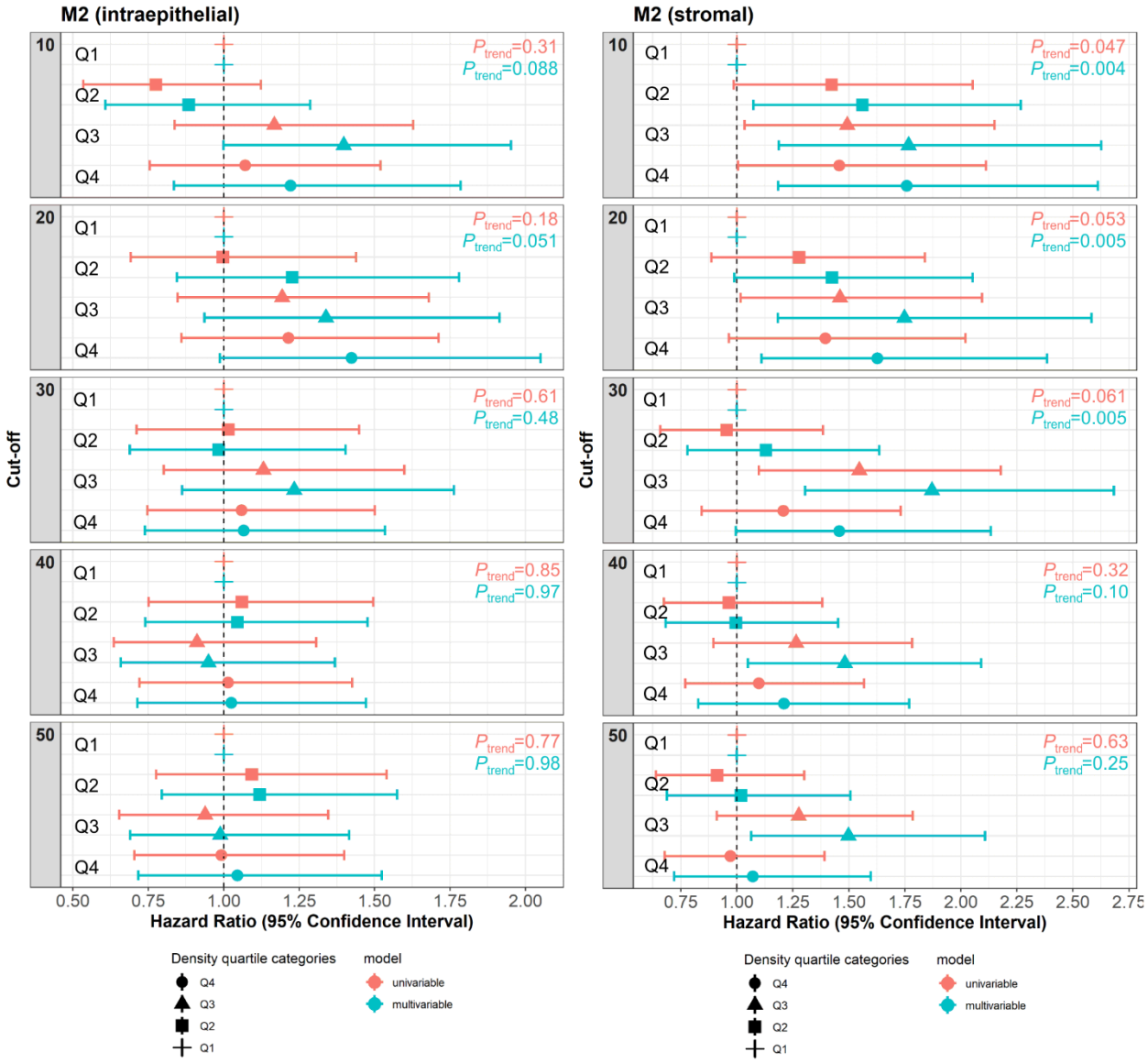


Figure S10. Forest plots of inverse probability weighting-adjusted Cox regression models of colorectal cancer specific survival according to the densities of intraepithelial and stromal M2-like polarized macrophages, with M2-like macrophages defined using different cut-offs (10-50%) of the M2-end of the macrophage polarization (M1:M2) index distribution. The multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer, tumor location, tumor differentiation, disease stage, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations, and long-interspersed nucleotide element-1 methylation level. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models.

