Supplementary Data

Supplementary Figure Legends

Figure S1. Representative images of aggregation of lymphocytes and a germinal center. Clinical significance of localization of TLSs.

A) Representative images of CD3 and CD20 immunohistostaining (×40). Scale bars, 100 µm.

B) Representative images of a germinal center (×40). The inset shows a higher magnification image of the boxed area (×400). Scale bars, 100 µm. Centroblasts (red arrowheads), centrocytes (blue arrowheads), FDCs (black arrowheads) and HEV (yellow dotted line).

C) Representative images of peritumoral TLSs (peri) and intratumoral TLSs (intra) (×40). Scale bars, 100 µm.

D) Kaplan–Meier analyses of recurrence-free survival and overall survival in ESCC by localization of TLSs. *P*-values were calculated by log-rank test.

Figure S2. Identification of cell types in ESCC by scRNA-seq.

A) Schematic overview of the experimental workflow.

B) UMAP plots colored by initial cluster.

C) UMAP plot showed the major cell types using canonical marker genes.

D) Heatmap showed the expression of the canonical marker genes in each major cell type.

E) Numbers of cells from each patient for cell types.

F) Box and whisker plots showed the cluster cell distributions in accordance with histological types (normal tissues (Normal), tumors without TLSs (TLS-) and tumors with TLSs (TLS+)). Box middle lines, median; box limits, upper and lower quartiles; box whiskers, 1.5× the interquartile range. Dunn's multiple comparisons test was performed. $^{**}p < 0.01$.

Figure S3. Trajectory analysis in CD8^{+} T cell subtypes.

Trajectory analysis of CD8^{+} T cells. The pseudotime trajectory was calculated with lighter colors indicating newer values.

Figure S4. Phenotypes in myeloid cells. Representative images of CD11c immunostaining.

A) UMAPs of myeloid cells, colored by signature scores of the DC-like, MDSC-like and TAM-like myeloid subsets.

B) Representative images of CD11c immunostaining in tumor tissues with or without TLS (×400). Scale bars, 50 µm.

Figure S5. Schematic view showing that SEMA4D on T cells stimulates DCs.

Figure S6. Schematic view showing that TLSs correlate with enhancement of anti-tumor immunity in ESCC.

Supplementary Table Legends.

Supplementary Table S1. Clinicopathological characteristics in patients of esophageal squamous cell carcinoma with and without TLS presence.

Supplementary Table S2. Primary antibody with CODEX.

Supplementary Table S3. Univariate and multivariate analyses of RFS and OS between TLS presence and clinicopathological characteristics in ESCC patients.

Supplementary Table S4. Summary of patient characteristics used in scRNA-seq in this study.

Supplementary Table S5. Components of each gene signature in our study.

