Additional file

Tumor-associated macrophages and Tregs influence and represent immune cell infiltration of muscle-invasive bladder cancer and predict prognosis

Table S1

Variable		**All patients n=101**	**Cluster 1 Treg high (n=10)**	**Cluster 2 Macrophage high (n=16)**	**Cluster 3 Immune low (n=75)**	***p***
Median Age (IQR)		68 (59-75)	69.5 (52-80)	71 (58-72)	68 (59-75)	0.9
Gender	Male	78 (77%)	7 (70%)	12 (75%)	59 (79%)	0.8
	Female	23 (23%)	3 (30%)	4 (25%)	16 (21%)	
Max. tumor-stage	pT2	24 (24%)	3 (30%)	3 (19%)	18 (24%)	0.9
	pT3	57 (56%)	6 (60%)	9 (56%)	42 (56%)	
	pT4	20 (20%)	1 (10%)	4 (25%)	15 (20%)	
pN Stage	pN0	49 (49%)	5 (50%)	7 (44%)	37 (49%)	0.9
	pN1/pNx	52 (51%)	5 (50%)	9 (56%)	38 (51%)	
Histological subtype	NOS	73 (72%)	6 (60%)	13 (81%)	54 (72%)	0.4
	Squamous	11 (11%)	2 (20%)	1 (6%)	8 (11%)	
	Micropapillary	6 (6%)	0	1 (6%)	5 (7%)	
	Neuroendocine	3 (3%)	0	0	3 (4%)	
	Sarcomatoid	2 (2%)	0	0	2 (3%)	
	Plasmacytoid	2 (2%)	0	0	2 (3%)	
	Other (2 Lymphoepithelial, 1 Glandular, 1 Giant cell)	4 (4%)	2 (20%)	1 (6%)	1 (1%)	
Adjuvant chemotherapy	No	67 (66%)	4 (40%)	11 (69%)	52 (69%)	0.2
	Yes	34 (34%)	6 (60%)	5 (31%)	23 (31%)	

Table S1: *Association of clusters with clinical and pathological patient characteristics. Level of significance (p-value) was calculated using Chi^2-test or ANOVA, respectively. IQR= interquartile range; NOS=not otherwise specified*

Table S2

		CD163 low (n=99)	**CD163 high (n=40)**	***p***
Gender	Female	19 (19%)	10 (25%)	0.5
	Male	80 (81%)	30 (75%)	
Age	Median (IQR)	68 (60-76)	71 (59-78)	0.2
Tumor stage	pT2	32 (32%)	7 (18%)	0.2
	pT3	51 (52%)	23 (58%)	
	pT4	16 (16%)	10 (25%)	
Lymph node stage	pN0	50 (51%)	17 (43%)	0.4
	pN+/pNx	49 (49%)	23 (57%)	
Adjuvant Chemotherapy	no	70 (71%)	30 (75%)	0.6
	yes	29 (29%)	10 (25%)	

Table S2: *Clinical and pathological patient characteristics did not differ significantly between low and high CD163+ macrophage infiltration.*

Table S3

		Hazard ratio univariate	p	Hazard ratio multivariate	p
CD3 T-cells (Stroma)	continuous	0.3 (0.06-1.3)	0.1		
CD4 T-cells (Stroma)	continuous	0.2 (0.0-1.9)	0.3		
CD8 T-cells (Stroma)	continuous	0.7 (0.1-3.2)	0.7		
Tregs (Stroma)	continuous	0.2 (0.03-0.8)	0.05	0.2 (0.0-1.3)	0.1
Macrophages (Stroma)	continuous	3.6 (1.1-10.6)	0.03	6.3 (1.8-21.0)	0.004
K-Means Clusters for Tregs + Macrophages in Stroma	2 vs. 1	4.6 (1.3-16.1)	0.02	3.1 (0.8-10.9)	0.08
	2 vs. 3	2.0 (1.1-3.6)	0.02	2.2 (1.2-4.1)	0.01
	3 vs. 1	2.3 (0.7-7.4)	0.2	1.4 (0.4-4.7)	0.6

Table S3: *Cox-Regression model for overall survival (OS) with immune cells counted only in the stroma. The stroma the stroma area was assigned using the algorithmic tissue separation of the inForm® Tissue Analysis Software. Variables with significant prediction on OS were added to the multivariate model adjusting for tumor, lymph node status and adjuvant chemotherapy. K-means clusters for macrophages and Tregs was performed using cells per square mm counted in the stroma. Cluster 1: Treg high; Cluster 2: macrophage high; Cluster 3: Treg low and macrophage low*

