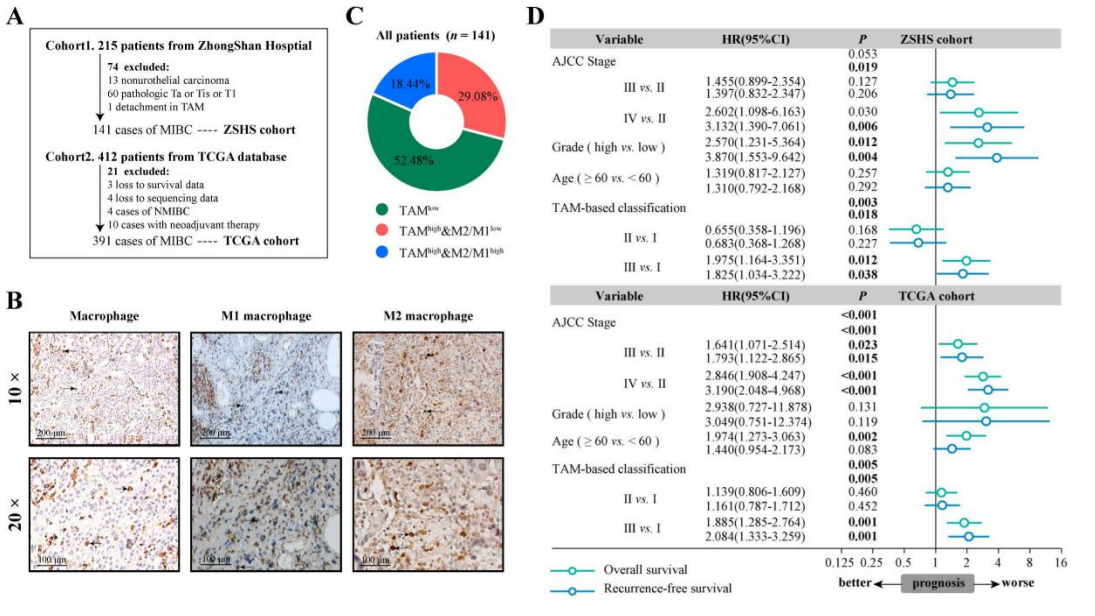
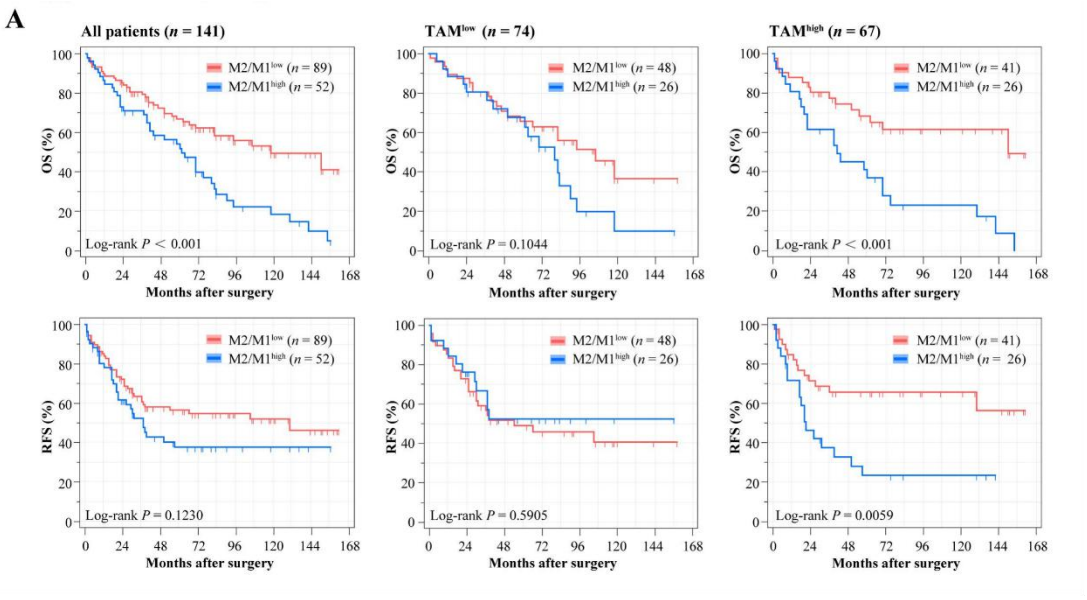
Infiltration and Polarization of Tumor-associated Macrophages Predict Prognosis and Therapeutic Benefit in Muscle-Invasive Bladder Cancer

