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Original article

Infiltration of CD3$^+$ and CD68$^+$ cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors[1]

Gottfrid Sjödahl, Ph.D.[a], Kristina Lövgren, B.Sc.[a], Martin Lauss, Ph.D.[a], Gunilla Chebil, M.D.[a], Oliver Patschan, M.D.[b], Sigurdur Gudjonsson, Ph.D., M.D.[b], Wiking Månsson, M.D.[b], Mårten Fernö, Ph.D.[a], Karin Leandersson, Ph.D.[c], David Lindgren, Ph.D.[c], Fredrik Liedberg, Ph.D., M.D.[b], Mattias Höglund, Ph.D.[a,*]

[a] *Department of Oncology, Clinical Sciences, Skåne University Hospital, Lund University, Lund, Sweden*
[b] *Department of Urology, Clinical Sciences, Skåne University Hospital, Lund University, Lund, Sweden*
[c] *Department of Laboratory Medicine, Center for Molecular Pathology, Skåne University Hospital, Lund University, Malmö, Sweden*

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Abstract

Objectives: Urothelial carcinoma (UC) aggressiveness is determined by tumor inherent molecular characteristics, such as molecular subtypes, as well as by host reactions directed toward the tumor. Cell types responsible for the host's response include tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs). The aim of the present investigation was to explore the immunological response in relation to UC molecular subtypes and to evaluate the prognostic effect of TIL and TAM counts in tissue sections from muscle-invasive (MI) tumors.

Methods and materials: Tissue microarrays with 296 tumors spanning all pathological stages and grades were analyzed with antibodies for CD3, CD8, FOXP3, CD68, and CD163. Cases were classified into the following molecular subtypes: urobasal, genomically unstable, and squamous cell carcinoma–like using a combination of immunohistochemistry and histology. The Cox regression and Kaplan-Meier analyses were performed with progression-free survival and disease-specific survival as end points.

Results: UC molecular subtypes demonstrate different degrees of immunological responses; the urobasal subtype induces a weak response, the genomically unstable subtype induces an intermediate response, and the squamous cell carcinoma–like subtype induces a strong response. These subtype specific responses are independent of tumor stage and include both TILs and TAMs. The presence of infiltrating CD3$^+$ TILs was significantly associated with good prognosis in the MI cases ($P < 0.01$). This positive association was modulated by the presence of CD68$^+$ TAMs. The strongest association with poor survival was observed for a high ratio between CD68 and CD3 ($P = 7 \times 10^{-5}$).

Conclusion: UC molecular subtypes induce immunological responses at different levels. A high CD68/CD3 ratio identifies a bad prognosis group among MI UC cases.

Keywords: Bladder cancer; Molecular subtypes; T cells; CD3 antigen; CD68 antigen; Prognosis

1. Introduction

The inherent molecular characteristics of urothelial carcinomas (UCs) determine their aggressiveness. For instance, low-stage low-grade UCs with a small propensity to progress show very different molecular profiles compared to muscle-invasive (MI) cases [1]. However, molecular profiles also differ among pathologically similar tumors. We recently showed that UCs may be classified into

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* Corresponding author. Tel.: +464-6-222-0393.
E-mail address: Mattias.Hoglund@medlu.se (M. Höglund).

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3 major molecular subtypes, urobasal (Uro), genomically unstable (GU), and squamous cell carcinoma–like (SCCL) tumors using immunohistochemistry (IHC) [2] and that Ta tumors are dominated by the Uro cases, T1 tumors by Uro and GU cases, and MI tumors by GU and SCCL cases. The tumor subtypes show distinct molecular profiles at both RNA [3] and protein [2] levels, as well as distinct survival characteristics. However, the outcome of a given tumor is also affected by host reactions toward the tumor cells. Tumor-infiltrating lymphocytes (TILs) are a heterogeneous group of immune cells that are abundant in tumors of different origin. Cytotoxic T cells (CTLs) are the main effector cells in antitumor T-cell immunity, and their activity is modulated by other T-cell subtypes, such as T-helper cells and regulatory T cells (Tregs). The effect of a particular immune response is determined by the balance between the various T-cell subtypes involved. It has previously been shown that tumor infiltration by both CTLs ($CD8^+$) and T cells in general ($CD3^+$) promotes survival in patients with UC [4,5]. Another important immune cell population present in tumors is tumor-associated macrophages (TAMs). As for T cells, macrophages may have activating (M1), or suppressive (M2) immune functions. TAMs have previously been suggested to be M2-like [6]. They secrete a wide range of cytokines and have an important role in immune suppression, angiogenesis, and tumor progression. Consequently, TAMs may have an overall tumor growth–promoting effect, counteracting the action of the T-cell response [7,8]. Thus, in the present investigation, we have evaluated the level of both TILs and TAMs in 296 cases of UC and related the immune response to UC molecular subtypes and clinical outcome.