Figure S1

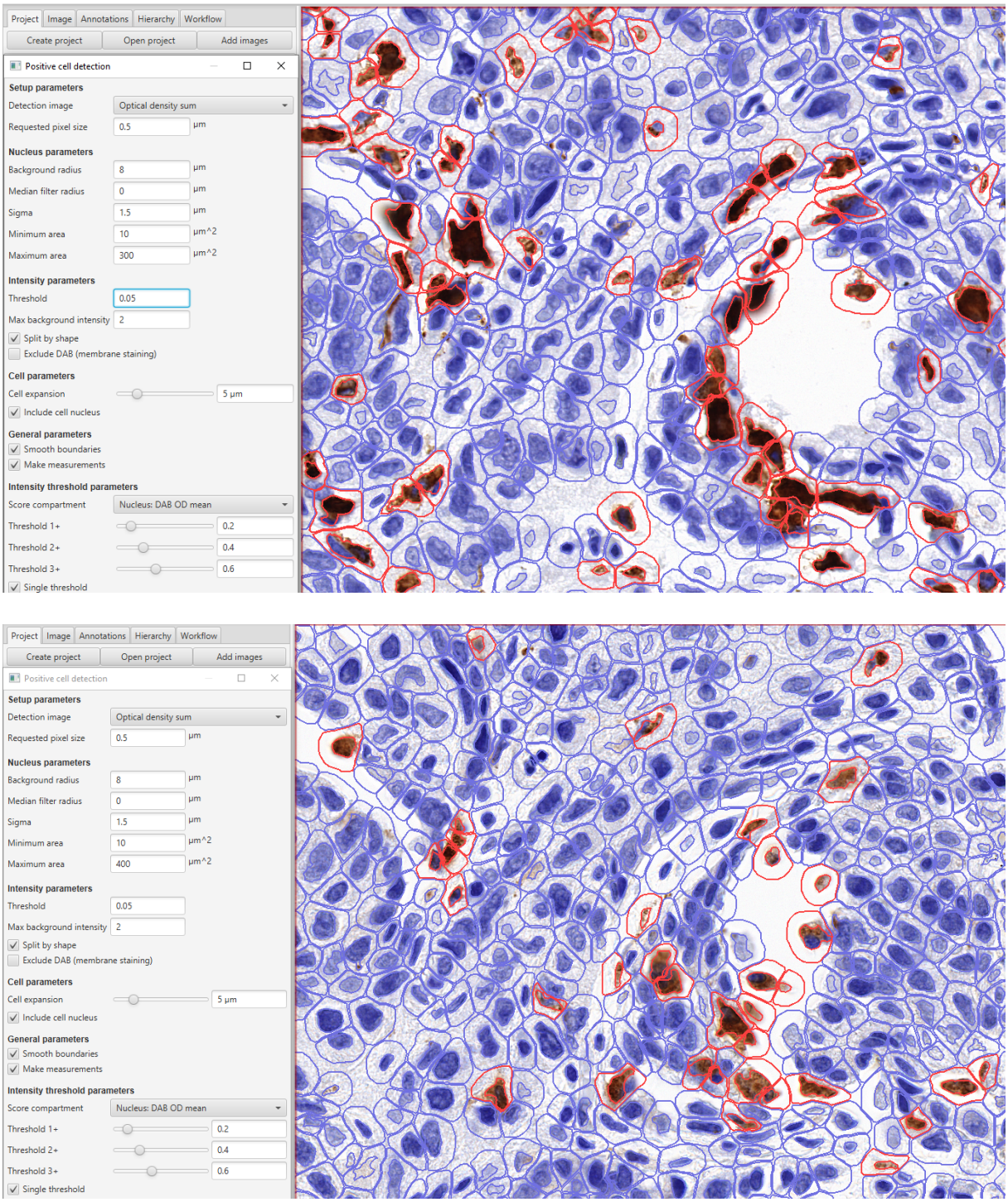

Figure S1: *QuPath Settings for positive cell detection for CD163 (upper image) and CD68 (lower image). Magnification 400x.*

