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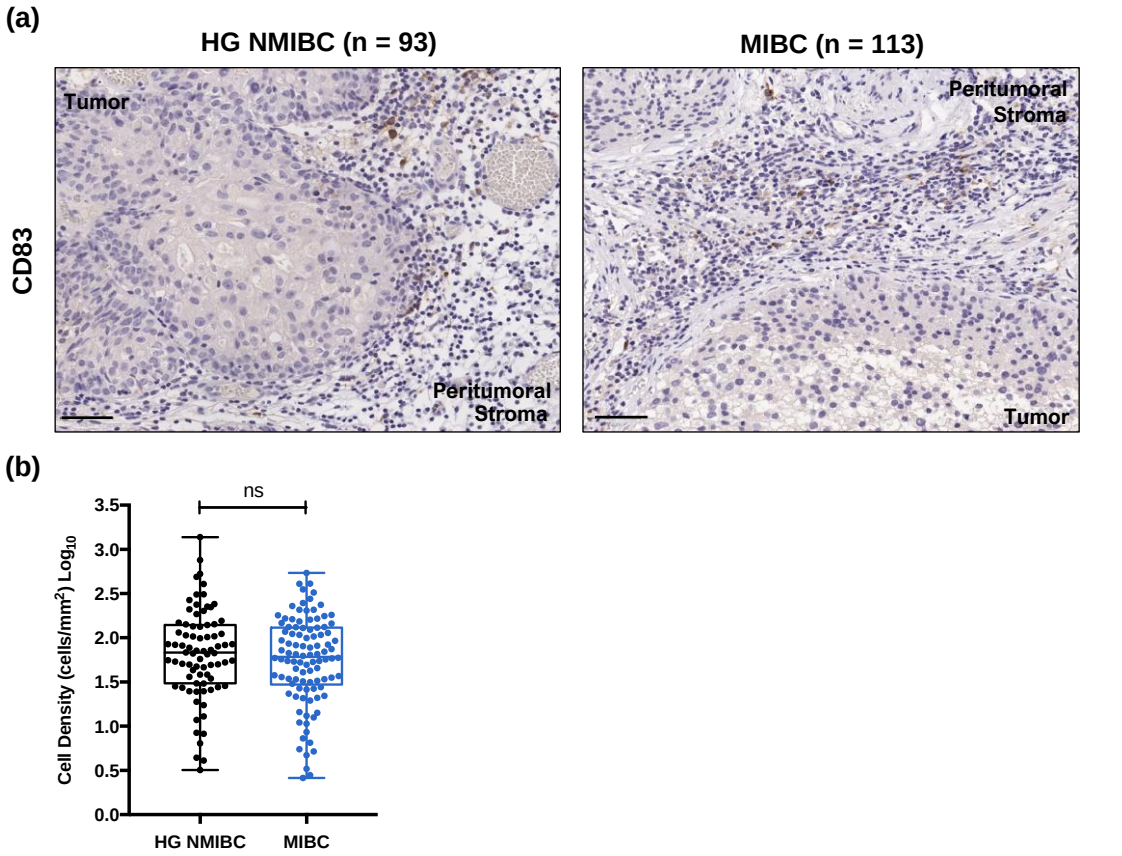
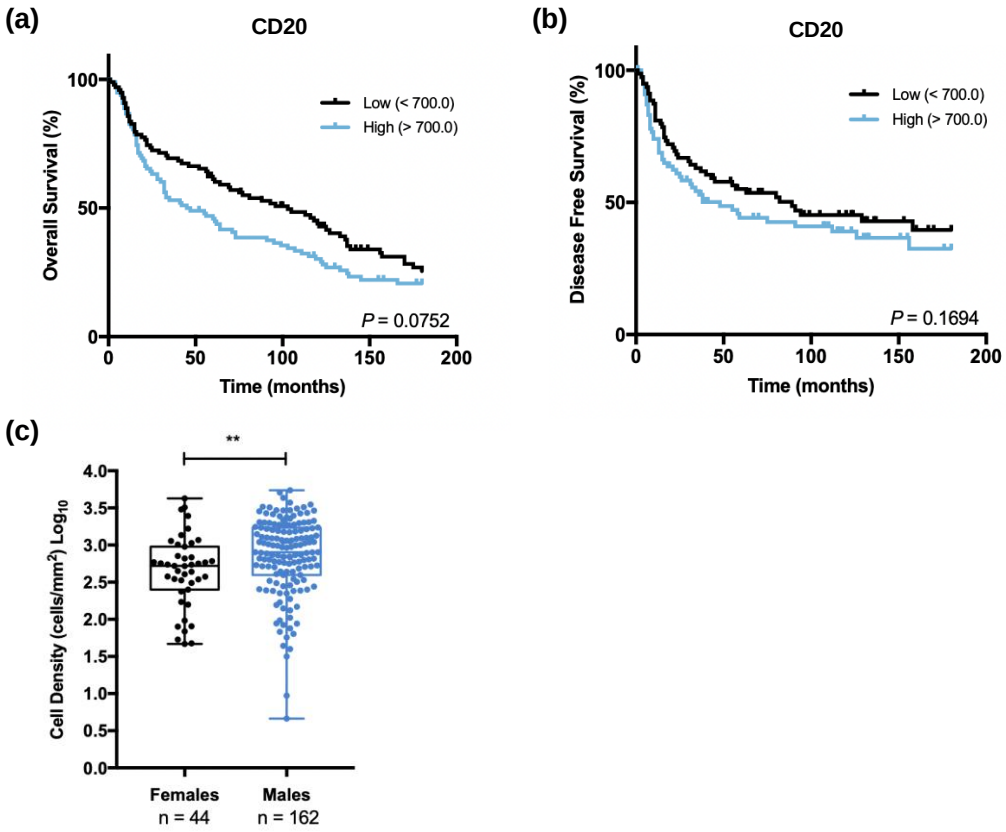
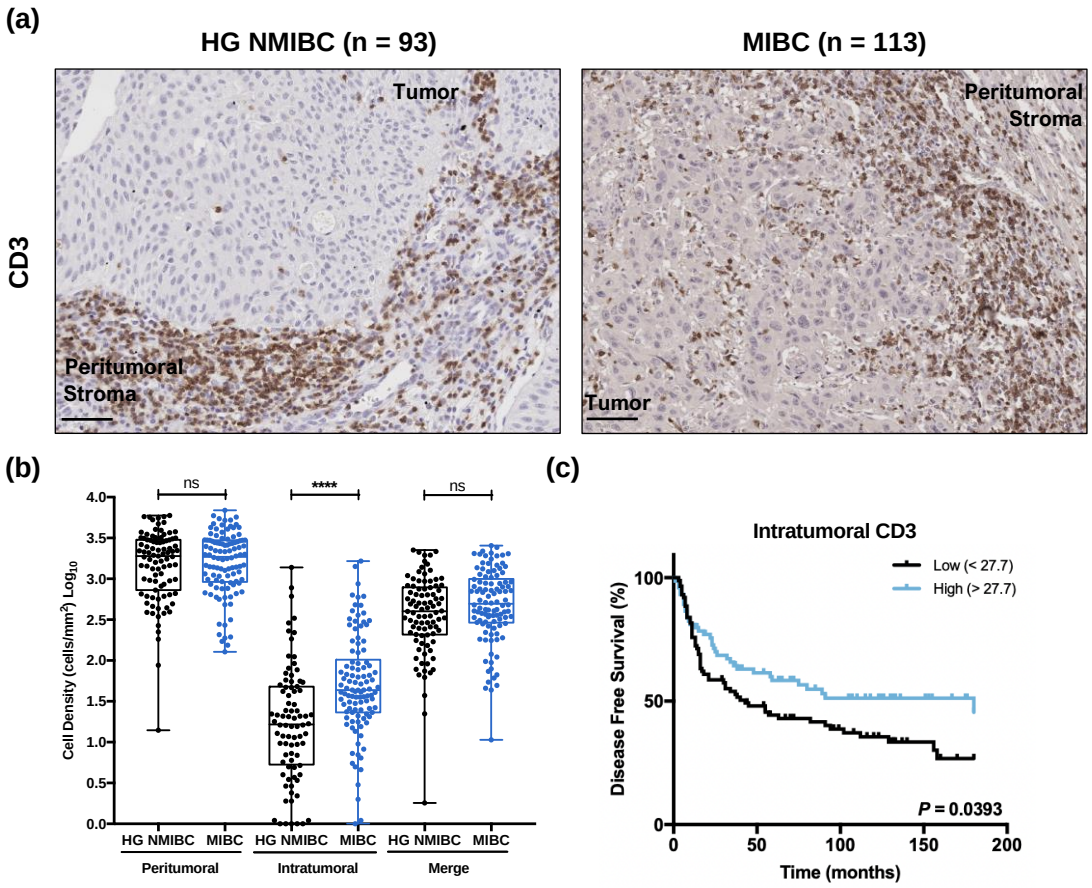
Supplementary figures



