Figure S1. Overview flowchart of the study.

CD11c CD45RO CD68 panCK PD-L1 DAPI   CD4 CD8A CD56 FoxP3 GranzymeB DAPI
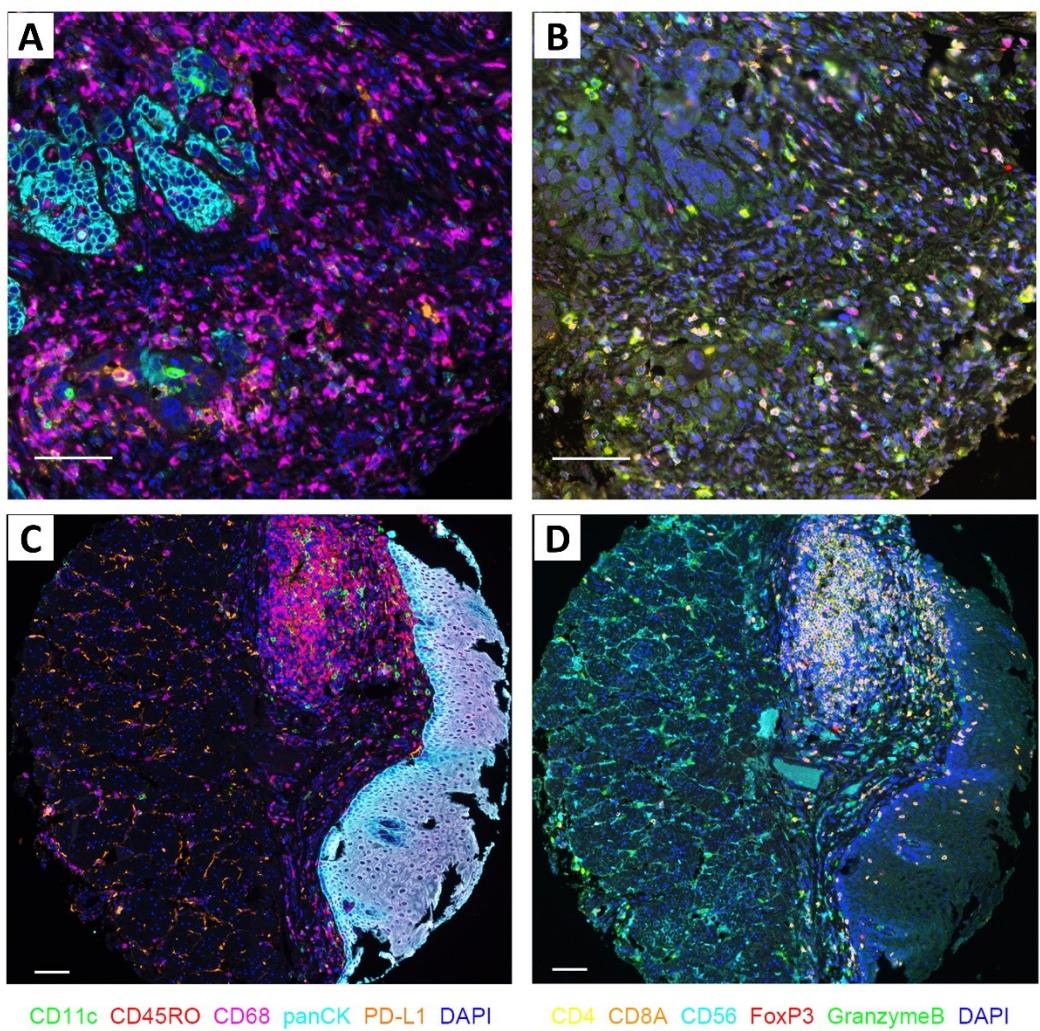
Figure S2. The representative images displaying different immune subpopulation in ESCC tumor specimens after multispectral merged imaging for panel A (A and C) and panel B (B and D).
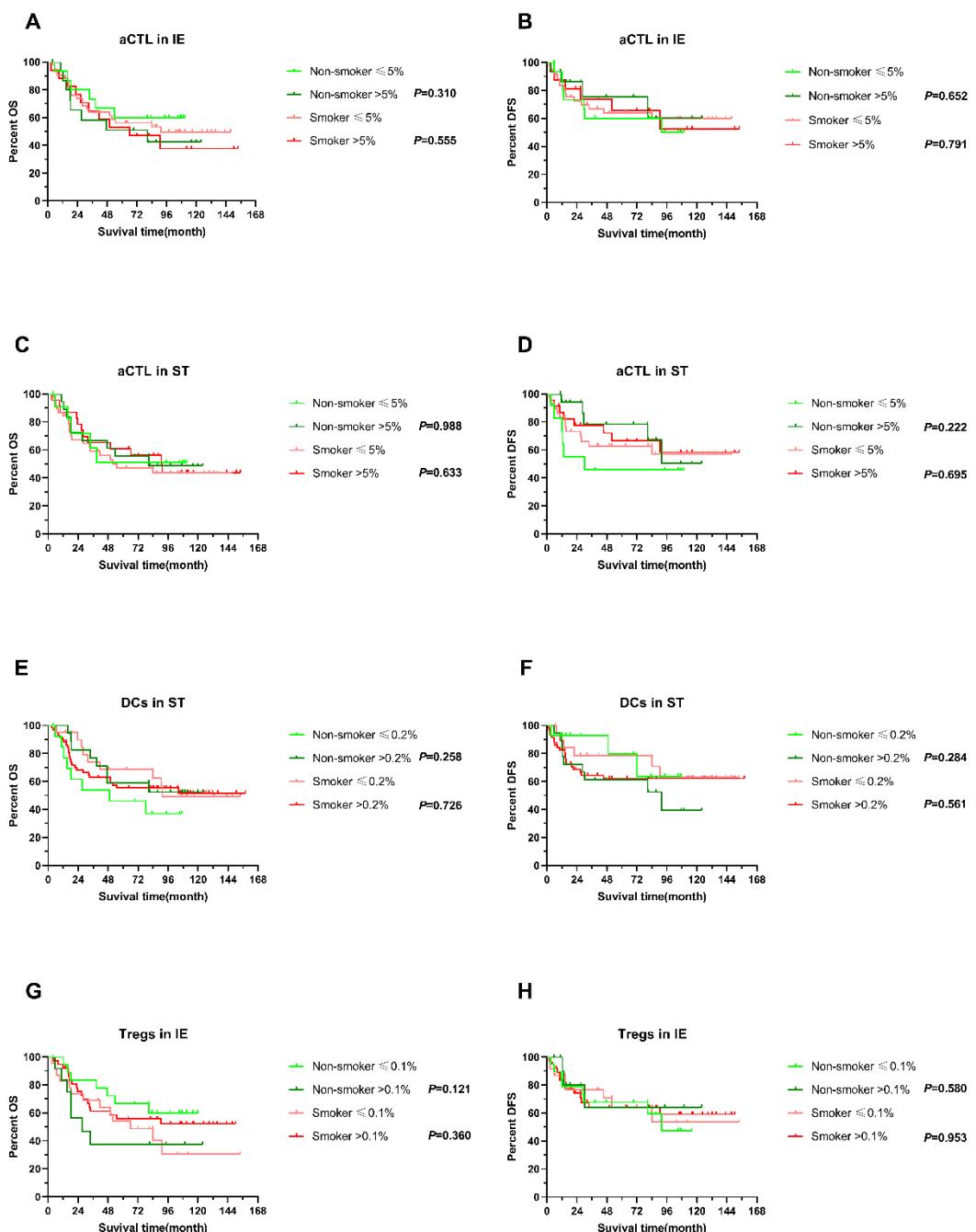
Figure S3. OS of stratified immune cell infiltration in tumor specimens between smoking and non-smoking ESCC patients.