Figure S2

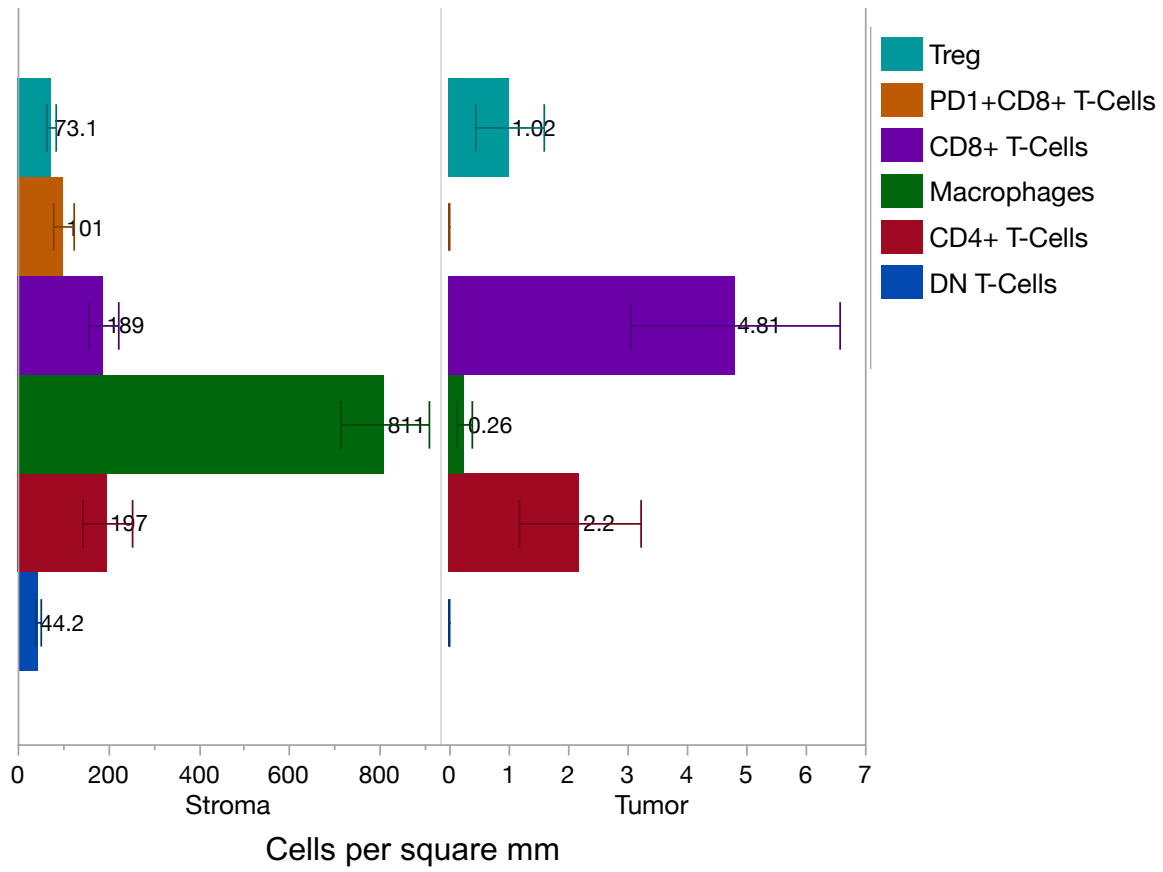

Figure S2: *Number of cells (per mm^2) detected in the stroma and tumor area using the algorithmic tissue separation of the inForm® Tissue Analysis Software.*