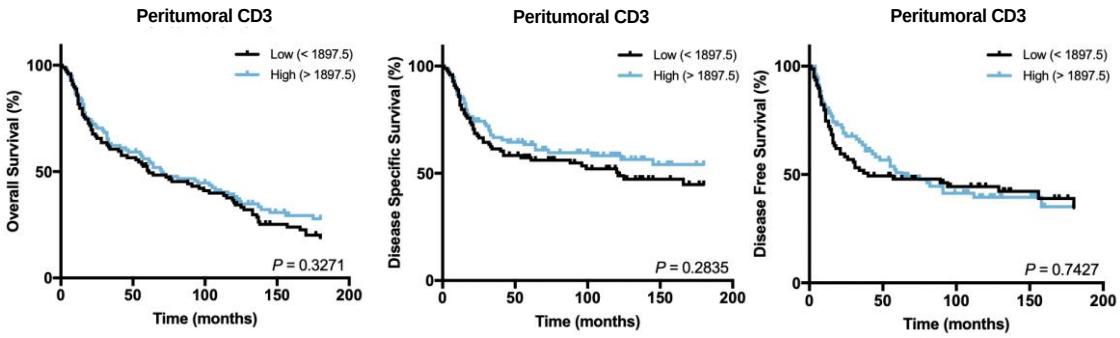
Supplementary figure 1 – CD83 expression is similar in HG NMIBC and MIBC. (a) Representative examples of CD83 staining in HG NMIBC and MIBC tissue specimens. Notice the absence of staining in the intratumoral area. Scale bar, 50 µm. **(b)** Distribution of $CD83^+$ cells density per mm^2 in HG NMIBC (n = 93) and MIBC (n = 113) samples. The number of positive cells per mm^2 was calculated with QuPath's *Positive Cell Detection* tool. *P*-values from the non-parametric Mann-Whitney *U*-test; ns $P > 0.05$. HG NMIBC – high-grade non-muscle invasive bladder cancer; MIBC – muscle-invasive bladder cancer.