Fig. S1

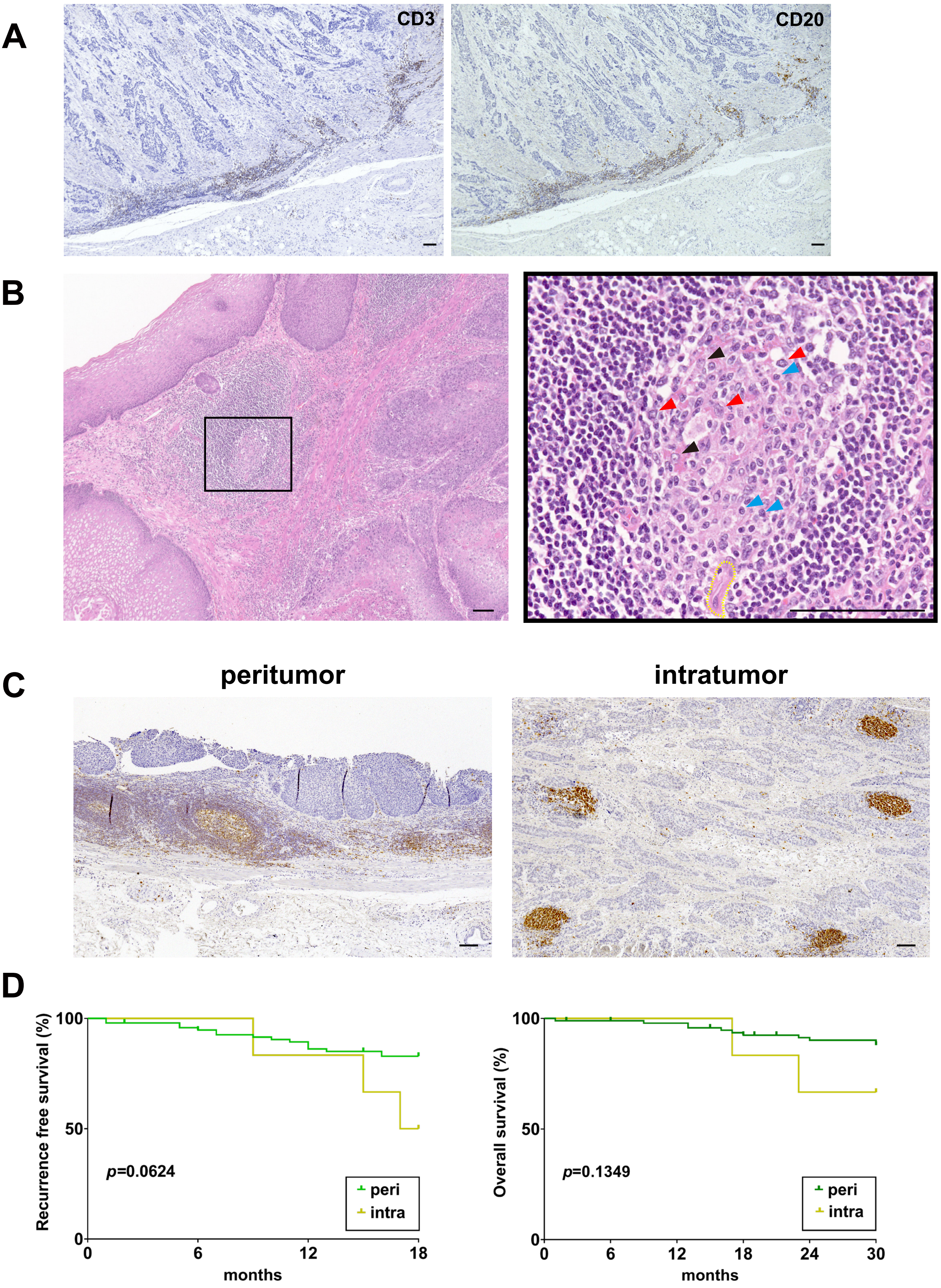
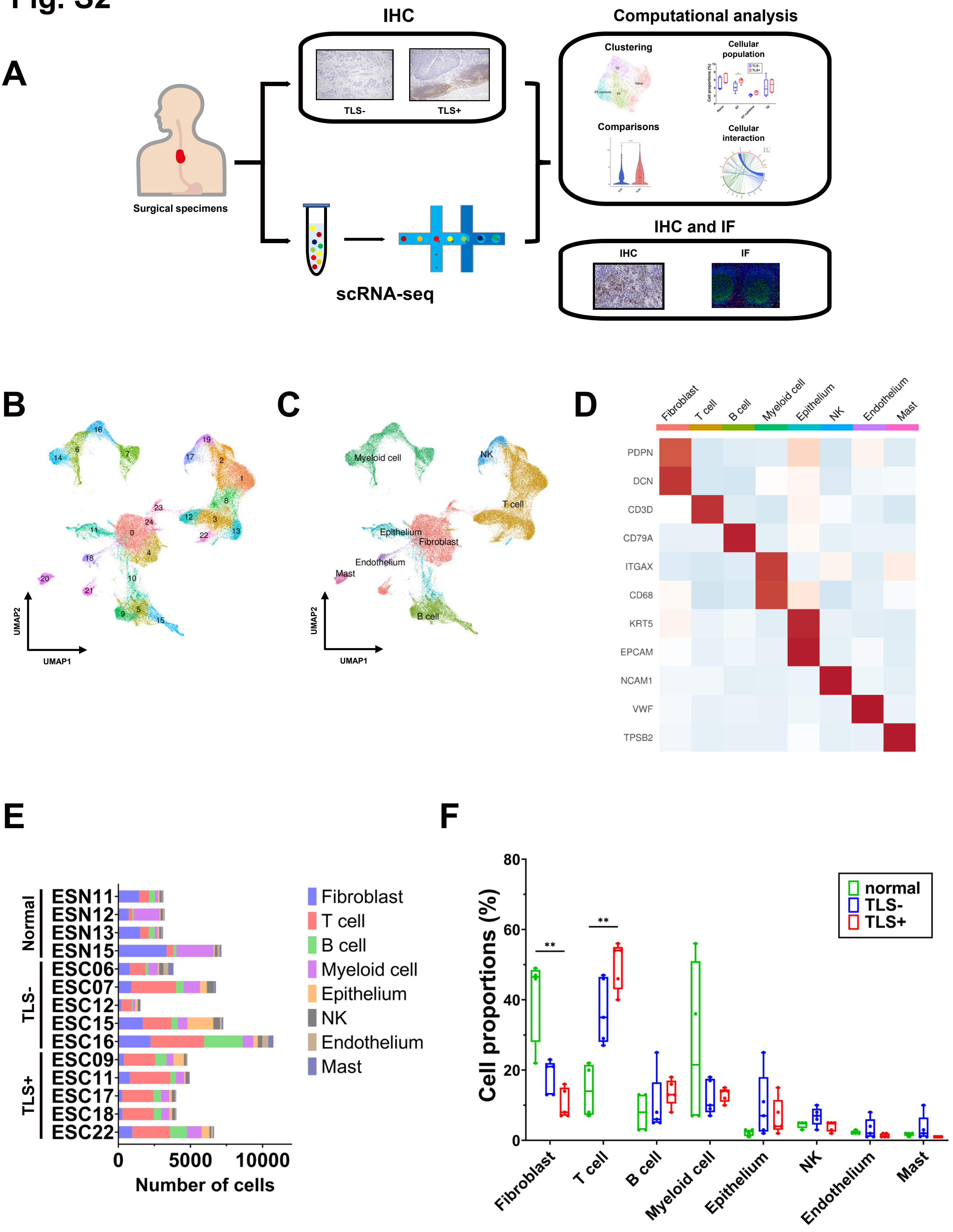