Figure S3

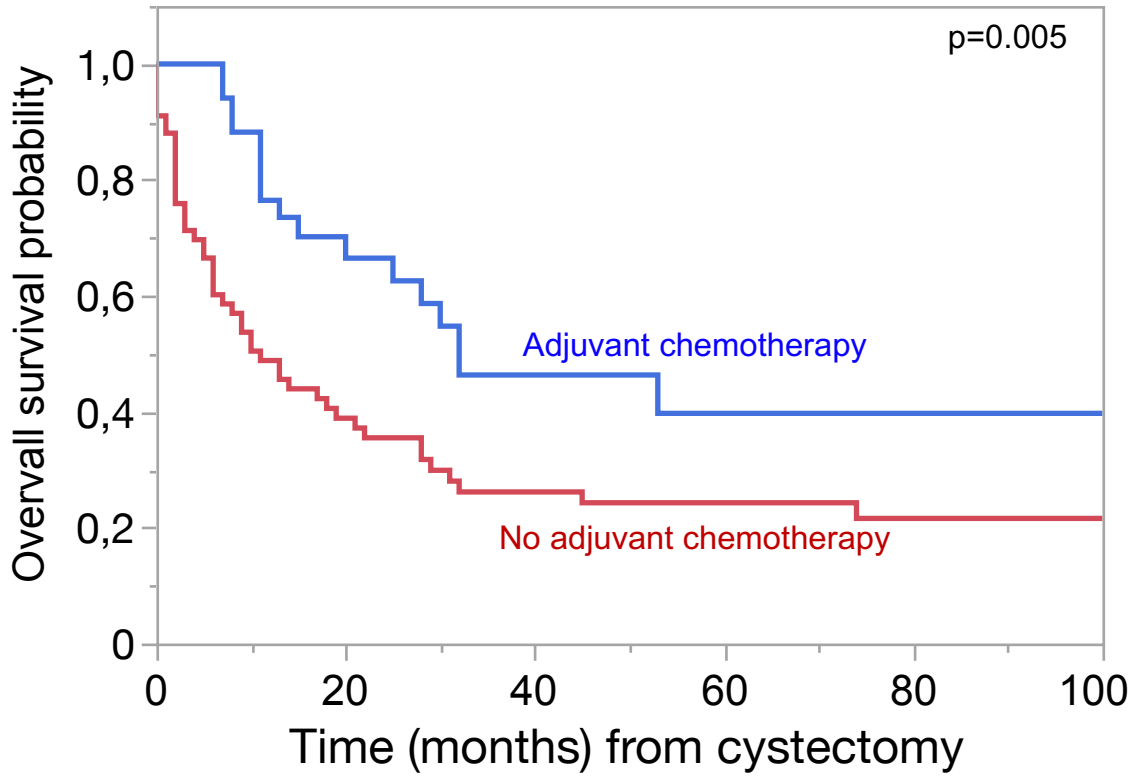

Figure S3: *Kaplan–Meier curves for overall survival probability for patients with (n=34) and without adjuvant chemotherapy (n=67); p(log-rank) = 0.005.*

Figure S4

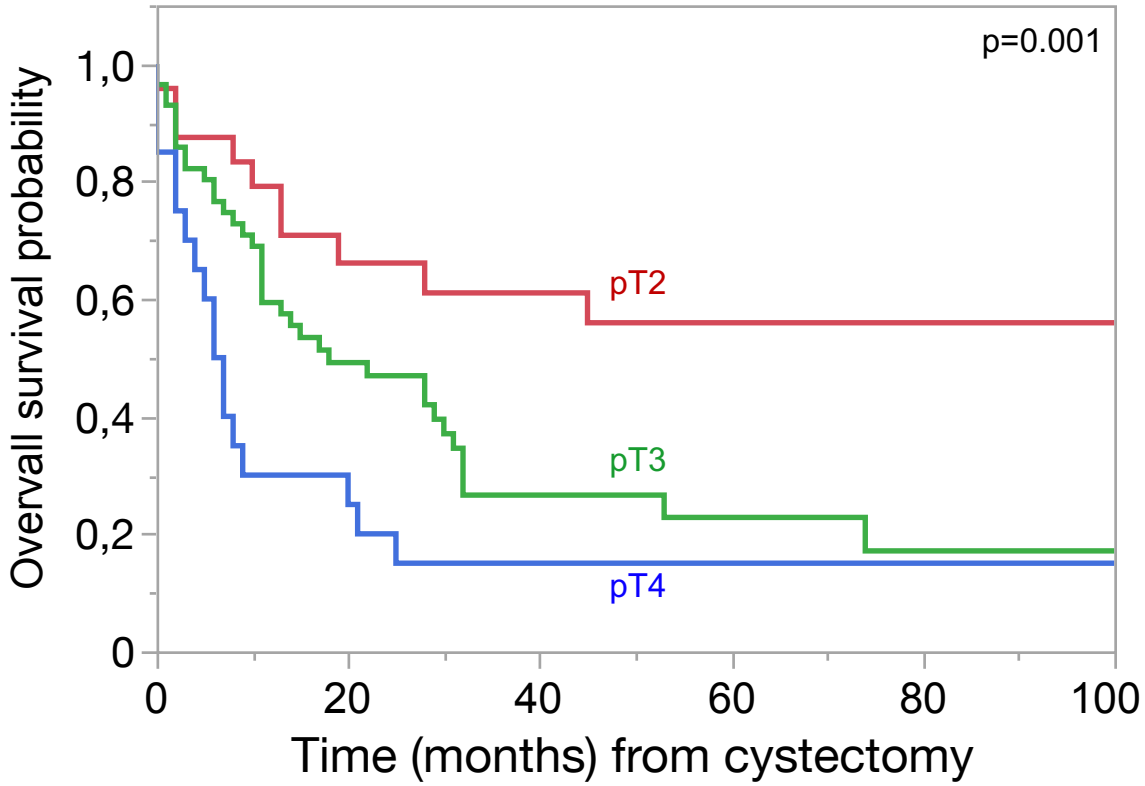

Figure S4: *Kaplan–Meier curves for overall survival probability stratified for patients by pathological tumor stage; p(log-rank) = 0.001.*