2. Materials and methods

2.1. Patient and sample selection

Tumor biopsies from transurethral resection of 296 patients diagnosed with UC in the Southern Sweden Healthcare Region between 2001 and 2009 were included. Patient and tumor data are summarized in Table 1. Of the patients with non-MI (NMI) tumors, 56 received bacillus Calmette-Guérin (BCG) treatment (6 instillations), of the patients with MI tumors 52 were treated with radical cystectomy, and of these 4 with additional neoadjuvant chemotherapy. The majority of the cases included were primary tumors; approximately 1 in 5 tumors (54 cases) had a history of bladder cancer. Median follow-up time was 51 months for patients with NMI disease and 70 months for patients with MI disease. The investigation was approved by the regional ethics committee (no. 2010/5). The tissue sections were reevaluated by a uropathologist (G.C.) using TNM 2009 [9], as staging and grading was performed according to the World Health Organization 1999 system.

Table 1
Distribution of molecular subtype and patient/tumor data across pathological stages

	Ta, $n = 112$	T1, $n = 89$	MI, $n = 93$	Tx, $n = 2$
Molecular subtype, no. of cases				
Urobasal	98	34	11	2
GU	9	43	46	0
SCC-like	0	4	34	0
Not classified	5	8	2	0
Patient/tumor data				
Age, mean years	69.1	70.8	71.7	75.0
Gender, no. of cases				
Male	77	72	69	2
Female	35	17	24	0
Tumor grade, no. of cases				
G1	42	0	0	0
G2	62	31	10	1
G3	8	58	83	1

2.2. Tissue microarrays and IHC

Tissue microarray blocks were constructed from 1.0-mm punches of formalin-fixed paraffin-embedded specimens of transurethral resection of the bladder using a manual array (TMA arrayer, Pathology Devices, Inc, Westminster, MD). Tissue punches were collected from areas with the highest grade on the corresponding sections and from areas without necrosis. Tissue microarray (TMA) sections were stained with antibodies against CD3, CD8, FOXP3, CD68, and CD163. As negative controls, the primary antibodies were omitted for each staining. Antibodies for CD3 (clone, F7.2.38; mouse; dilution, 1:200; product M7254; Dako), CD8 (clone, C8/144B; mouse; dilution, 1:50; product M7103; Dako), FOXP3 (clone, 236A/E7; mouse; dilution, 1:1,000; product ab20034; Abcam), CD163 (clone, 10D6; mouse; dilution, 1:250; product NCL-CD163; Novocastra), and CD68 (clone, EBM11; mouse; dilution, 1:1,500; product M0718; Dako) were used. All markers showed discrete cellular-staining patterns. Each TMA core was given a score of 0 to 5 based on the average count of positive cells per tissue area. The scores 0 to 5 corresponded approximately to the bins 0 to 20, 20 to 50, 50 to 100, 100 to 300, 300 to 500, and >500 positive cells per TMA core. The range of scoring intervals was defined to capture the observed variation for each individual marker. For each marker, 1 case per bin was manually counted, and these cases were used as a reference in the evaluation process. The evaluations were done manually by 2 observers. When the opinions differed, both the observers discussed the case and reached an agreement. For most cases ($n = 287$), 2 cores were available, and the mean of the 2 cores was used. Marker scores were recorded for the entire core and separately for the intratumoral and stromal compartments when the distinction between both the compartments was clear. The evaluations were performed on digitalized

images, scanned using a ScanScope CS scanner (Aperio) at ×40 magnification.

2.3. Tumor classification and statistical analysis

Tumor cases were classified into the molecular subtypes Uro, GU, and SCCL as described in Sjödahl et al. [2] using the variables urothelial-like growth pattern, World Health Organization 1999 grade 3, and CCNB1, CCND1, and KRT5 IHC data. Anti–α-smooth muscle actin antibody was used to facilitate the identification of tumor stroma as described previously [2]. Mann-Whitney *U* test or Kruskal-Wallis rank sum test was used to test marker score differences between the sample groups. The Cox regression model was used to investigate association with outcome. All markers were analyzed for univariate association to progression-free survival for NMI cases and disease-specific survival (DSS) for MI cases. Progression of NMI included progression to MI or to cystectomy. Only patients with MI tumors who received curative treatment, i.e., cystectomy, were included in the analysis of DSS ($n = 52$). Pathological data from cystectomy specimens were not used as such information is not available at the time of treatment decision. Instead, clinical stage was used in the multivariate analysis of MI tumors. As all but 2 of the 52 cystectomized cases were G3, grade was not considered informative in the Cox regression analyses. CD68 and CD3 were tested also in a bivariate model of DSS to dissect the independence of associations. *P*-values for Kaplan-Meier analyses were estimated by log-rank tests. All statistical tests were performed using *R*.