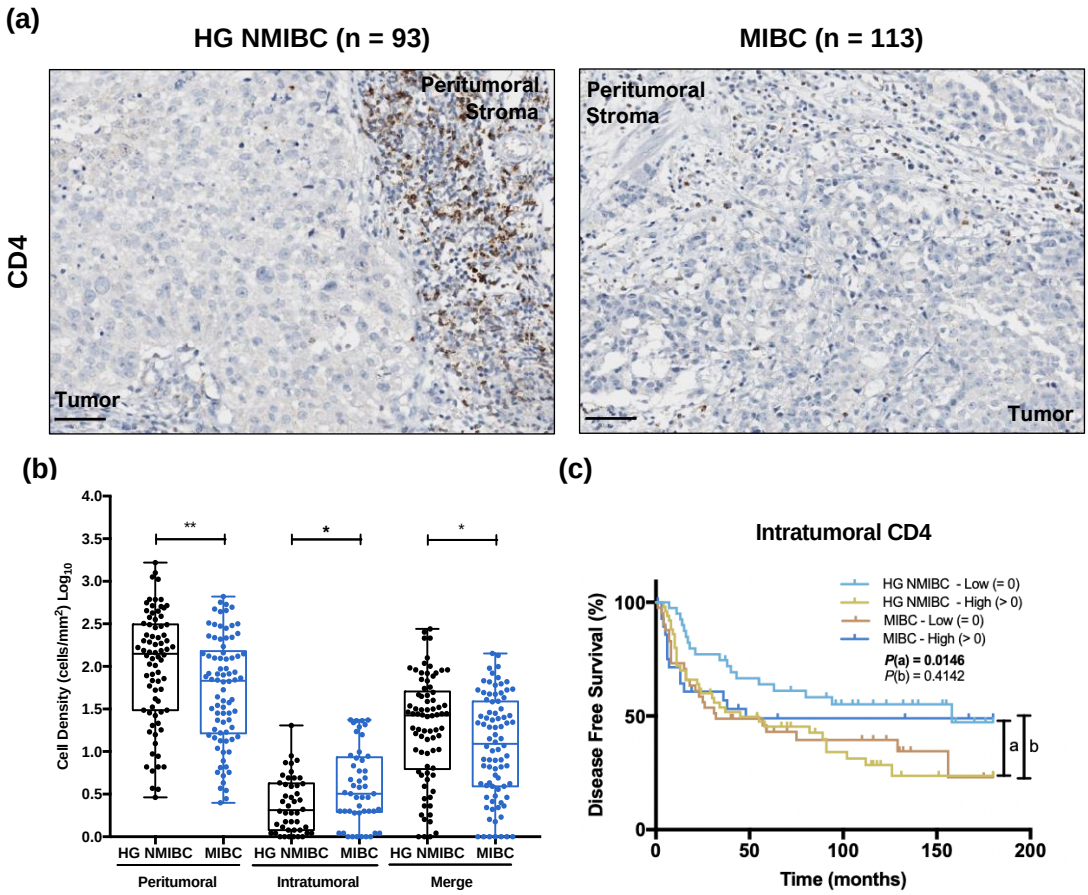
Supplementary figure 2 – Higher CD20 expression tends to associate with shortened OS and DFS. Kaplan-Meier estimates of OS **(a)** and DFS **(b)** in BlCa patients stratified by CD20 expression. Dichotomization into low and high expression was based on the median value. **(c)** Distribution of $CD20^{+}$ cells density in samples from female (n = 44) and male (n = 162) individuals. $^{**}P < 0.01$.