Figure S5

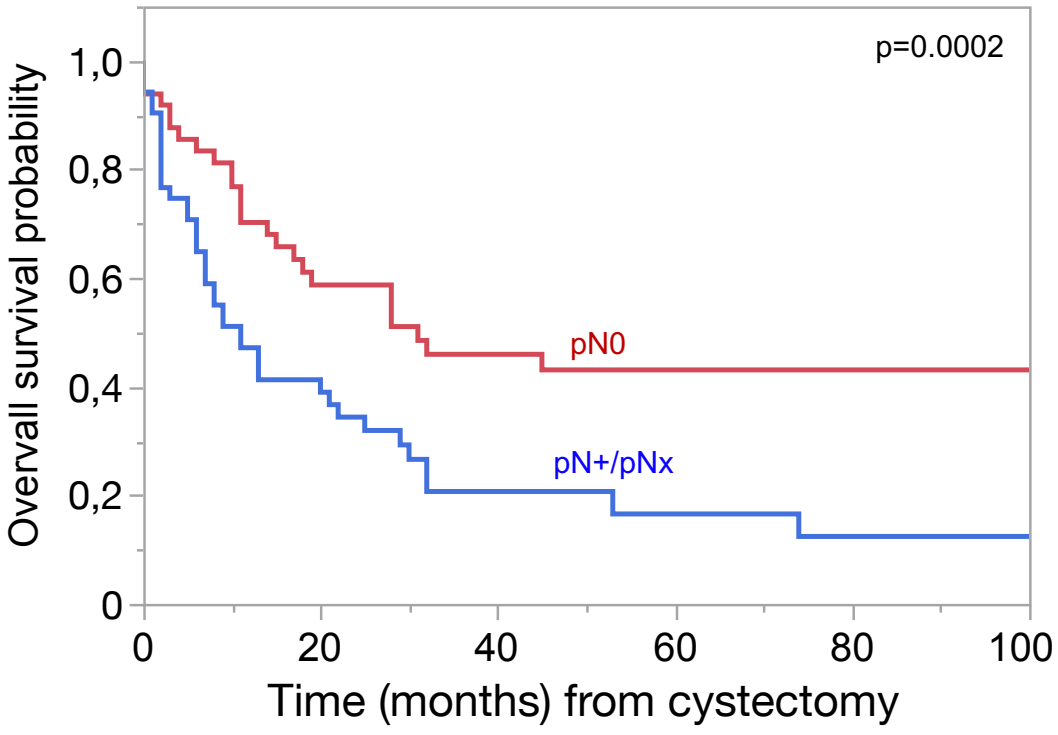

Figure S5: *Kaplan–Meier curves for overall survival probability stratified for patients by pathological lymph node stage; p(log-rank) = 0.0002.*