Table S1 Clinical characteristics of patients with normal specimens

Variables	Total	Smoking status, n(%)		P
		Non-smoker (n=17)	Current smoker (n=12)	
Gender				**0.001**^{**}
Male	19	7 (36.8)	12 (63.2)	
Female	10	10 (100.0)	0 (0.0)	
Age				0.999
≤ 58 yrs	9	5 (55.6)	4 (44.4)	
> 58yrs	20	12 (60.0)	8 (40.0)	
Tumor size				0.408
≤ 3.5cm	21	11 (52.4)	10 (47.6)	
> 3.5cm	8	6 (75.0)	2 (25.0)	
T stage				0.273
T1-2	11	8 (72.7)	3 (27.3)	
T3	18	9 (50.0)	9 (50.0)	
N stage				0.999
N0	15	9 (60.0)	6 (40.0)	
N1-3	14	8 (57.1)	6 (42.9)	
TNM stage				0.471
I-II	17	11 (64.7)	6 (35.3)	
III-IV	12	6 (50.0)	6 (50.0)	
G stage				0.488
W/D [a]	3	1 (33.3)	2 (66.7)	
M/D	15	8 (53.3)	7 (46.7)	
P/D	11	8 (72.7)	3 (27.3)	

[a] W/D, well-differentiated; M/D, moderately differentiated; P/D, poorly differentiated.

[**] $P<0.01$.

Table S2 Clinical characteristics of patient with cancer specimens

| Variables | Total | Smoking status, n(%) | | P |
		Non-smoker (n=34)	Current smoker (n=83)	
Age				0.226
≤ 58 yrs†	61	15(24.6)	46(75.4)	
> 58yrs	56	19(33.9)	37(66.1)	
Tumor size				0.489
≤ 3.5cm	54	14(25.9)	40(74.1)	
> 3.5cm	63	20(31.7)	43(68.3)	
T stage				0.092
T1-2	29	12(41.4)	17(58.6)	
T3	88	22(25.0)	66(75.0)	
N stage				
N0	56	14(25.0)	42(75.0)	0.354
N1-3	61	20(32.8)	41(67.2)	
TNM stage				0.869
I-II	64	19(29.7)	45(70.3)	
III-IV	53	15(28.3)	38(71.7)	
G stage				0.087
W/D ‡	30	8(26.7)	22(73.3)	
M/D	61	14(23.0)	47(77.0)	
P/D	26	12(46.2)	14(53.8)	

† Median age.

