

## Localization and density of tertiary lymphoid structures associate with molecular subtype and clinical outcome in colorectal cancer liver metastases

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**Table of contents**

Supplementary methods .....  
3

Supplementary figures ..... 4

Supplementary tables .....  
12

## **Supplementary methods**

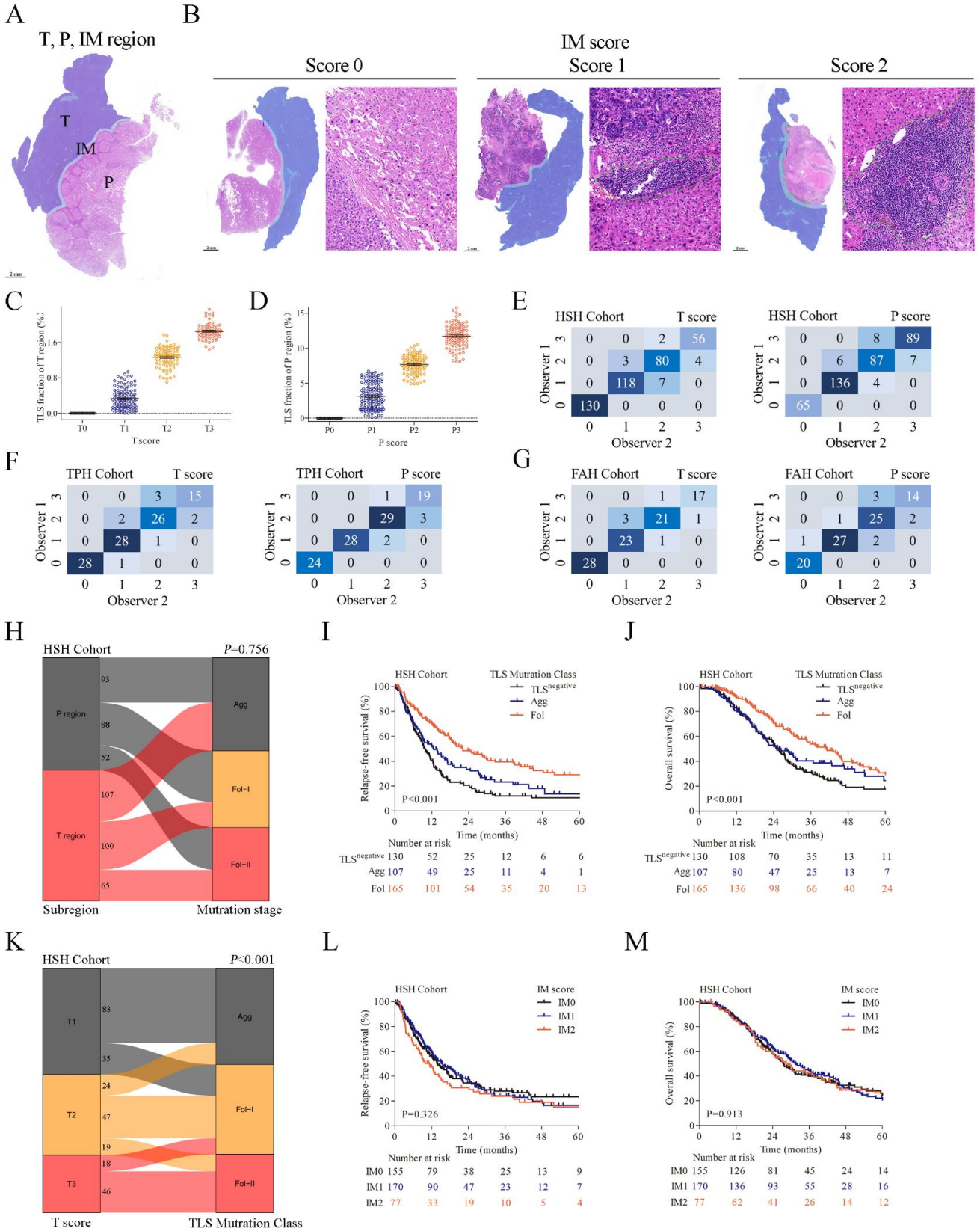
### **Evaluation of TLS abundance in the IM region**

The subregions of WSI were shown in Supplementary Fig. 1A. TLS abundance in the IM region (IM score) was divided into three groups: (1) score 0: without TLS in the IM region; (2) score 1: the area of TLSs in the IM region is less than 50% and (3) score 2: the area of TLSs in the IM region is more than 50% (Supplementary Fig. 1B).