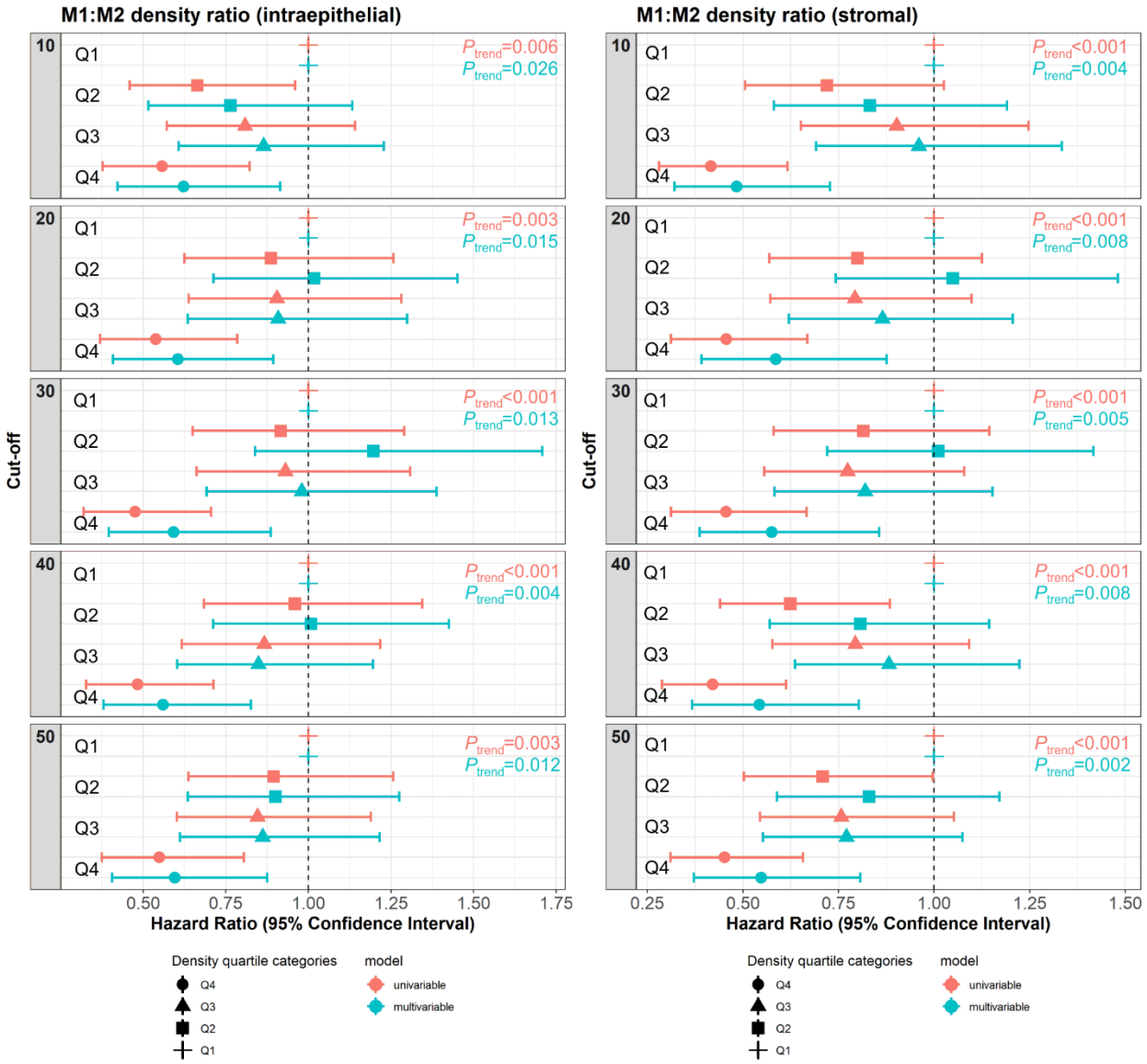


Figure S11. Forest plots of inverse probability weighting-adjusted Cox regression models of colorectal cancer specific survival according to the intraepithelial and stromal M1:M2-density ratio with M1-like and M2-like macrophages defined using different cut-offs (10-50%) of both tails of the macrophage polarization (M1:M2) index distribution. The multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer, tumor location, tumor differentiation, disease stage, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations, and long-interspersed nucleotide element-1 methylation level. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models.