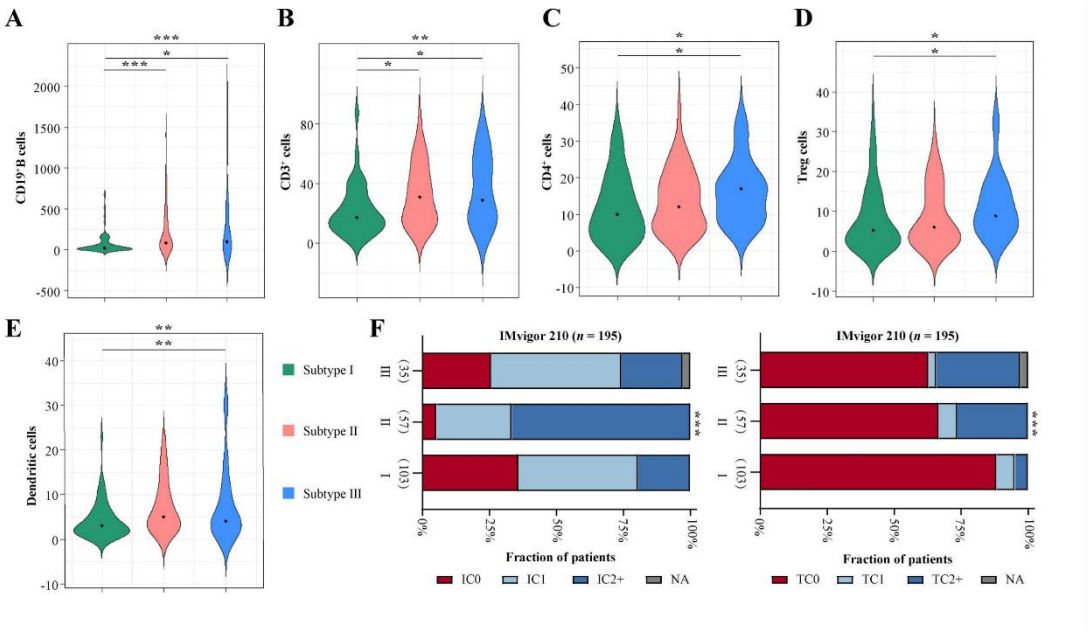
Supplementary Materials



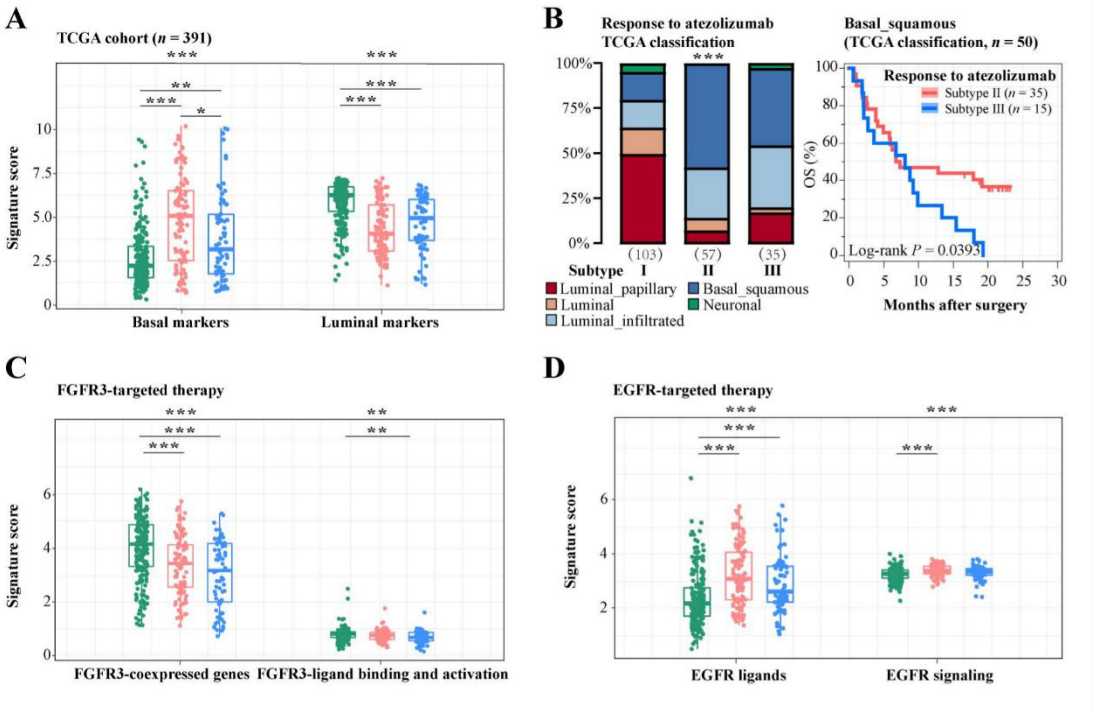
Supplementary Figure 1. Patients enrollment and representative immunohistochemistry images of macrophages. (A) Comprehensive information of patients enrolled in this study. (B) Representative immunohistochemistry images of TAMs (left), M1 macrophage (middle) and M2 macrophage (right). Positive cells were indicated by arrowheads. (C) Immune stratification based on TAMs infiltration and M2/M1 ratio in ZSHS cohort. (D) Univariate Cox results of clinicopathological characteristics and immune stratification based on TAMs infiltration and M2/M1 ratio in ZSHS and TCGA cohort.



Supplementary Figure 2. Kaplan-Meier analyses for M2/M1 ratio according to TAMs infiltration. (A) Kaplan Meier curves analyses of OS and RFS for M2/M1 ratio according to TAM infiltration in ZSHS cohort. Data were analyzed by log-rank test.



Supplementary Figure 3. Association of immune cells with stratification based on TAMs infiltration and M2/M1 ratio. (A-E) The association between immune stratification and immune cells infiltration assessed by IHC staining in ZSHS cohort (n = 141). (F) Quantification analyses of TAMs infiltration and M2/M1 ratio across PD-L1 expression and distribution in IMvigor210 cohort. Data were analyzed by Kruskal-Wallis test. *P < 0.05, **P < 0.01 and ***P < 0.001. IC, immune cells; TC, tumor cells.



Supplementary Figure 4. Correlations of molecular features with TAMs infiltration and M2/M1 ratio in TCGA and IMvigor210 trial. (A) Association of luminal and basal signature score with immune stratification based on TAMs infiltration and M2/M1 ratio in TCGA cohort. (B) Quantification analyses of TAMs infiltration