### **Verification of the TLS scoring system**

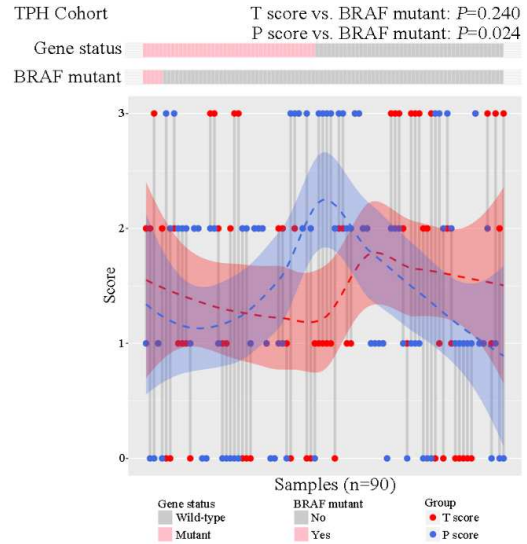
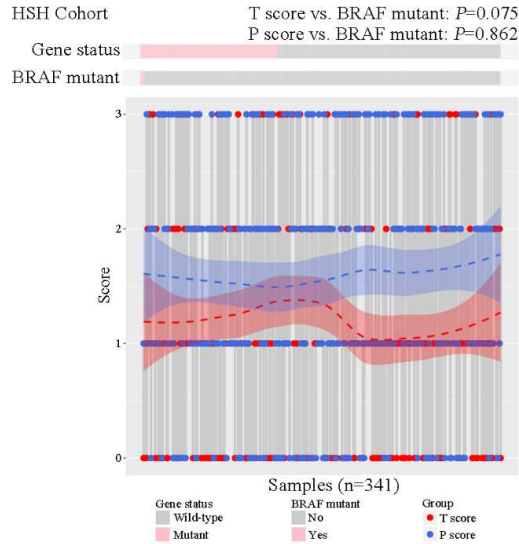
The subregions of TLSs in Cohort D were assessed by pathologists and the TLS fraction in the T region and P region (area of TLSs/area of the scoring subregion) was calculated by computer. As shown in Supplementary Fig. 1C and D, T score 0, 1, 2 and 3 corresponded to TLS fractions 0%, 0%-0.8%, 0.8%-1.6% and 1.6%-100%, respectively, while P score 0, 1, 2 and 3 corresponded to TLS fractions 0%, 0%-5%, 5%-10% and 10%-100%, respectively. These findings further demonstrated that this TLS scoring system could represent the abundance of TLSs.

Supplementary figures

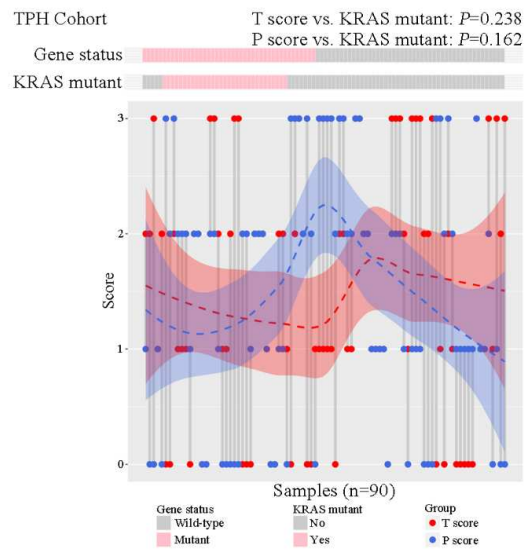
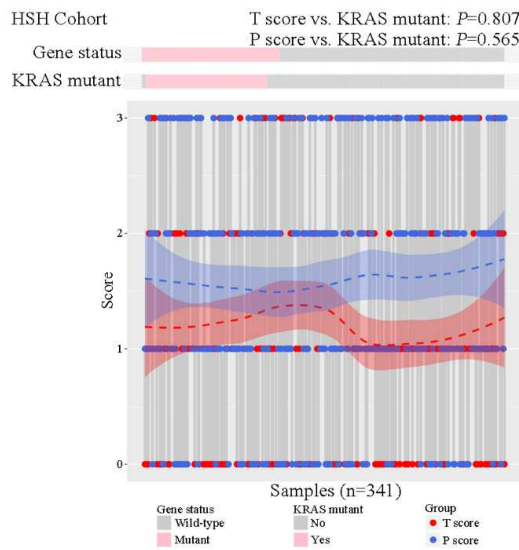