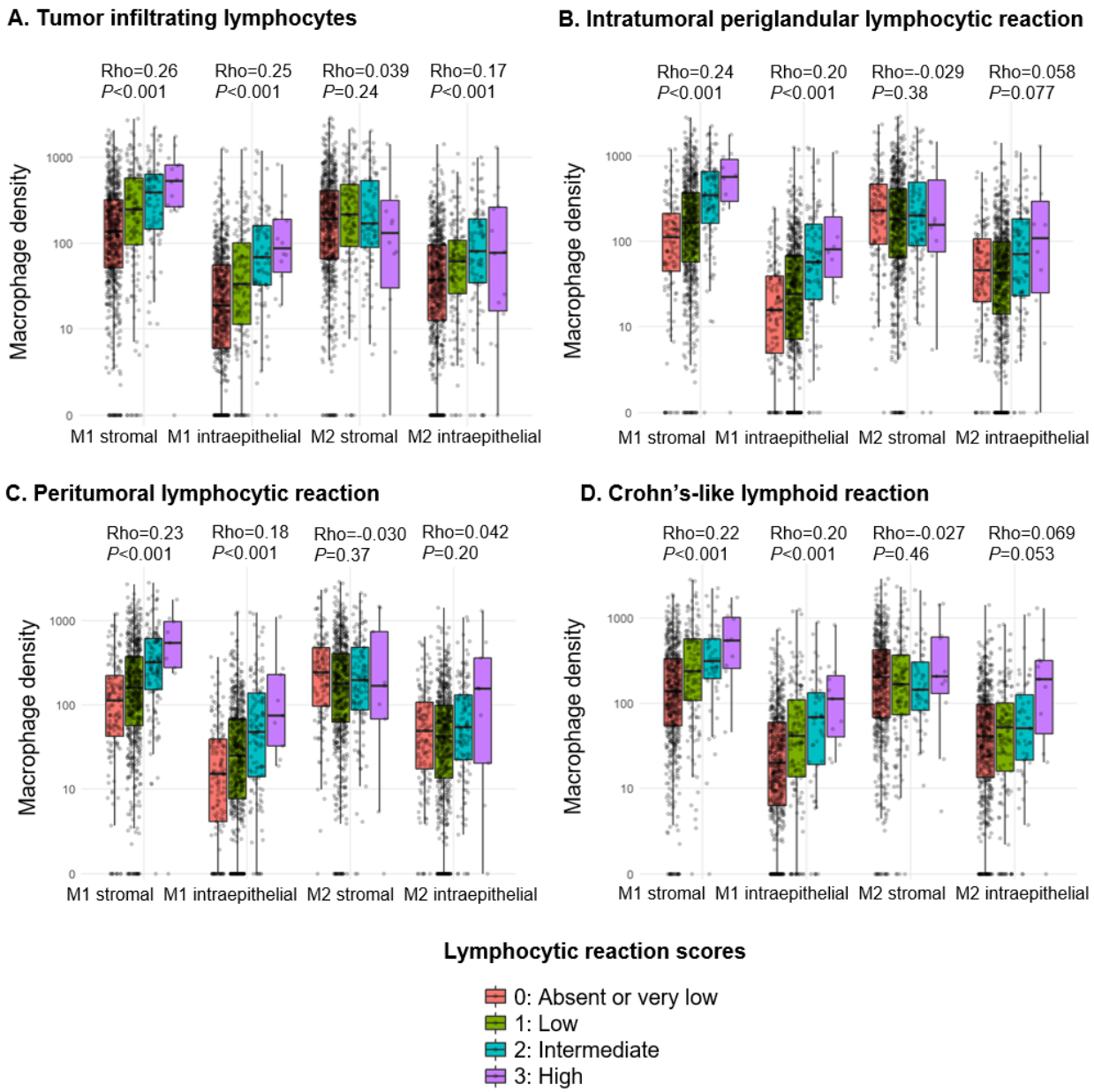


Figure S12. Densities of M1-like and M2-like macrophages in relation to histologic lymphocytic reaction patterns. A. Tumor infiltrating lymphocytes. B. Intratumoral periglandular lymphocytic reaction. C. Peritumoral lymphocytic reaction. D. Crohn's-like lymphoid reaction.

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