A
CD3
CD20
B
C
peritumor
intratumor
D
Recurrence free survival (%)
100
50
0
0
6
12
18
months
p=0.0624
peri
intra
Overall survival (%)
100
50
0
0
6
12
18
24
30
months
p=0.1349
peri
intra

Fig. S2



A
IHC
Computational analysis
TLS-
TLS+
Clustering
Cellular population
Comparisons
Cellular interaction
Surgical specimens
scRNA-seq
IHC and IF
IHC
IF
B
UMAP2
UMAP1
C
Myeloid cell
NK
T cell
Epithelium
Fibroblast
Endothelium
Mast
B cell
UMAP2
UMAP1
D
Fibroblast
T cell
B cell
Myeloid cell
Epithelium
NK
Endothelium
Mast
PDPN
DCN
CD3D
CD79A
ITGAX
CD68
KRT5
EPCAM
NCAM1
VWF
TPSB2
E
Normal
TLS-
TLS+
ESN11
ESN12
ESN13
ESN15
ESC06
ESC07
ESC12
ESC15
ESC16
ESC09
ESC11
ESC17
ESC18
ESC22
0
5000
10000
Number of cells
Fibroblast
T cell
B cell
Myeloid cell
Epithelium
NK
Endothelium
Mast
F
Cell proportions (%)
80
60
40
20
0
**
**
normal
TLS-
TLS+
Fibroblast
T cell
B cell
Myeloid cell
Epithelium
NK
Endothelium
Mast