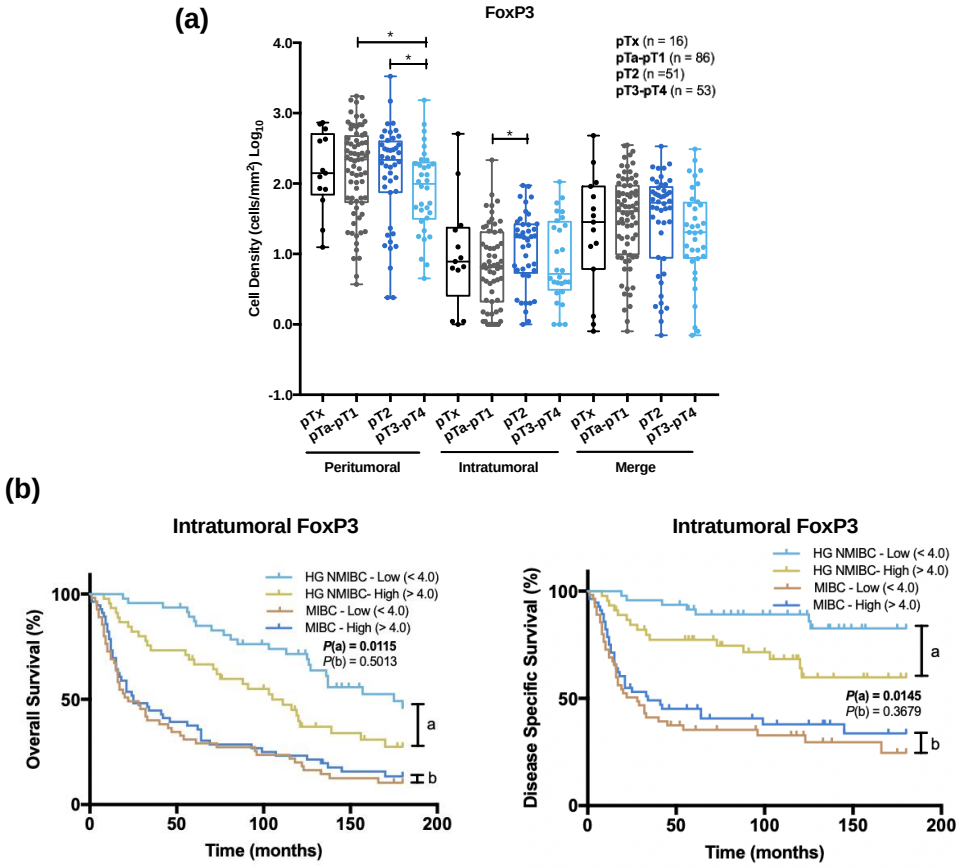
Supplementary figure 3 – Differential distribution of CD3$^+$ cells within the tumor landscape. Tumor infiltration of CD3$^+$ cells benefits patients' prognosis. (a) Representative examples of CD3 staining in HG NMIBC and MIBC tissue specimens. Notice the increased intratumoral infiltration in MIBC samples. Scale bar, 50 µm. **(b)** Distribution of CD3$^+$ cells density in HG NMIBC (n = 93) and MIBC (n = 113) samples. The number of positive cells per mm^2 was calculated with QuPath's *Positive Cell Detection* tool. "Merge" refers to the whole-slide evaluation, considering peritumoral and intratumoral regions together. *P*-values from the non-parametric Mann-Whitney *U*-test; ns $P > 0.05$, ****$P < 0.0001$. **(c)** Kaplan-Meier estimates of DFS in BlCa patients stratified by intratumoral CD3 expression. Dichotomization into low and high expression was based on the median value. *P*-values were assessed by the log-rank test. HG NMIBC – high-grade non-muscle invasive bladder cancer; MIBC – muscle-invasive bladder cancer.