**Supplementary Fig. 1.** Additional information of the TLS scoring system. (A) Representatively schematic diagram of intra-tumor (T), peri-tumor (P) and invasive margin (IM) in whole side image (WSI). Peri-tumor region was highlighted in deep blue and invasive margin was highlighted in light blue. (B) Representative WSIs of H&E staining for TLS scoring system (IM score). (C, D) The distribution of TLS fraction with different T score (C) and P score (D) in the HSH Cohort. TLS fraction: area of TLSs/area of the scoring subregion. (E-G) Detailed T score, P score and IM score in the HSH Cohort (E), TPH Cohort (F) and FAH Cohort (G), respectively. (H) Correlation between T region, P region and maturation stages of TLSs in the HSH Cohort. (I, J) Kaplan-Meier curves showing relapse-free survival (I) and overall survival (J) of CRCLM patients stratified by TLSs Maturation Class in the HSH Cohort. (K) Correlation between T score and TLSs Maturation Class in the HSH Cohort. (L, M) Kaplan-Meier curves showing relapse-free survival (L) and overall survival (M) of CRCLM patients stratified by IM score in the HSH Cohort.

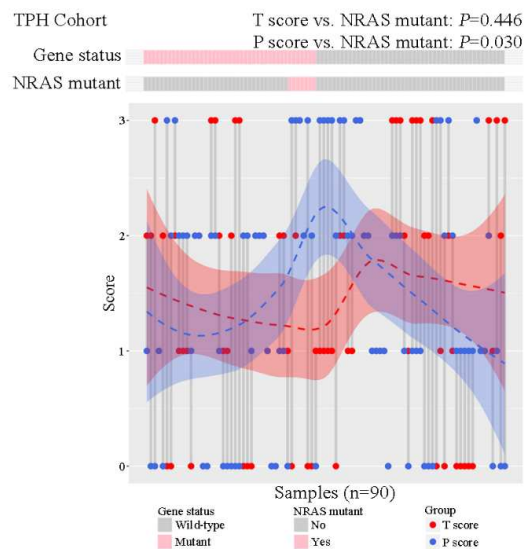
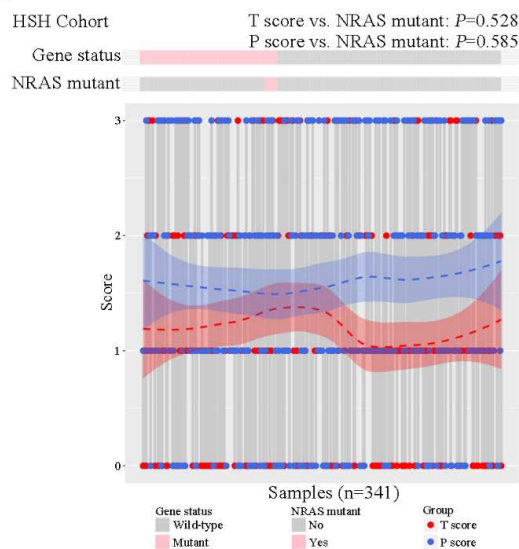
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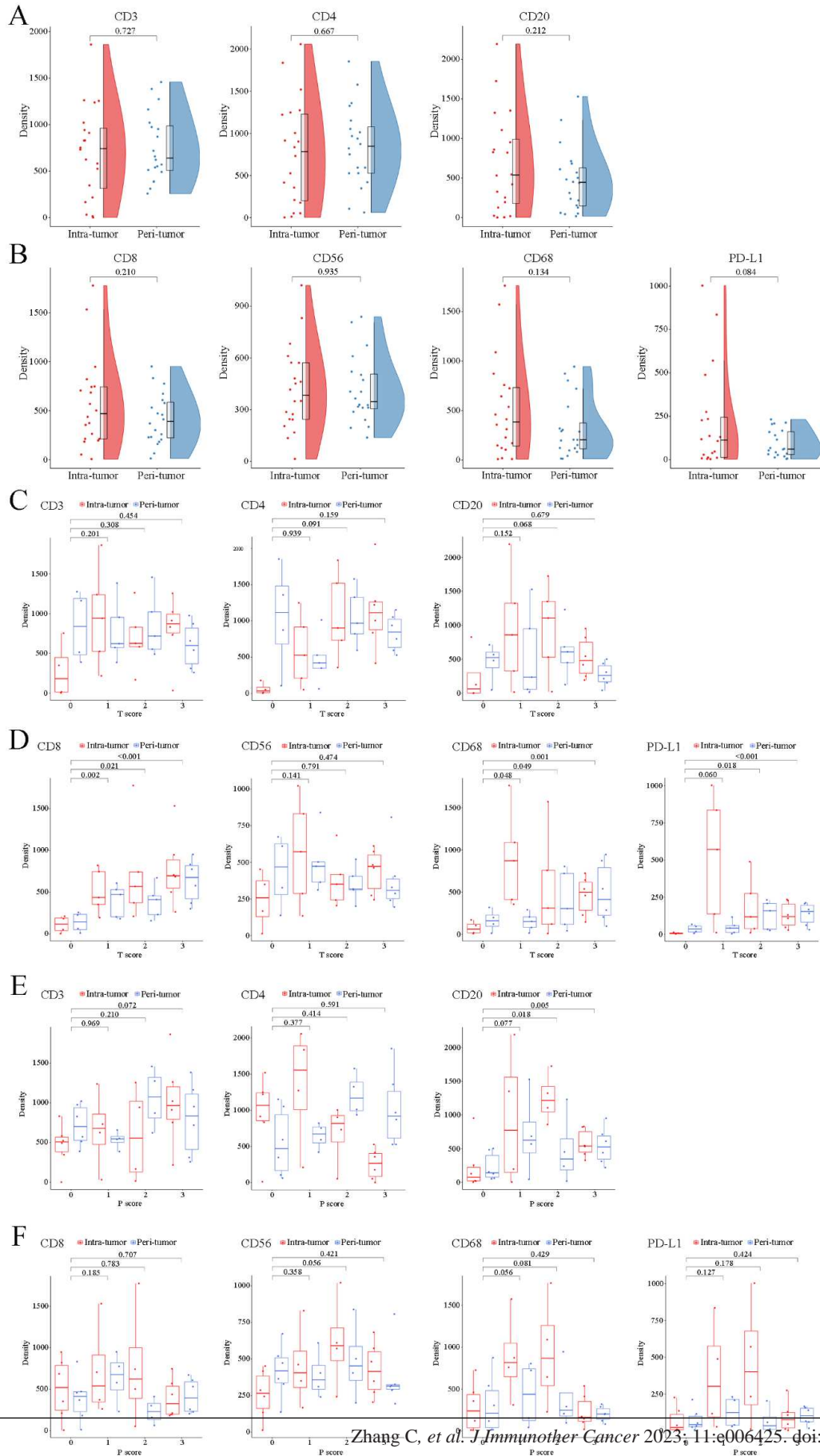
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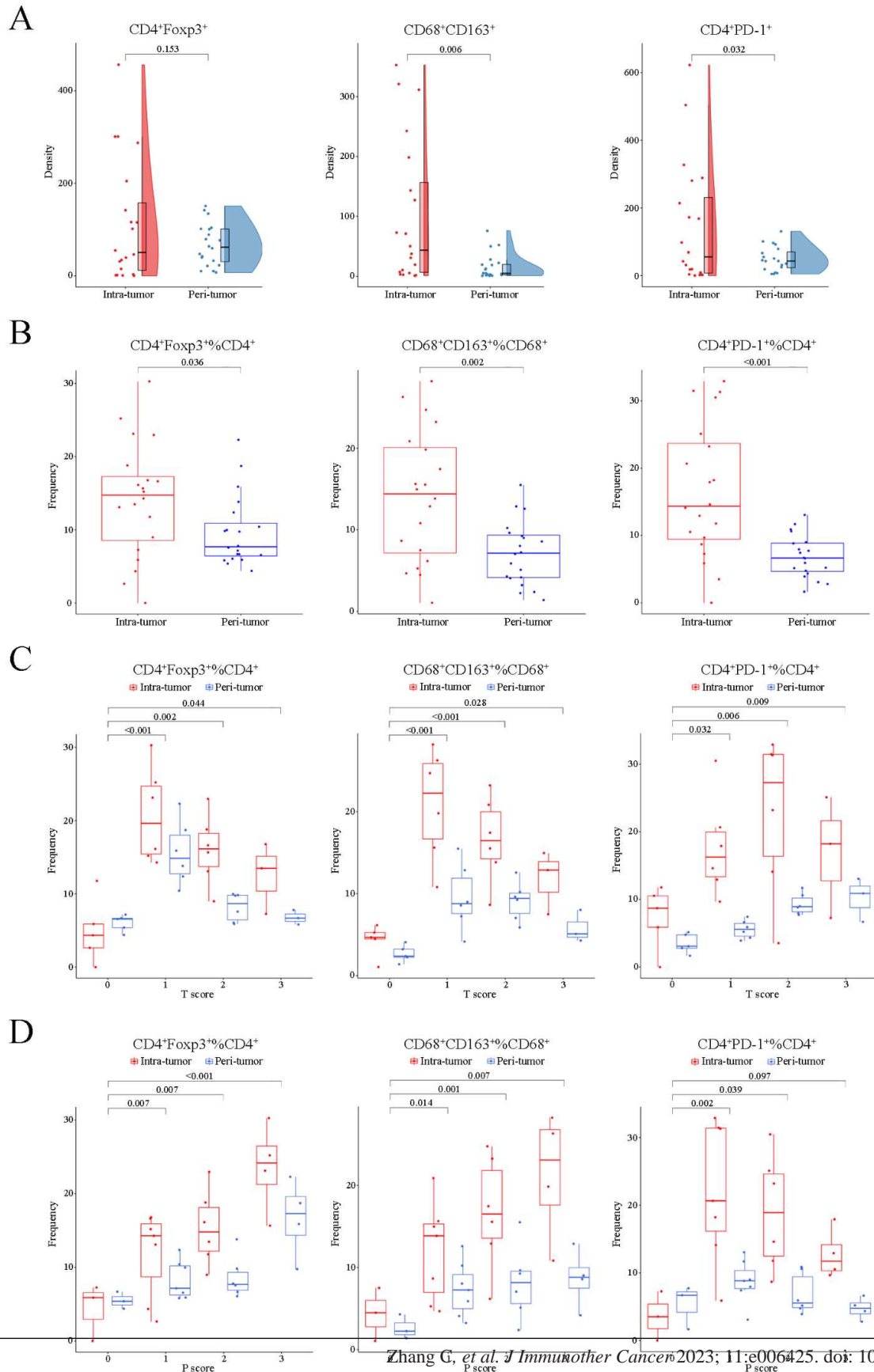