Fig. S3

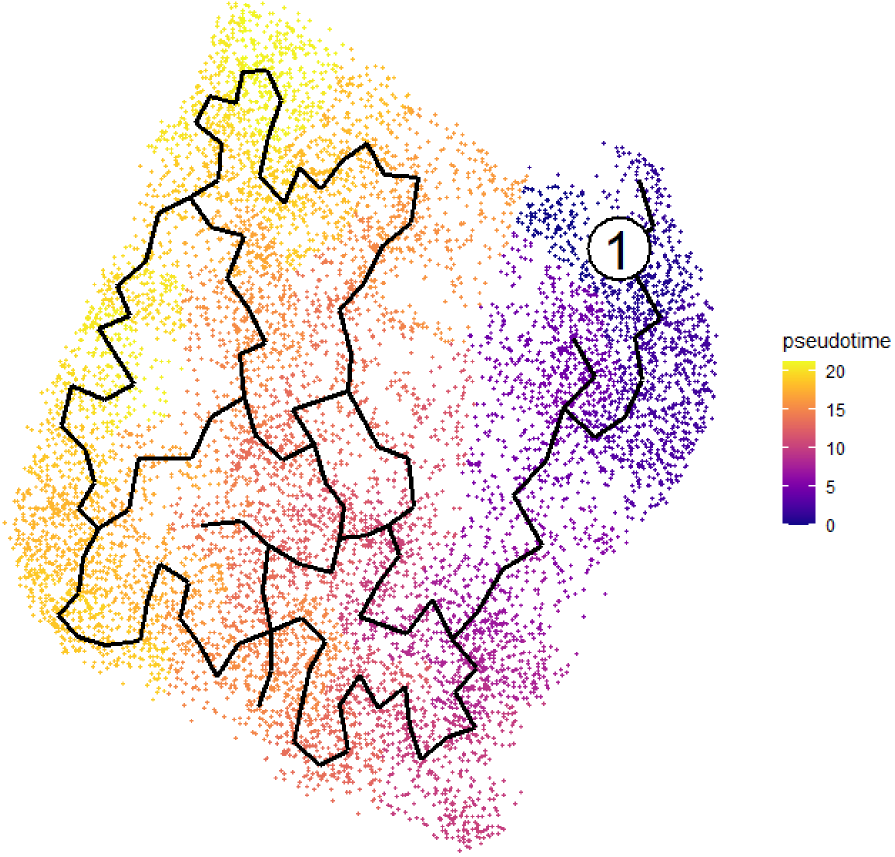
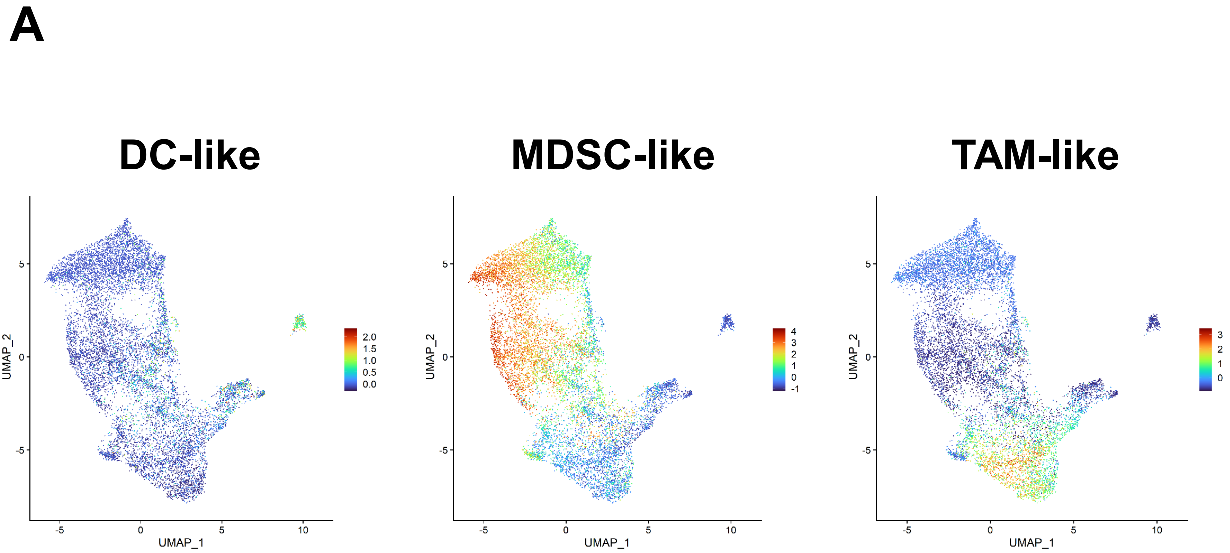