and M2/M1 ratio across molecular classification systems in IMvigor210 cohort (left). Kaplan Meier curves for OS stratified according to immune stratification in basal/squamous patients in IMvigor210 cohort (right). (C-D) Association of FGFR3-targeted therapies (C), and EGFR-targeted therapies (D) signature score with immune stratification based on TAMs infiltration and M2/M1 ratio. Data were analyzed by log-rank test. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Supplementary Table 1. Patient characteristics in ZSHS and TCGA cohort

Factors	ZSHS cohort (*n* = 141)						TCGA cohort (*n* = 391)					
	Patients	%	Subtype I	Subtype II	Subtype III	*P**	Patients	%	Subtype I	Subtype II	Subtype III	*P**
	141	100	74	41	26		391	100	206	114	71	
Age (year)						0.464						**<0.001**
<60	52	36.9	30	15	7		84	21.5	60	17	7	
≥60	89	63.1	44	26	19		307	78.5	146	97	64	
Gender						0.620						0.064
Male	117	83.0	60	36	21		285	72.9	160	75	50	
Female	24	17.0	14	5	5		106	27.1	46	39	21	
Grade						0.052						**0.003**
Low grade	24	17.0	18	4	2		20	5.1	19	1	0	
High grade	117	83.0	56	37	24		369	94.4	186	112	71	
pT stage						0.606						**0.001**
pT2	90	63.8	51	25	14		113	28.9	73	26	14	
pT3	30	21.3	14	10	6		189	48.3	79	71	39	
pT4	21	14.9	9	6	6		56	14.3	30	13	13	
pN stage						0.212						0.341
pN0	133	94.3	72	38	23		228	58.3	122	69	37	
pN+	8	5.7	2	3	3		123	31.5	59	38	26	
LVI						**0.012**						0.211
Absent	52	36.9	35	8	9		125	32.0	71	35	19	
Present	89	63.1	39	33	17		142	36.3	73	36	33	
AJCC stage						0.346						**0.009**
II	87	61.7	50	24	13		125	32.0	82	28	15	
III	46	32.6	22	14	10		137	35.0	60	47	30	
IV	8	5.7	2	3	3		129	33.0	64	39	26	
ACT						0.260						\
Applied	69	48.9	32	21	16		\	\	\	\	\	
Not applied	72	51.1	42	20	10		\	\	\	\	\	