**Supplementary Fig. 2.** Correlation between gene mutation and T score and P score. (A) Relevance of T score and P score to BRAF mutation in the HSH and TPH Cohorts. (B) Relevance of T score and P score to KRAS mutation in the HSH and TPH Cohorts. (C) Relevance of T score and P score to NRAS mutation in the HSH and TPH Cohorts. The pick represents a mutation of gene, while the gray represents a wild-type of gene at the top. The connected red and blue circles on each column represent a patient's T score and P score, respectively. The red and blue curves represent the overall distribution of T score and P score of patients, respectively.





**Supplementary Fig. 3.** The immune composition of TLSs of CRCLM patients in the HSH Cohort. (A, B) Quantification of the density of CD3, CD4, CD20, CD8, CD56, CD56, CD68 and PD-L1 in intra-tumor and peri-tumor TLSs. (C-F) Quantification of the density of CD3, CD4, CD20, CD8, CD56, CD56, CD68 and PD-L1 in intra-tumor and peri-tumor TLSs with different T score (C, D) and P score (E, F), respectively.



**Supplementary Fig. 4.** The immune composition of TLSs of CRCLM patients in the TPH Cohort. (A, B) Quantification of the density (A) and frequency (B) of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells, CD4<sup>+</sup>PD-1<sup>+</sup> Tfh cells and CD68<sup>+</sup>CD163<sup>+</sup> M2 macrophages in intra-tumor and peri-tumor TLSs. (C, D) Quantification of the frequency of CD4<sup>+</sup>PD-1<sup>+</sup> Tfh cells and CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells within CD4<sup>+</sup> T cells and CD68<sup>+</sup>CD163<sup>+</sup> M2 macrophages within CD68<sup>+</sup> macrophages in intra-tumor and peri-tumor TLSs with different T score (C) and P score (D).

**Supplementary tables**

Supplementary Table 1. Information of the antibodies used in our research.

Antibodies	Source	Catalogue
CD3	Bioss	bs-10498R
CD4	Abcam	ab133616
CD20	Dako	IR604
Foxp3	Abcam	ab20034
PD-1	CST	86163S
CD8	Abcam	ab178089
CD56	Abcam	ab75813
CD68	Abcam	ab213363
CD163	Abcam	ab182422
PD-L1	CST	13684S

Supplementary Table 2. Univariate and multivariate analysis of relapse-free survival of CRCLM patients in HSH Cohort