Fig. S4


A
DC-like
MDSC-like
TAM-like
UMAP_2
UMAP_1
5
0
-5
-5
0
5
10
2.0
1.5
1.0
0.5
0.0
4
3
2
1
0
-1
3
2
1
0

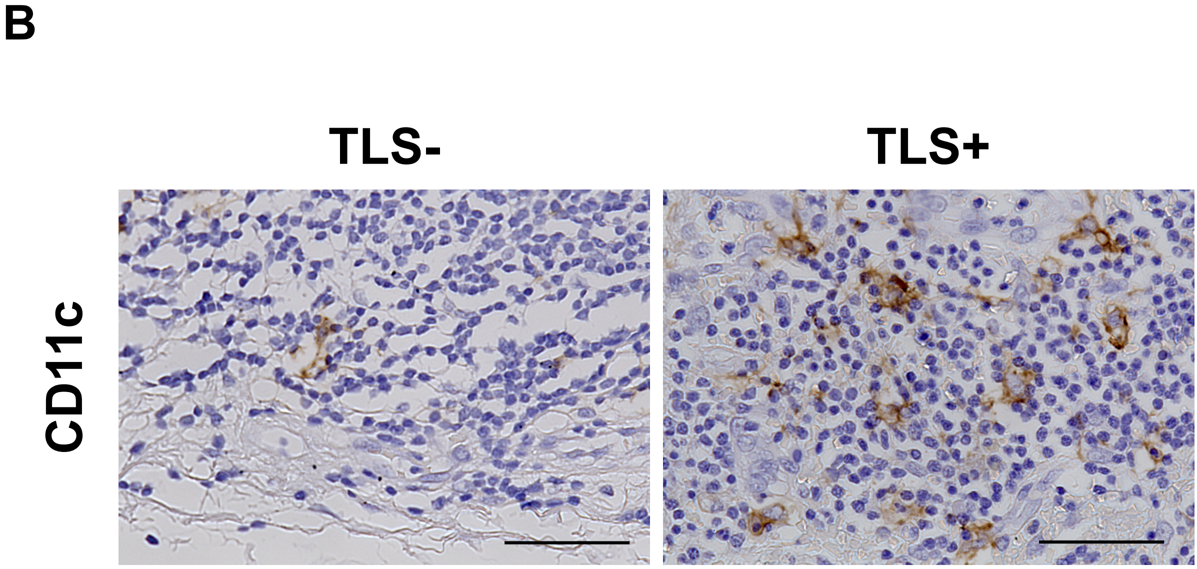