Abbreviations: LVI: lymphatic vessel invasion; AJCC: American Joint Committee on Cancer; ACT: adjuvant chemotherapy (cis-platinum based therapy).

**P* value was used from Chi-square test; significant P value < 0.05 was shown in bold.

Supplementary Table 2. Description of the gene signature

Signature*	Genes	Source
Luminal markers	CYP2J2, ERBB2, ERBB3, FGFR3, FOXA1, GATA3, GPX2, KRT18, KRT19, KRT20, KRT7, KRT8, PPARG, XBP1, UPK1A, UPK2	PMID: 30096301
Basal markers	CD44, CDH3, KRT1, KRT14, KRT16, KRT5, KRT6A, KRT6B, KRT6C	PMID: 30096301
FGFR3-coexpressed genes	FGFR3, TP63, IRS1, SEMA4B, PTPN13, TMPRSS4	PMID: 22553347
FGFR3-ligand binding and activation	FGF1, FGF16, FGF17, FGF18, FGF2, FGF20, FGF23, FGF4, FGF5, FGF8, FGF9, FGFR3, GALNT3	Reactome hsa190239
EGFR ligands	EGFR, AREG, AREGB, EREG, HBEGF, TGFA	PMID: 25009231
EGFR signaling	AREG, BTC, CBL, EGF, EGFR, EPGN, EREG, GAB1, GRB2, HBEGF, HRAS, HSP90AA1, KRAS, NRAS, PIK3CA, PIK3R1, PLCG1, RPS27A, SHC1, SOS1, TGFA, UBA52, UBB, UBC	Reactome hsa1643713
Immune checkpoint	CD274, CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2, TIGIT	PMID: 31563503
Antigen presenting machinery	B2M, HLA-A, HLA-B, HLA-C, TAP1, TAP2	PMID: 27855702

*Signature score was defined as the mean of normalized expression of related genes; normalized gene expression was calculated as $\log_2$(FPKM+1).