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Sex (male vs. female)	1.054 (0.822-1.352)	0.678		
Age, years (60< vs. ≥60)	0.869 (0.687-1.099)	0.242		
Occurrence of metastases (metachronous vs. synchronous)	1.187 (0.921-1.530)	0.186		
Primary tumor location (left vs. right)	0.836 (0.642-1.090)	0.185		
CEA (ng/ml) (5< vs. ≥5)	1.562 (1.185-2.060)	0.002	1.386 (1.030-1.864)	0.031
CA19-9 (U/ml) (40< vs. ≥40)	1.323 (1.041-1.682)	0.022	1.020 (0.783-1.331)	0.881
CA125 (U/ml) (35< vs. ≥35)	1.162 (0.902-1.498)	0.245		
Size, cm (5< vs. ≥5)	1.325 (1.008-1.742)	0.043	1.048 (0.746-1.473)	0.785
Tumor number (single vs. multiple)	1.675 (1.315-2.132)	<0.001	1.392 (1.053-1.902)	0.029
Liver fibrosis (yes vs. no)	1.058 (0.707-1.584)	0.784		
Tumor grade (G1-2 vs. G3)	1.236 (0.948-1.613)	0.118		
T stage (Tis-2 vs. T3-4)	1.350 (1.029-1.770)	0.030	0.861 (0.611-1.213)	0.392
Lymph node metastasis (yes vs. no)	1.345 (1.121-1.670)	0.035	1.172 (1.011-1.383)	0.042
Differentiation		0.621		
Poor	reference			
Moderate	1.124 (0.806-1.569)	0.491		
Well	1.072 (0.734-1.486)	0.841		
Gene status (wild-type vs. mutant)	1.123 (0.865-1.458)	0.383		
Neoadjuvant chemotherapy (yes vs. no)	1.372 (1.080-1.742)	0.010	1.254 (1.022-1.637)	0.035
TLS Maturation Class		<0.001		
TLS <sup>negative</sup>	reference			
Agg	1.805 (1.157-2.818)	0.009		
Fol-I	2.396 (1.550-3.705)	<0.001		
Fol-II	3.181 (2.098-4.822)	<0.001		
T score		<0.001		<0.001
0	reference		reference	
1	1.414 (1.089-2.046)	0.014	1.424 (1.097-1.983)	0.025
2	2.288 (1.485-3.502)	<0.001	1.718 (1.371-2.032)	<0.001
3	2.945 (1.944-4.461)	<0.001	2.204 (1.631-2.815)	<0.001
P score		<0.001		<0.001
0	reference		reference	
1	0.716 (0.403-0.904)	0.017	0.647 (0.311-1.015)	0.058
2	0.516 (0.386-0.689)	<0.001	0.323 (0.198-0.503)	<0.001
3	0.385 (0.212-0.408)	<0.001	0.211 (0.117-0.327)	<0.001

Abbreviations: CRCLM, colorectal cancer liver metastases; HR, hazard ratio; CI, confidential interval; CEA, carcinoembryonic antigen.

Supplementary Table 3. Univariate and multivariate analysis of overall survival of CRCLM patients in HSH Cohort

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Sex (male vs. female)	1.135 (0.877-1.469)	0.335		
Age, years (60< vs. ≥60)	0.901 (0.707-1.149)	0.401		
Occurrence of metastases (metachronous vs. synchronous)	1.073 (0.827-1.392)	0.598		
Primary tumor location (left vs. right)	1.054 (0.796-1.396)	0.712		
CEA (ng/ml) (5< vs. ≥5)	1.372 (1.032-1.825)	0.030	1.310 (1.002-1.714)	0.041
CA19-9 (U/ml) (40< vs. ≥40)	1.211 (0.946-1.551)	0.128		
CA125 (U/ml) (35< vs. ≥35)	0.992 (0.764-1.288)	0.952		
Size, cm (5< vs. ≥5)	1.166 (0.881-1.543)	0.283		
Tumor number (single vs. multiple)	1.743 (1.357-2.239)	<0.001	1.323 (1.013-1.729)	0.040
Liver fibrosis (yes vs. no)	1.390 (0.939-2.059)	0.100		
Tumor grade (G1-2 vs. G3)	1.500 (1.144-1.967)	0.003	1.171 (0.874-1.569)	0.290
T stage (Tis-2 vs. T3-4)	1.386 (1.050-1.829)	0.021	0.956 (0.702-1.302)	0.775
Lymph node metastasis (yes vs. no)	1.465 (1.099-1.953)	0.009	1.326 (1.056-1.787)	0.049
Differentiation		0.114		
Poor	reference			
Moderate	1.385 (0.979-1.960)	0.065		
Well	1.040 (0.775-1.397)	0.793		
Gene status (wild-type vs. mutant)	1.117 (0.851-1.467)	0.424		
Neoadjuvant chemotherapy (yes vs. no)	1.609 (1.253-2.065)	<0.001	1.572 (1.216-2.033)	0.001
TLS Maturation Class		<0.001		
TLS <sup>negative</sup>	reference			
Agg	3.785 (2.341-6.122)	<0.001		
Fol-I	3.224 (1.943-5.351)	<0.001		
Fol-II	3.110 (1.892-5.112)	<0.001		
T score		<0.001		<0.001
0	reference		reference	
1	1.692 (1.231-2.775)	0.037	1.318 (1.041-1.768)	0.046
2	2.749 (1.730-4.369)	<0.001	2.024 (1.486-2.327)	0.011
3	2.888 (1.836-4.542)	<0.001	2.233 (1.695-3.283)	<0.001
P score		<0.001		<0.001
0	reference		reference	
1	0.693 (0.511-0.941)	0.019	0.701 (0.515-1.003)	0.038
2	0.445 (0.330-0.599)	<0.001	0.473 (0.334-0.602)	<0.001
3	0.177 (0.102-0.309)	<0.001	0.209 (0.115-0.383)	<0.001

Abbreviations: CRCLM, colorectal cancer liver metastases; HR, hazard ratio; CI, confidential interval; CEA, carcinoembryonic

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Supplementary Table 5. Correlation between the presence of TLS and the gene mutation of KRAS, NRAS and TP53 in GSE159216.