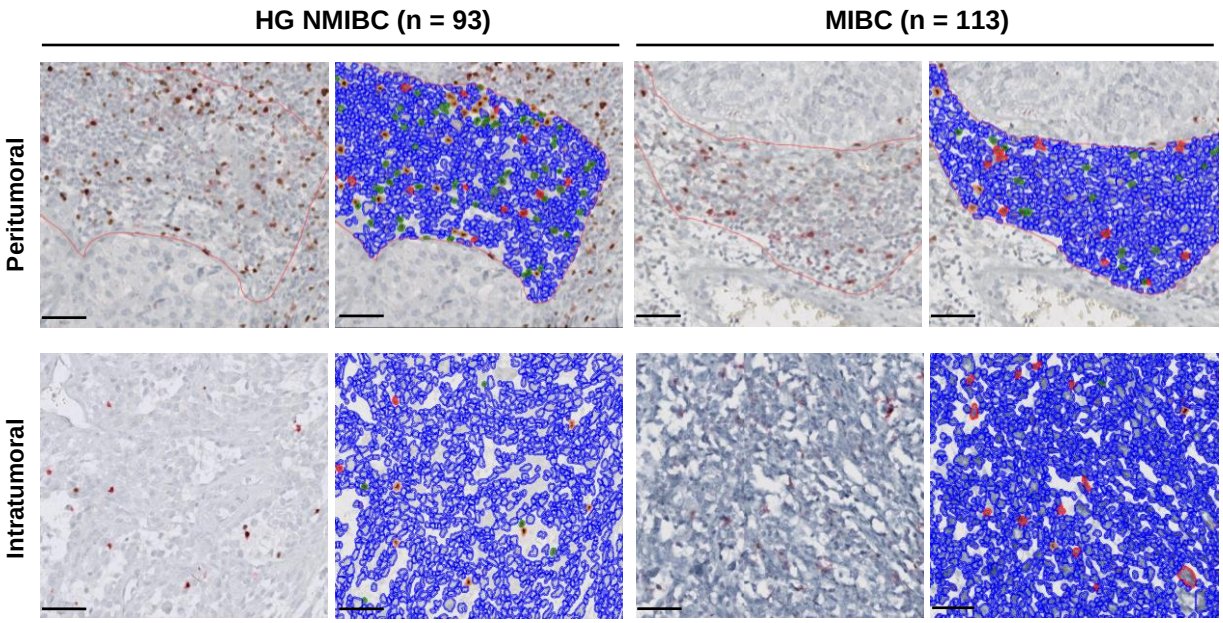
Supplementary figure 4 – Peritumoral CD3^{+} cells do not influence patients' prognosis. Kaplan-Meier estimates of OS, DSS and DFS in BlCa patients stratified by peritumoral CD3 expression. Dichotomization into low and high expression was based on the median value.



Supplementary figure 5 – CD4 distribution pattern differs between tissue compartments and intratumoral infiltration correlates with worse prognosis, especially in NMIBC. (a) Representative examples of CD4 staining in HG NMIBC and MIBC tissue specimens. Notice the increased peritumoral staining in HG NMIBC samples and the slightly higher tumor infiltration in MIBC. Scale bar, 50 μm. **(b)** Distribution of $CD4^+$ cells density in HG NMIBC (n = 93) and MIBC (n = 113) samples. The number of positive cells per mm^2 was calculated with QuPath's *Positive Cell Detection* tool. "Merge" refers to the whole-slide evaluation, considering peritumoral and intratumoral regions together. *P*-values from the non-parametric Mann-Whitney *U*-test; $*P < 0.05$, $**P < 0.01$. **(c)** Stratified Kaplan-Meier analyses of DFS according to intratumoral CD4 expression and "HG NMIBC vs MIBC" classification. a – Comparison between "HG NMIBC Low" and "HG NMIBC High"; b –Comparison between "MIBC Low" and "MIBC High". Dichotomization into low and high expression was based on the median value. *P*-values were assessed by the log-rank test. HG NMIBC – high-grade non-muscle invasive bladder cancer; MIBC – muscle-invasive bladder cancer.