B
TLS-
TLS+
CD11c

Fig. S5

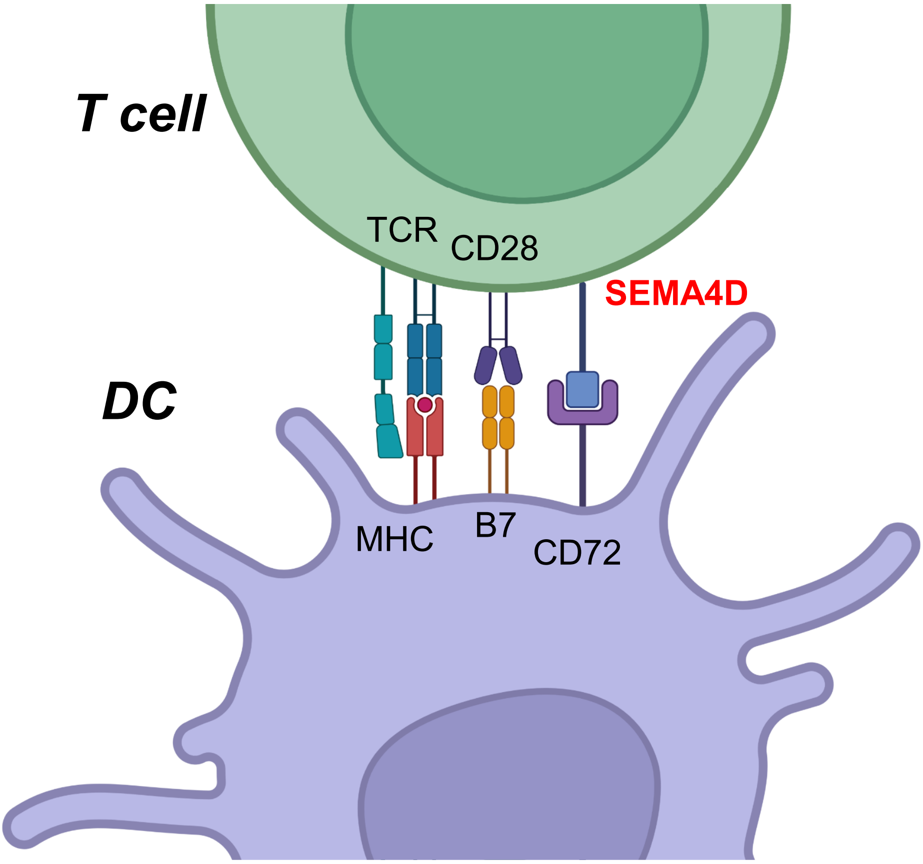
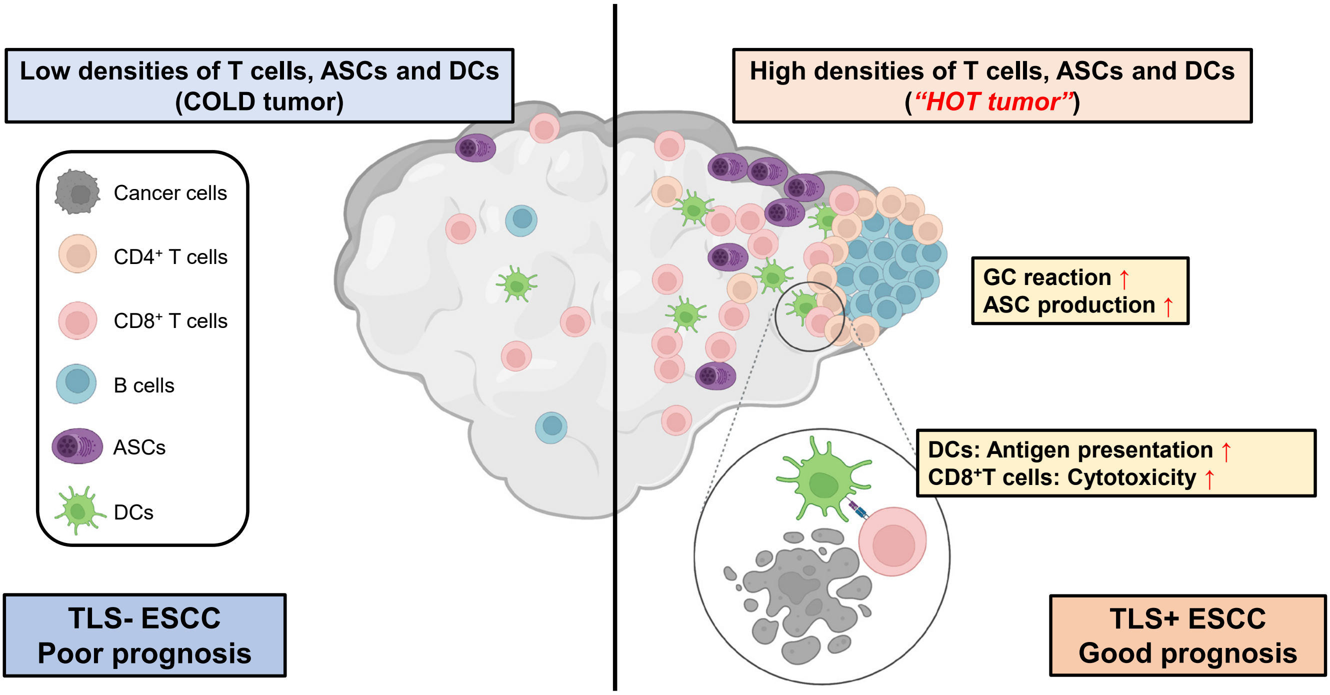