Figure S6

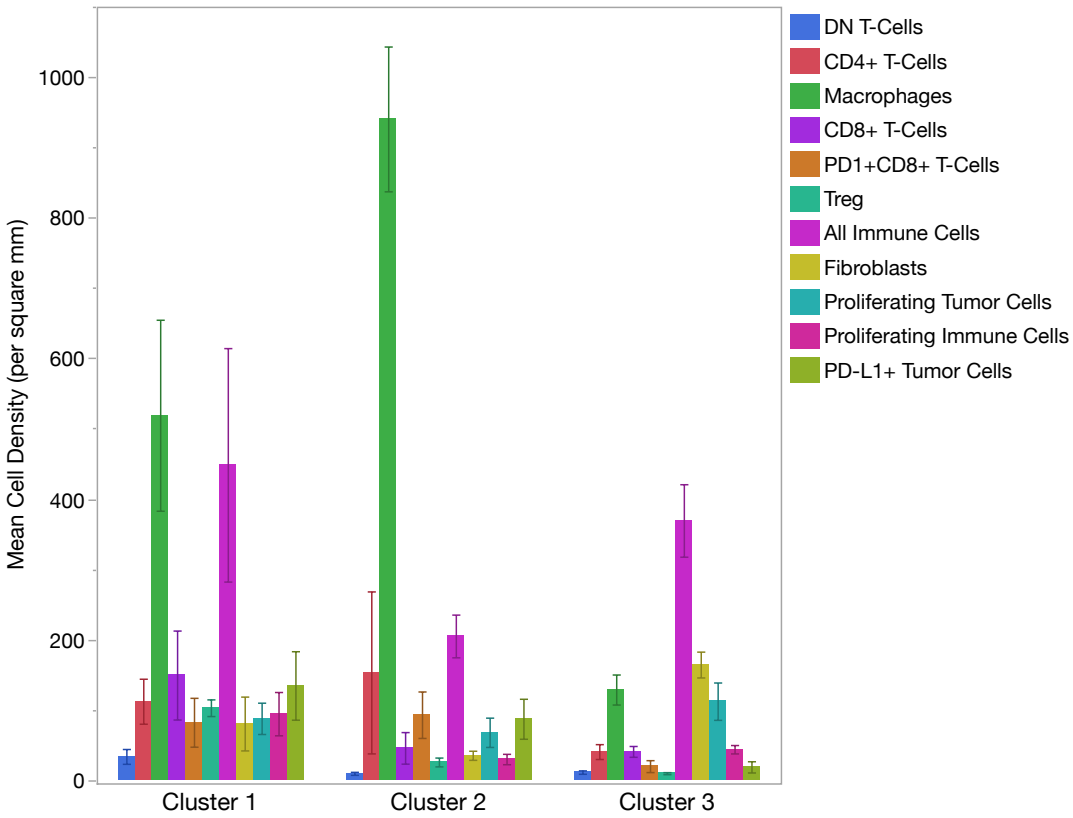

Figure S6: *Cell types (mean cell density/mm2) represented in the three clusters. Error bars show standard errors of means.*

Figure S7

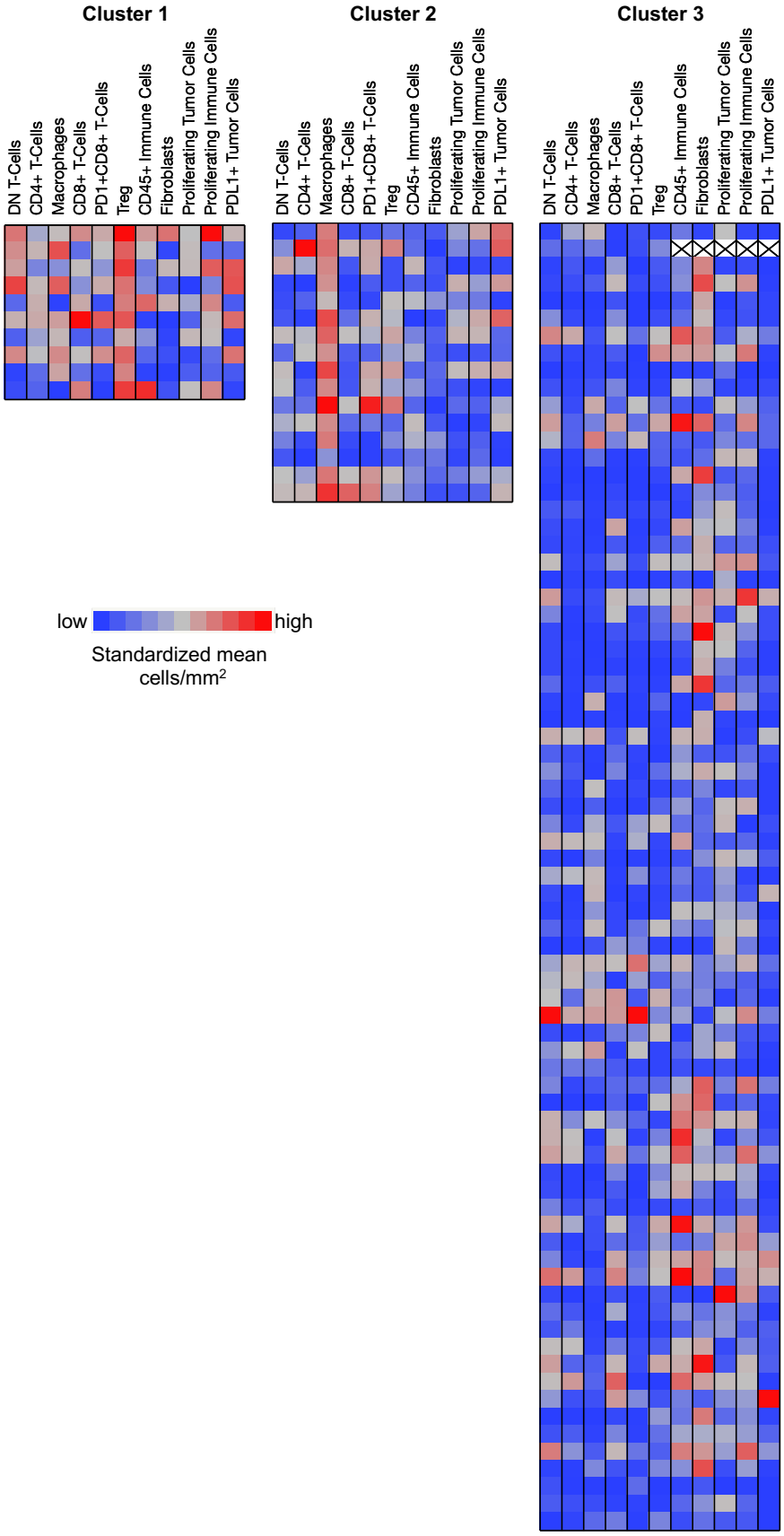

Figure S7: *Heatmap of Z-Scores (z = xi −mean(x)/ st.dev(x)) for each cell type and each sample.*