Supplementary figure 6 – Different distribution of FoxP3$^+$ cells between tissue compartments. Tumor-infiltrating FoxP3$^+$ cells negatively influence prognosis, particularly in HG NMIBC. (a) Distribution of FoxP3$^+$ cells density in pTx (n = 16), pTa-pT1 (n = 86), pT2 (n = 51) and pT3-pT4 (n = 53) neoplasms. "Merge" refers to the whole-slide evaluation, considering peritumoral and intratumoral regions together. *P*-values from the non-parametric Mann-Whitney *U*-test; only significant associations ($P < 0.05$) are denoted for simplification purposes - *$P < 0.051$. **(b)** Stratified Kaplan-Meier analyses of OS and DSS according to intratumoral FoxP3 expression and "HG NMIBC vs MIBC" classification. a – Comparison between "HG NMIBC Low" and "HG NMIBC High"; b – Comparison between "MIBC Low" and "MIBC High". Dichotomization into low and high expression was based on the median value. *P*-values were assessed by the log-rank test. HG NMIBC – high-grade non-muscle invasive bladder cancer; MIBC – muscle-invasive bladder cancer.



Supplementary figure 7 - Representative examples of peritumoral and intratumoral FoxP3/ICOS double-staining in HG NMIBC and MIBC tissue specimens. Each image is followed by the respective evaluation obtained with QuPath's *Object classification* tool (green = single $FoxP3^+$ cells; red = single $ICOS^+$ cells; orange = $FoxP3^+ICOS^+$ cells). Notice the increased proportions of peritumoral $FoxP3^+ICOS^+$ cells in HG NMIBC samples and intratumoral single $ICOS^+$ cells in MIBC samples. Scale bar, 50 μm. HG NMIBC – high-grade non-muscle invasive bladder cancer; MIBC – muscle-invasive bladder cancer.

Supplementary tables

Supplementary table 1. Cox-regression analyses to assess the predictive value of immunological markers for OS.

OS	Unadjusted		Adjusted*	
	HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**
CD83				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.92 (0.66-1.28)	0.622	0.93 (0.67-129)	0.650
CD20				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.34 (0.968-1.864)	0.078	1.06 (0.75-1.48)	0.751
CD68				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.45 (1.06-2.00)	**0.022**	0.90 (0.63-1.29)	0.560
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.29 (0.93-1.79)	0.122	0.91 (0.64-1.28)	0.574
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.34 (0.96-1.85)	0.081	0.89 (0.62-1.26)	0.506
CD163				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.23 (0.89-1.69)	0.207	0.77 (0.54-1.09)	0.143
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.21 (0.87-1.67)	0.258	1.00 (0.717-1.38)	0.985
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.47 (1.06-2.04)	**0.020**	1.07 (0.76-1.50)	0.708
CD3				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.10 (0.80-1.51)	0.573	0.74 (0.52-1.04)	0.081
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.85 (0.61-1.80)	0.330	0.92 (0.67-1.28)	0.640
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.97 (0.70-1.35)	0.972	0.93 (0.67-1.29)	0.682
CD8				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.09 (0.79-1.51)	0.583	0.769 (0.55-1.08)	0.131
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.02 (073-1.41)	0.916	0.96 (0.69-1.33)	0.783
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.14 (0.82-1.58)	0.444	0.89 (0.64-1.25)	0.512
CD4				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.92 (0.67-1.27)	0.617	0.96 (0.70-1.33)	0.830
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.90 (0.65-1.25)	0.526	1.14 (0.82-1.59)	0.450

Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.91 (0.66-1.27)	0.591	1.12 (0.80-1.56)	0.505
FoxP3				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.245 (0.90-172)	0.175	1.15 (0.83-1.59)	0.406
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.91 (0.65-1.26)	0.563	1.02 (0.73-1.42)	0.926
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.00 (0.72-1.39)	0.984	1.07 (0.77-1.49)	0.675
ICOS				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.20 (0.87-1.65)	0.268	0.83 (0.59-1.18)	0.301
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.82 (0.59-1.5)	0.256	0.83 (0.59-1.15)	0.261
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.76 (0.55-1.06)	0.109	0.61 (0.43-0.86)	**0.004**
FoxP3 + ICOS				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.24 (0.90-1.71)	0.197	1.12 (0.81-1.54)	0.497
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.84 (0.61-1.18)	0.318	0.94 (0.68-1.32)	0.734
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.08 (0.78-1.50)	0.650	1.06 (0.76-1.47)	0.739
PD-L1				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.91 (0.66-1.25)	0.557	1.05 (0.76-1.45)	0.753

*Multivariable model, adjusted for “HG NMIBC vs MIBC” classification. OS – Overall Survival; HR – Hazard Ratio; CI – Confidence Interval.