Fig. S6


Low densities of T cells, ASCs and DCs
(COLD tumor)
High densities of T cells, ASCs and DCs
("HOT tumor")
Cancer cells
CD4⁺ T cells
CD8⁺ T cells
B cells
ASCs
DCs
GC reaction ↑
ASC production ↑
DCs: Antigen presentation ↑
CD8⁺T cells: Cytotoxicity ↑
TLS- ESCC
Poor prognosis
TLS+ ESCC
Good prognosis

Supplementary Table S1. Clinicopathological characteristics in patients of esophageal squamous cell carcinoma with and without TLS presence.

		TLS+	TLS-	***p*** [a]
		No. (%)		
Characteristics	180	144	36	
Sex				0.795
	Male	123 (85.4)	30 (83.3)	
	Female	21 (14.6)	6 (16.7)	
Age at surgery				0.877
	<63[b]	54 (37.5)	13 (36.1)	
	≥63	90 (62.5)	23 (36.9)	
pT stage[c]				**0.0196**
	T1/T2	105 (72.9)	19 (52.8)	
	T3/T4	39 (27.1)	17 (47.2)	
Lymph node metastasis (Negative vs Positive)				**0.0294**
	Negative	69 (47.9)	10 (27.8)	
	Positive	75 (52.1)	26 (72.2)	
pStage (UICC)[d]				**0.0029**
	I/II	80 (55.6)	10 (27.8)	
	III/IV	64 (44.4)	26 (72.2)	

[a] Chii-square test or Fisher’s exact test.

[b] Mean age.

[c, d] According to the Union for International Cancer Control (UICC) staging system.

Supplementary Table S2. Primary antibody with CODEX.

Primary antibody	Catalog	Clone	Flourophore
CD3e	4450030	EP449E	Cy5
CD8	4250012	C8/144B	Atto550
CD20	4450018	L26	Alexa Fluor™ 750
Pan-Cytokeratin	4450020	AE-1/AE-3	Alexa Fluor™ 750
Ki67	4250019	B56	Atto550
CD107a	4350001	H4A3	Cy5

Supplementary Table S3. Univariate and multivariate analyses of RFS and OS between TLS presence and clinicopathological characteristics in ESCC patients.