Figure S8

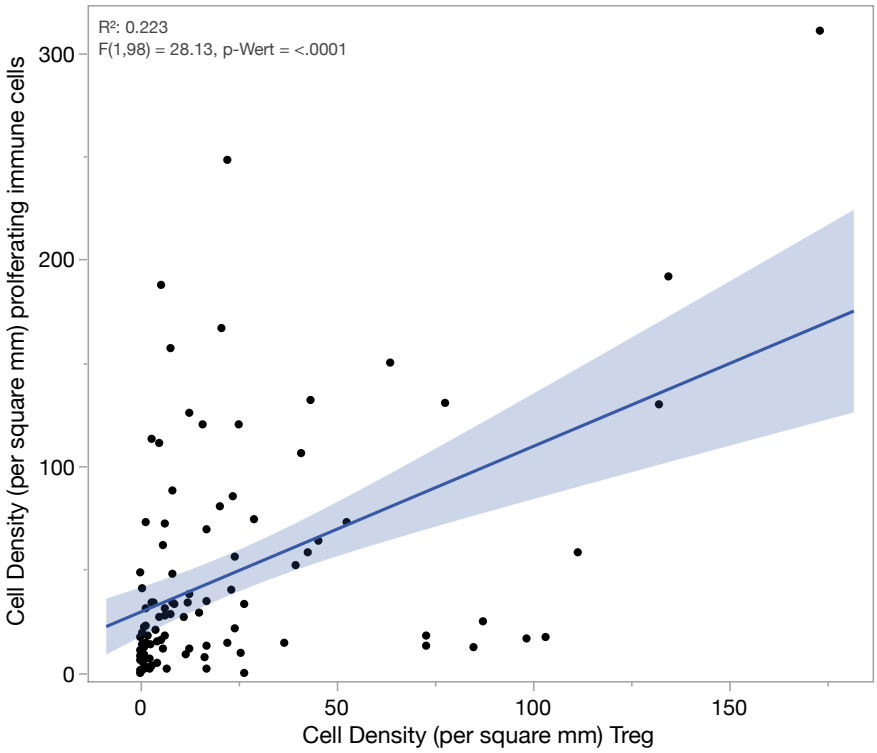

Figure S8: *Correlation plot of the cell density of Treg (FoxP3+) vs. proliferating immune cells (CD45+Ki67+).*