Supplementary table 2. Cox-regression analyses to assess the predictive value of immunological markers for DSS.

DSS	Unadjusted		Adjusted*	
	HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**
CD83				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.94 (0.62-1.42)	0.770	0.92 (0.61-1.40)	0.702
CD20				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.69 (1.11-2.57)	**0.015**	1.19 (0.77-1.84)	0.426
CD68				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.71 (1.14-2.56)	**0.009**	0.90 (0.58-1.40)	0.626
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.54 (1.02-2.34)	**0.040**	0.94 (0.61-1.46)	0.785
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.58 (1.04-2.38)	**0.030**	0.88 (0.57-1.38)	0.585
CD163				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.33 (0.89-1.99)	0.161	0.73 (0.48-1.12)	0.151
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.17 (0.78-1.76)	0.447	0.90 (0.60-1.37)	0.646
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.509 (1.00-2.28)	**0.048**	0.973 (0.63-150)	0.900
CD3				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.11 (0.74-1.66)	0.616	0.65 (0.42-0.99)	**0.043**
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.80 (0.53-1.21)	0.287	0.84 (0.56-1.27)	0.406
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.90 (0.60-1.36)	0.612	0.83 (0.55-1.25)	0.373
CD8				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.16 (0.78-1.74)	0.456	0.78 (0.51-1.18)	0.237
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.11 (0.73-1.68)	0.618	1.00 (0.66-1.52)	1.000
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.30 (0.86-1.97)	0.217	0.95 (0.62-1.45)	0.806
CD4				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.99 (0.67-1.48)	0.976	1.10 (0.74-1.65)	0.628
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.87 (0.58-1.31)	0.505	1.20 (0.79-1.83)	0.386
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.84 (0.56-1.26)	0.399	1.10 (0.74-1.67)	0.652
FoxP3				

Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.17 (0.78-1.76)	0.444	1.06 (0.71-1.59)	0.779
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.78 (0.52-1.19)	0.256	0.90 (0.59-1.37)	0.626
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.87 (0.57-1.32)	0.505	0.94 (0.62-1.43)	0.776
ICOS				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.18 (0.79-1.78)	0.411	0.74 (0.48-1.13)	0.159
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.80 (0.52-1.21)	0.293	0.81 (0.54-1.24)	0.338
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.71 (0.47-1.09)	0.117	0.54 (0.36-0.84)	**0.005**
FoxP3 + ICOS				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.04 (0.69-1.56)	0.849	0.93 (0.62-1.40)	0.728
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.61 (0.40-0.93)	**0.023**	0.71 (0.46-1.09)	0.114
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.82 (0.54-1.24)	0.343	0.80 (0.53-1.22)	0.308
PD-L1				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.10 (0.73-1.63)	0.658	1.34 (0.89-2.00)	0.160

*Multivariable model, adjusted for “HG NMIBC vs MIBC” classification. DSS – Disease-Specific Survival; HR – Hazard Ratio; CI – Confidence Interval.

Supplementary table 3. Cox-regression analyses to assess the predictive value of immunological markers for DFS.

DFS	Unadjusted		Adjusted*	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
CD83				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.11 (0.73-1.69)	0.629	1.11 (0.73-1.70)	0.619
CD20				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.27 (0.84-1.93)	0.253	1.24 (0.81-1.90)	0.316
CD68				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.94 (0.62-1.42)	0.770	0.84 (0.51-1.37)	0.476
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.12 (0.74-1.69)	0.595	1.06 (0.68-1.65)	0.793
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.12 (0.74-1.68)	0.604	1.05 (0.66-1.68)	0.822
CD163				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.14 (0.75-1.71)	0.544	1.09 (0.68-1.75)	0.708
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.22 (0.81-1.84)	0.339	1.19 (0.78-1.81)	0.410
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.20 (0.80-1.81)	0.381	1.16 (0.75-1.80)	0.496
CD3				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.65 (0.42-0.99)	**0.044**	0.58 (0.37-0.91)	**0.018**
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.98 (0.64-1.47)	0.909	0.98 (0.65-1.49)	0.943
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.85 (0.56-1.29)	0.452	0.84 (0.56-1.28)	0.424
CD8				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.60 (0.39-0.91)	**0.017**	0.54 (0.35-0.84)	**0.007**
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.68 (0.45-1.04)	0.075	0.67 (0.44-1.03)	0.066
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.62 (0.40-0.94)	**0.027**	0.58 (0.37-0.90)	**0.015**
CD4				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.24 (.82-1.87)	0.305	1.25 (0.83-1.89)	0.283
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.32 (0.87-2.00)	0.186	1.40 (0.91-2.14)	0.125
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.31 (0.87-1.99)	0.197	1.38 (0.90-2.10)	0.141
FoxP3				

Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.77 (0.51-1.18)	0.229	0.76 (0.50-1.16)	0.207
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.00 (0.66-1.52)	0.989	1.01 (0.66-1.54)	0.959
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.90 (0.59-1.37)	0.625	0.91 (0.60-1.38)	0.643
ICOS				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.98 (0.65-1.49)	0.942	0.94 (0.60-1.46)	0.772
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.18 (0.78-1.80)	0.436	1.18 (0.77-1.80)	0.448
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.85 (0.56-1.29)	0.447	0.82 (0.53-1.26)	0.369
FoxP3 + ICOS				
Intratumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.02 (0.67-1.54)	0.93	1.01 (0.67-1.53)	0.959
Peritumoral				
Low	1.00 (Reference)		1.00 (Reference)	
High	1.00 (0.66-1.54)	0.965	1.02 (0.67-1.55)	0.941
Merge				
Low	1.00 (Reference)		1.00 (Reference)	
High	0.88 (0.58-1.33)	0.531	0.87 (0.57-1.32)	0.514
PD-L1				
Low	1.00 (Reference)		1.00 (Reference)	
High	2.04 (1.33-3.14)	**0.001**	2.07 (1.35-3.19)	< **0.001**

*Multivariable model, adjusted for “HG NMIBC vs MIBC” classification. DFS – Disease-Free Survival; HR – Hazard Ratio; CI – Confidence Interval.

Supplementary table 4. Primary antibodies used in IHC.

Antibody	Company	Clone	Species	Dilution	Antigen Retrieval	Positive Control	Detection Kit/ Detection System
CD3	Leica Biosystems (NCL-L-CD3-565)	LN10	Mouse	1/200 (1h incubation)	EDTA buffer pH9, 20 min, MW (700W)	Tonsil	*ultra*View Universal DAB Detection Kit / n.a.
CD20	Leica Biosystems (NCL-L-CD20-L26)	L26	Mouse	1/150 (1h incubation)	EDTA buffer pH9, 20 min, MW (700W)	Tonsil	*ultra*View Universal DAB Detection Kit / n.a.
CD68	Dako (M0876)	PG-M1	Mouse	1/150 (1h incubation)	EDTA buffer pH9, 20 min, MW (700W)	Tonsil	*ultra*View Universal DAB Detection Kit / n.a.
CD83	abcam (ab275021)	EPR23809-19	Rabbit	1/300 (1h incubation)	EDTA buffer pH9, 20 min, MW (700W)	Tonsil	*ultra*View Universal DAB Detection Kit / n.a.
CD163	Leica Biosystems (NCL-L-CD163)	10D6	Mouse	Pre-diluted (1h incubation)	Sodium Citrate buffer pH6, MW (700W)	Spleen	*ultra*View Universal DAB Detection Kit / n.a.
ICOS	Cell Signaling Technology (#89601)	D1K2TTM	Rabbit	1/200 (1h incubation)	CC1, 64 min, 95ºC	Colon Carcinoma	*ultra*View Universal Alkaline Phosphatase Red Detection Kit / BenchMark ULTRA
PD-L1	Abcam (ab228462)	SP142	Rabbit	1/25 (1h incubation)	EDTA buffer pH9, 20 min, MW (700W)*	Tonsil, Lung, Placenta	optiView DAB Detection Kit / BenchMark ULTRA
CD8	Roche (#5937248001)	SP57	Rabbit	Pre-diluted (16 min incubation)	CC1, 64 min, 95ºC	Tonsil	*ultra*View Universal DAB Detection Kit / BenchMark ULTRA
CD4	Leica Biosystems (NCL-L-CD4-368)	4B12	Mouse	1/100 (32 min incubation)	ER2, 60 min, 95º C	Tonsil	optiView DAB Detection Kit / BenchMark ULTRA
FoxP3	Abcam (ab20034)	236A/E7	Mouse	1/100 (1h incubation)	CC1, 64 min, 95ºC	Tonsil	*ultra*View Universal DAB Detection Kit / BenchMark ULTRA

*Manual Antigen Retrieval. CC1 – Cell Conditioning 1; EDTA - Ethylenediamine tetraacetic acid; ER2 - Epitope Retrieval 2; n.a. – non applicable; MW - Microwave; ON – Overnight.