Supplementary Table 3. Immunohistochemistry (IHC) antibodies

Cells	Antibodies information	Diluted	Evaluation
Macrophages	Anti-CD68 antibody (Monoclone; Rb; Abcam; ab955)	1:400	Cell count (200x)
M1 macrophages	Anti-CD68 antibody (Monoclone; Ms; DAKO; IR609)	Ready-to-use	Cell count (200x)
	Anti-HLA-DR antibody (Monoclone; Rb; Abcam; ab2511)	1:250	
M2 macrophages	Anti-MRC1 antibody (Monoclone; Rb; Sigma; HPA004114)	1:500	Cell count (200x)
NK cells	Anti-CD56 antibody (Monoclone; Ms; DAKO; M7304)	Ready-to-use	Cell percentage (per specimen)
B cells	Anti-CD19 antibody (Monoclone; Ms; Abcam; ab31947)	1:400	Cell count (200x)
T cells	Anti-CD3 antibody (Monoclone; Rb; Abcam; ab16669)	1:200	Cell count (200x)
$CD8^+$ T cells	Anti-CD8 alpha antibody (Monoclone; Ms; Abcam; ab17147)	1:100	Cell count (200x)
$CD4^+$ T cells	Anti-CD4 antibody (Monoclone; Ms; Abcam; ab67001)	1:50	Cell count (200x)
Th1 cells	Anti-CD4 antibody (Monoclone; Ms; Abcam; ab67001)	1:50	Cell count (per specimen)
	Anti-T-bet antibody (Monoclone; Rb; Abcam; ab150440)	1:500	
Th2 cells	Anti-CD4 antibody (Monoclone; Ms; Abcam; ab67001)	1:50	Cell count (per specimen)
	Anti-GATA3 antibody (Monoclone; Rb; Abcam; ab186371)	1:100	
Treg cells	Anti-FOXP3 antibody (Monoclone; Ms; Abcam; ab22510)	1:100	Cell count (200x)
Mast cells	Anti-Mast Cell Tryptase antibody (Monoclone; Ms; Abcam; ab2378)	1:10000	Cell count (per specimen)
Neutrophils	Anti-CD66b antibody (Polyclone; Rb; Abcam; ab197678)	1:1000	Cell count (per specimen)
Dendritic cells	Anti-HLA-DR antibody (Monoclone; Ms; Abcam; ab20181)	1:200	Cell count (200x)
	Anti-CD11c antibody (Monoclone; Rb; Abcam; ab52632)	1:200	
PD-1	Anti-PD1 antibody (Monoclone; Ms; Abcam; ab52587)	1:100	Cell count (200x)
PD-L1	Anti-PD-L1 antibody (Monoclone; Rb; Abcam; ab228415)	1:500	Cell count (200x)
CTLA-4	Anti-CTLA-4 antibody (Monoclone; Ms; Santa-Cruz; sc-376016)	1:100	Cell count (200x)
TIM-3	Anti-TIM 3 antibody (Polyclone; Rb; Abcam; ab185703)	1:100	Cell count (200x)
LAG-3	Anti-LAG-3 antibody (Monoclone; Rb; Abcam; ab209236)	1:500	Cell count (200x)
TIGIT	Anti-TIGIT antibody (Monoclone; Rb; Abcam; ab243903)	1:100	Cell count (200x)
GZMB	Anti-Granzyme B antibody (Monoclone; Rb; Abcam; ab4059)	1:200	Cell count (200x)
PRF-1	Anti-Perforin antibody (Monoclone; Ms; Abcam; ab75573)	Ready-to-use	Cell count (200x)
IFN-γ	Anti-Interferon gamma antibody (Monoclone; Rb; Abcam; Ab9657)	1:300	Cell count (200x)
IL-10	Anti-IL-10 antibody (Monoclone; Ms; Abcam; ab134742)	1:200	Cell count (200x)
TGF-β	Anti-LAP antibody (Polyclone; Go; R&D; AB-246-NA)	1:100	Cell count (200x)

Supplementary Table 4. Association of TAM-based classification and PD-L1 IC in univariate and multivariate analyses

Cox regression analyses	Univariate			Multivariate		
(n = 195)	**HR**	**95%CI**	***P****	**HR**	**95%CI**	***P****
IC level			**0.005**			0.088
IC1 vs. IC0	0.953	0.629-1.445	0.821	0.962	0.627-1.477	0.859
IC2+ vs. IC0	0.508	0.319-0.809	**0.004**	0.610	0.367-1.011	0.055
TAM-based classification			**0.001**			**0.007**
II vs. I	0.587	0.378-0.912	**0.018**	0.713	0.441-1.154	0.168
III vs. I	1.555	1.020-2.370	**0.040**	1.649	1.074-2.532	**0.022**

Abbreviations: IC: immune cells.

*Significant *P* value < 0.05 was shown in bold.

Supplementary Table 5. Association of TAM-based classification and TMB in univariate and multivariate analyses

Cox regression analyses	Univariate			Multivariate		
(n = 195)	**HR**	**95%CI**	***P****	**HR**	**95%CI**	***P****
TMB (high vs. low)	0.512	0.337-0.777	**0.002**	0.571	0.371-0.878	**0.011**
TAM-based classification			**0.001**			0.064
II vs. I	0.587	0.378-0.912	**0.018**	0.713	0.440-1.154	0.168
III vs. I	1.555	1.020-2.370	**0.040**	1.486	0.880-2.511	0.139

Abbreviations: TMB: tumor mutation burden.

*Significant *P* value < 0.05 was shown in bold.