Figure S9

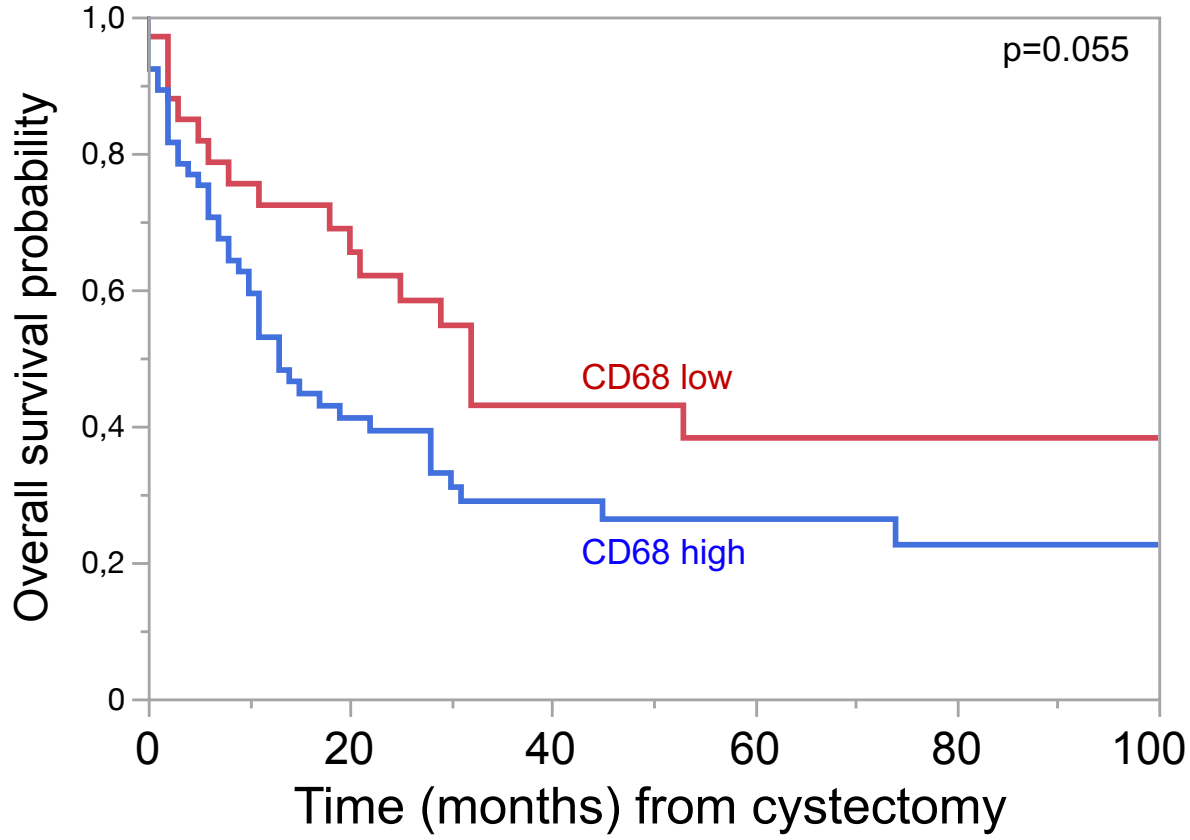

Figure S9: *Kaplan–Meier curves for overall survival probability stratified for patients by the macrophage marker CD68 with a cut-off 5%/all cells; p(log-rank) = 0.055.*

Figure S10

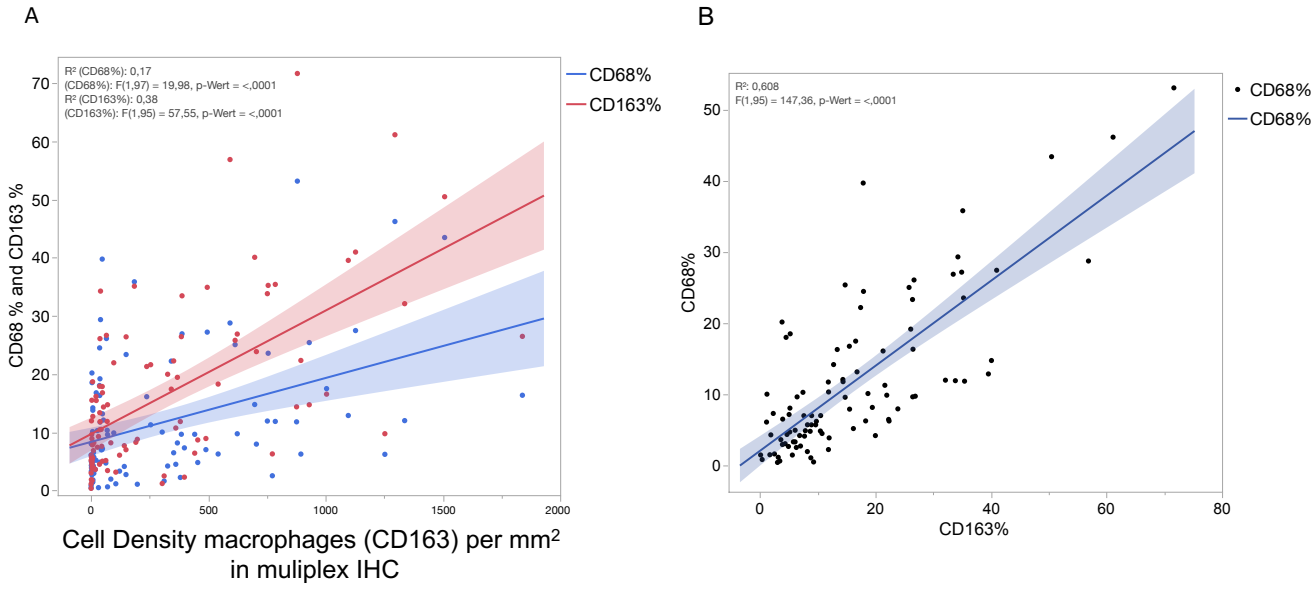

Figure S10: *Correlation plot of routine IHC for CD163 and CD68 vs. CD163 in the multiplex IHC panel (A). And Correlation between CD68 and CD163 expression in routine IHC (B).*