Variables	KRAS		P value	NRAS		P value	TP53		P value
	WT	Mut		WT	Mut		WT	Mut	
TLS (%)			0.232			0.214			0.900
TLS+	70 (40.9)	58 (33.9)		124 (72.5)	4 (2.3)		37 (21.7)	91 (53.2)	
TLS-	28 (16.4)	15 (8.8)		39 (22.8)	4 (2.3)		12 (7.0)	31 (18.1)	

Abbreviations: WT, wild-type; Mut, mutation.

Supplementary Table 6. Clinical characteristics and TLS scores of CRCLM patients in HSH and TPH Cohorts for multiplex immunohistochemistry.

No.	T score	P score	Occurrence of metastases	Primary tumor location	CEA, ng/ml	CA19-9, U/ml	CA125, U/ml	Size, cm	Tumor number	Liver fibrosis	Tumor grade	T stage	Lymph node metastasis	Differentiation	Gene status	Neoadjuvant chemotherapy	Immune Class
HSH Cohort																	
1	0	0	Synchronous	Left	1.9	10.0	22.4	4.0	Multiple	No	G2	T4	Yes	Moderate	Wild-type	Yes	Class III
2	0	3	Metachronous	Left	50.0	34.4	32.1	3.5	Multiple	No	G2	T4	Yes	Poor	Mutant	Yes	Class I
3	0	3	Synchronous	Left	3.6	31.8	6.4	1.5	Multiple	No	G2	T2	Yes	Moderate	Wild-type	Yes	Class I
4	0	3	Synchronous	Left	20.3	1540.0	25.4	2.0	Multiple	No	G2	T2	Yes	Poor	Mutant	No	Class I
5	1	0	Metachronous	Left	7.2	37.5	18.0	2.5	Single	No	G3	T2	No	Well	Wild-type	No	Class IV
6	1	1	Synchronous	Right	1438.0	0.6	12.6	13.0	Single	No	G3	T2	No	Moderate	Wild-type	No	Class III
7	1	2	Synchronous	Right	11.4	76.0	58.9	2.0	Single	No	G2	T3	Yes	Moderate	Wild-type	No	Class II
8	1	2	Metachronous	Left	81.1	25.7	89.4	2.8	Multiple	Yes	G2	T2	Yes	Poor	Wild-type	Yes	Class II
9	1	3	Synchronous	Left	5.9	58.2	61.1	5.0	Multiple	No	G3	T2	No	Well	Wild-type	Yes	Class I
10	2	0	Metachronous	Left	4.4	8.1	11.6	5.0	Multiple	No	G2	T3	Yes	Poor	Wild-type	Yes	Class IV
11	2	0	Synchronous	Left	30.5	975.4	7.1	6.0	Single	No	G3	T2	No	Moderate	Wild-type	No	Class IV
12	2	1	Synchronous	Left	12.1	12.7	20.1	2.0	Single	No	G3	T2	Yes	Poor	Mutant	Yes	Class III
13	2	2	Synchronous	Left	57.8	0.6	8.8	3.8	Multiple	No	G2	T2	Yes	Moderate	Wild-type	Yes	Class II
14	2	3	Synchronous	Right	18.4	0.9	17.5	3.0	Multiple	No	G1	T3	Yes	Moderate	Mutant	No	Class I
15	3	0	Synchronous	Right	8.4	86.1	5.3	1.4	Single	No	G2	T2	Yes	Well	Mutant	No	Class IV
16	3	0	Metachronous	Left	4.9	11.2	11.2	3.0	Single	Yes	G2	T2	No	Poor	Wild-type	No	Class IV
17	3	1	Metachronous	Left	17.7	70.2	28.4	2.6	Single	No	G2	T2	No	Moderate	Wild-type	Yes	Class IV
18	3	1	Synchronous	Left	101.0	20.8	12.0	6.5	Multiple	No	G3	T4	Yes	Moderate	Wild-type	No	Class IV
19	3	2	Synchronous	Left	42.2	118.5	98.4	2.7	Single	No	G2	T2	No	Moderate	Wild-type	No	Class III
20	3	3	Synchronous	Right	0.9	435.0	46.6	3.0	Multiple	No	G3	T2	Yes	Poor	Wild-type	Yes	Class II
TPH Cohort																	