‡ W/D, well-differentiated; M/D, moderately differentiated; P/D, poorly differentiated.

Table S3 Correlation between age and immune infiltration in patient of ESCC

Variables†	Non-smokers			Smokers		
	n	r_s‡	*P*	n	r_s	*P*
IE TAM	32	-0.01	0.970	79	0.33	**0.003****
ST TAM	32	-0.10	0.600	78	0.36	**0.001****
IE DC	32	-0.14	0.458	79	0.10	0.372
ST DC	32	-0.27	0.135	78	0.10	0.370
IE Tmem	32	0.09	0.632	79	-0.10	0.357
ST Tmem	32	0.06	0.746	78	-0.19	0.089
IE Treg	31	0.39	**0.032***	60	-0.16	0.217
ST Treg	30	0.07	0.711	61	0.00	0.988
IE Th	31	-0.29	0.115	60	-0.03	0.828
ST Th_s	30	-0.06	0.738	61	0.07	0.617
IE CTL	31	0.31	0.087	60	0.09	0.477
ST CTL	30	0.21	0.269	61	0.00	0.990
IE aCTL	31	0.28	0.129	60	0.09	0.515
ST aCTL	30	0.06	0.736	61	0.01	0.987
IE aCTL/CTL	31	-0.22	0.230	60	0.02	0.891
ST aCTL/CTL	30	-0.30	0.111	61	-0.03	0.818
IE NK	31	-0.37	**0.043***	60	0.25	0.058
ST NK	30	-0.28	0.139	61	0.39	**0.002****

† IE, intraepithelial. ST, stromal.   ‡r_s, Spearman correlation coefficient.

* *P*<0.05, ** *P*<0.01

Table S4 Correlation between age, smoking characteristics, and infiltrated cell in tissues of smoking ESCC patients

Infiltrating cell†	n	Age		Pack-years of smoking		Age-adjusted pack-years of smoking		Smoking year		Age-adjusted smoking year	
		r_s‡	*P*	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*
IE TAM	79	0.33	**0.003****	0.11	0.347	0.04	0.733	0.26	**0.020***	0.12	0.312
ST TAM	78	0.36	**0.001****	0.25	**0.029***	0.19	0.105	0.47	**<0.001****	0.35	**0.002****
IE DC	79	0.10	0.372	0.08	0.472	0.06	0.590	-0.02	0.855	-0.09	0.461
ST DC	78	0.10	0.37	0.15	0.188	0.13	0.252	0.09	0.433	0.04	0.707
IE Tmem	79	-0.11	0.357	-0.13	0.27	-0.11	0.355	-0.18	0.104	-0.15	0.181
ST Tmem	78	-0.19	0.089	0.07	0.566	0.11	0.328	-0.06	0.595	0.05	0.696
IE Treg	60	-0.16	0.217	-0.25	0.056	-0.21	0.118	-0.23	0.081	-0.16	0.214
ST Treg	61	0.00	0.988	-0.12	0.358	-0.13	0.338	-0.06	0.629	-0.08	0.563
IE Th	60	-0.03	0.828	-0.01	0.972	0.01	0.963	0.03	0.802	0.06	0.633
ST Th	61	0.07	0.617	0.05	0.704	0.03	0.808	0.13	0.303	0.12	0.371
IE CTL	60	0.09	0.477	0.15	0.26	0.12	0.354	0.10	0.444	0.06	0.676
ST CTL	61	0.00	0.99	0.08	0.56	0.08	0.543	0.08	0.561	0.09	0.489
IE aCTL	60	0.09	0.515	0.15	0.263	0.13	0.347	0.16	0.234	0.13	0.321
ST aCTL	61	0.00	0.987	0.01	0.953	0.01	0.948	0.01	0.971	0.01	0.958
IE aCTL/CTL	60	0.02	0.891	0.06	0.627	0.06	0.642	0.14	0.294	0.16	0.227
ST aCTL/CTL	61	-0.03	0.818	-0.06	0.648	-0.05	0.687	-0.05	0.728	-0.04	0.792
IE NK	60	0.25	0.058	-0.04	0.737	-0.15	0.266	0.14	0.278	-0.01	0.943
ST NK	61	0.39	**0.002****	0.00	0.994	-0.13	0.317	0.18	0.168	-0.04	0.759

† IE, intraepithelial. ST, stromal. r_s, Spearman correlation coefficient

* *P*<0.05, ** *P*<0.01