	Univariate		Multivariate	
	HR (95% CI)	P value[a]	HR (95% CI)	P value[a]
RFS				
Sex (male vs female)	1.1 (0.51-2.5)	0.77	1.0 (0.46-2.3)	0.94
Age (<63[b] vs ≥63)	1.2 (0.72-2.1)	0.43	1.1 (0.60-1.9)	0.84
T stage[c] (T1, 2 vs T3, 4)	**0.44 (0.26-0.76)**	**0.003**	0.71 (0.39-1.3)	0.27
Lymph node metastasis (negative vs positive)	**0.32 (0.17-0.60)**	**0.0005**	**0.40 (0.20-0.80)**	**0.0092**
TLS (positive vs negative)	**0.35 (0.20-0.61)**	**0.0002**	**0.42 (0.23-0.75)**	**0.0032**
OS				
Sex (male vs female)	1.3 (0.46-3.7)	0.62	1.4 (0.48-4.0)	0.54
Age (<63[b] vs ≥63)	1.0 (0.52-2.0)	0.97	0.93 (0.47-1.9)	0.85
T stage[c] (T1, 2 vs T3, 4)	0.52 (0.27-1.0)	0.051	0.79 (0.38-1.6)	0.52
Lymph node metastasis (negative vs positive)	**0.39 (0.18-0.82)**	**0.013**	0.45 (0.20-1.0)	0.054
TLS (positive vs negative)	**0.35 (0.17-0.69)**	**0.0027**	**0.38 (0.19-0.78)**	**0.0079**

[a] Cox regression analysis.

[b] Mean age.

[c] According to the Union for International Cancer Control (UICC) staging system.

RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; Bold; Significant *p* values.

Supplementary Table S4. Summary of patient characteristics used in scRNA-seq in this study.

Sample ID	Age	Sex	TNM (UICC)[a]	Stage (UICC)[a]	TLS	Histology
ESC06	41	Female	pT1aN0M0	IA	negative	SCC
ESC07	52	Male	pT2N1M0	IIIA	negative	SCC
ESC09	80	Female	ypT1bN0M0	IB	positive	SCC
ESC11	70	Female	ypT2N0M0	IIA	positive	SCC
ESC12	61	Male	ypT3N2M0	IIIB	negative	SCC
ESC15	71	Male	ypT3N3M0	IVA	negative	SCC
ESC16	65	Male	ypT2N0M0	IIA	negative	SCC
ESC17	62	Male	pT1aN2M0	IIIA	positive	SCC
ESC18	74	Female	pT1bN0M0	IB	positive	SCC
ESC22	70	Female	ypT4aN2M0	IVA	positive	SCC
ESN11 (derived from ESC11 patient)	70	Female	--	--		Normal tissue
ESN12 (derived from ESC12 patient)	61	Male	--	--		Normal tissue
ESN13	65	Male	--	--		Normal tissue
ESN15 (derived from ESC15 patient)	71	Male	--	--		Normal tissue

[a] According to the Union for International Cancer Control (UICC) staging system.

Supplementary Table S5. Components of each gene signature in our study.

Gene set	Source	Genes																					
naive signature	Sinjab A, et al. 2021	CCR7	IL7R	LEF1	SELL	TCF1																	
cytokine signature	Chen B, et al. 2021	CSF3	IL1A	IL1B	IL1RN	IL1R1	IL1R2	OSM	PTGS2	TNF	IL6	IL11	IL24	LIF	IL17C	IL23A	TNFSF15	GSDMB	GSDMD	RELA	RELB	SOCS3	CEBPB
GCB signature	Wieland A, et al. 2021	TCL1A	CXCR5	AICDA	MME																		
ASC signature	Wieland A, et al. 2021	CD38	MZB1	XBP1	PRDM1																		
DC activation	Zhang Q, et al. 2019	FSCN1	BIRC3	LAMP3	CCL19	LAD1	MARCKS	TNFAIP2	CCR7	CCL22	MARCKSL	EBI3	TNFRSF11	NUB1	INSM1	RAB9A	LY75	SIAH2	POGLUT1	KDM2B	MGLL	TXN	MLLT6
		KIF2A	GRSF1	FAM49A	PLEKHG1	SOCS2	RFTN1	AC009812.4	BMP2K	NAV1	IL7R	ID2	CCL17	PPP1R9B	NRP2	TUBB6	ARNTL2	UVRAG	TXNDC11	MREG	BTG1		
DC migration	Maier B, et al. 2020	CCR7	MYO1G	CXCL16	ADAM8	ICAM1	FSCN1	MARCKS															
Antigen presentation	Hornburg M, et al. 2021	HLA-A	HLA-B	HLA-C	HLA-DQA1	TAP1	PSMB9																