1	0	0	Synchronous	Left	3.7	19.5	22.2	4.5	Multiple	No	G2	T2	Yes	Moderate	Mutant	Yes	Class III
2	0	1	Synchronous	Left	46.5	71.4	8.2	3.0	Multiple	Yes	G2	T3	Yes	Moderate	Wild-type	Yes	Class II
3	0	2	Metachronous	Left	81.2	182.7	69.6	8.0	Multiple	Yes	G2	T4	Yes	Poor	Mutant	No	Class I
4	0	2	Synchronous	Left	329.7	192.6	90.5	4.5	Multiple	Yes	G2	T3	Yes	Moderate	Wild-type	Yes	Class I
5	0	3	Metachronous	Left	2.5	50.1	37.3	5.0	Multiple	No	G2	T2	Yes	Well	Wild-type	Yes	Class I
6	1	1	Metachronous	Right	4.2	11.9	13.2	3.0	Single	No	G2	T3	No	Moderate	Wild-type	No	Class III
7	1	1	Metachronous	Left	21.6	61.0	12.8	2.5	Multiple	No	G2	T3	Yes	Moderate	Mutant	No	Class III
8	1	2	Synchronous	Right	2.7	8.0	11.4	3.5	Single	No	G3	T2	Yes	Well	Wild-type	No	Class II
9	1	2	Synchronous	Left	90.9	200.4	311.3	7.0	Multiple	No	G3	T4	Yes	Moderate	Mutant	No	Class II
10	1	3	Metachronous	Right	2.5	27.0	39.4	1.5	Single	No	G2	T2	Yes	Well	Wild-type	Yes	Class I
11	1	3	Synchronous	Left	1957.0	156.7	8.2	8.5	Multiple	No	G3	T4	Yes	Moderate	Wild-type	Yes	Class I
12	2	0	Metachronous	Left	4.9	38.4	40.1	2.5	Single	Yes	G2	T2	No	Moderate	Wild-type	No	Class IV
13	2	1	Metachronous	Right	53.5	32.4	9.1	6.0	Single	Yes	G3	T3	No	Poor	Mutant	Yes	Class III
14	2	1	Synchronous	Right	4.5	8.5	14.4	2.5	Multiple	No	G2	T2	No	Moderate	Wild-type	Yes	Class III
15	2	2	Metachronous	Left	1.52	30.0	40.3	3.0	Multiple	No	G2	T2	No	Moderate	Mutant	No	Class II
16	2	2	Synchronous	Left	328.5	784.0	618.3	7.5	Multiple	Yes	G3	T4	Yes	Poor	Wild-type	No	Class II
17	2	3	Metachronous	Right	142.8	67.6	40.1	6.5	Single	No	G3	T3	Yes	Poor	Wild-type	No	Class I
18	3	0	Metachronous	Left	6.3	31.2	9.5	4.0	Single	No	G2	T3	Yes	Moderate	Mutant	No	Class IV
19	3	0	Metachronous	Left	46.6	39.8	40.6	3.5	Multiple	No	G2	T3	No	Moderate	Wild-type	Yes	Class IV
20	3	1	Metachronous	Left	4.6	8.5	11.6	1.5	Single	No	G2	T2	No	Well	Wild-type	Yes	Class IV

Abbreviations: CRCLM, colorectal cancer liver metastases; CEA, carcinoembryonic antigen.

Supplementary Table 7. Corresponding relationships among T score, P score, Immune score, and Immune Class.

T score	P score	Immune score (RFS)	Immune score (OS)	Immune Class
0	3	-2.31	-2.7	Class I
1	3	-1.79	-2.03	
0	2	-1.54	-1.8	
2	3	-1.27	-1.36	
1	2	-1.02	-1.13	Class II
0	1	-0.77	-0.9	
3	3	-0.75	-0.69	
2	2	-0.5	-0.46	
1	1	-0.25	-0.23	Class III
0	0	0	0	
3	2	0.02	0.21	
2	1	0.27	0.44	
1	0	0.52	0.67	Class IV
3	1	0.79	1.11	
2	0	1.04	1.34	
3	0	1.56	2.01	

Abbreviations: RFS, recurrence-free survival; OS, overall survival.

Immune score (RFS) =  $(0.52 \times \text{T score}) + (-0.77 \times \text{P score})$

Immune score (OS) =  $(0.67 \times \text{T score}) + (-0.90 \times \text{P score})$