3. Results

We produced a TMA consisting of 296 cases (Table 1) and applied antibodies for CD3 (T cells in general), CD8 (CTLs), FOXP3 (Tregs), CD68 (TAMs in general), and CD163 (M2-like TAMs). As expected from the subcellular localization of the target proteins, antibodies against CD3, CD8, and CD163 showed membranous staining; FOXP3 nuclear staining; and CD68 showed cytoplasmic staining. The size and morphology of labeled cells were consistent with T cells and macrophages, respectively. Tumor cell positivity was only observed for CD68 and CD163 and only in a small subset of UCs (14 cases, 5%, for CD68, and 18 cases, 6%, for CD163) in which staining was generally weak and cytoplasmic. Overall, the markers CD3, CD8, CD68, and CD163 showed pair-wise strong correlations $R > 0.7$ (Table 2), i.e., T-cell marker scores were correlated with macrophage marker scores. This suggests that the immunological response in most cases involves both T cells and macrophages. The coordinated response among T cells and macrophages was also evident in cases where a clear separation between tumor parenchyma and stromal compartments was visible; either all labeled cells, irrespectively of marker, were localized to the stroma, or to both stroma and tumor parenchyma (Fig. 1). We observed cases with both moderate and strong positivity (infiltration) in stroma only and cases with strong positivity in both compartments (Fig. 1). However, no cases with strong positivity in tumor parenchyma only were observed.

Table 2
Pearson correlation coefficients between T-cell and macrophage markers

	CD3	CD8	FOXP3	CD68	CD163
CD3	1.00	–	–	–	–
CD8	0.79	1.00	–	–	–
FOXP3	0.70	0.62	1.00	–	–
CD68	0.72	0.74	0.60	1.00	–
CD163	0.75	0.78	0.60	0.88	1.00

We next applied the previously described IHC/histopathology classifier [2] to group cases into the 3 major molecular subtypes of UC, Uro, GU, and SCCL (Fig. 2). The subtypes represent major clinicopathological entities of UC with characteristic molecular and prognostic features [3] and only show moderate overlap with pathological tumor stage (Table 1). In Fig. 2, we have plotted the immune-marker scores for each tumor subtype when present in Ta, T1, and MI cases. Ta cases show low counts for all markers and no differences were observed between Ta Uro and Ta GU cases, except for a modest difference in CD68 counts. However, significant differences were seen between Uro, GU, and SCCL cases within stage T1, particularly with respect to macrophage counts. T-cell markers showed a similar but less pronounced pattern. The same pattern of differential labeling was also observed among MI cases. To further substantiate the relationship between molecular subtype and infiltration, we created linear models where the influence of tumor stage and molecular subtype on marker scores was evaluated. When correcting for stage, molecular subtype has a significant influence on scores for all the markers (CD3, $P = 0.014$; CD8, $P < 2 \times 10^{-6}$; FOXP3, $P < 2 \times 10^{-4}$; CD68, $P < 4 \times 10^{-5}$; CD163, $P < 3 \times 10^{-6}$). Conversely, when correcting for molecular subtype, pathological stage had a significant influence on 3 of the markers (CD3, $P = 0.0021$; CD68, $P < 2 \times 10^{-5}$; CD163, $P < 6 \times 10^{-5}$).

Hence, both molecular subtypes and pathological stage are associated with different levels of immunological responses. Notably, Uro cases show the weakest, GU the intermediate, and SCCL the strongest response, irrespective of tumor stage, suggesting that the relative strength of the induced response is intrinsic to the molecular subtype.

Next, we wanted to investigate the prognostic significance of tumor-infiltrating immunological cells. Scores for stromal, intratumoral, and total biopsy were analyzed separately when the distinction could be made. In no instance did the stromal or intratumoral score show stronger association with any outcome than the total score, irrespective of marker. We analyzed immune cell marker association to progression-free survival in NMI tumors. Although the low number of progression events ($n = 24$) in this

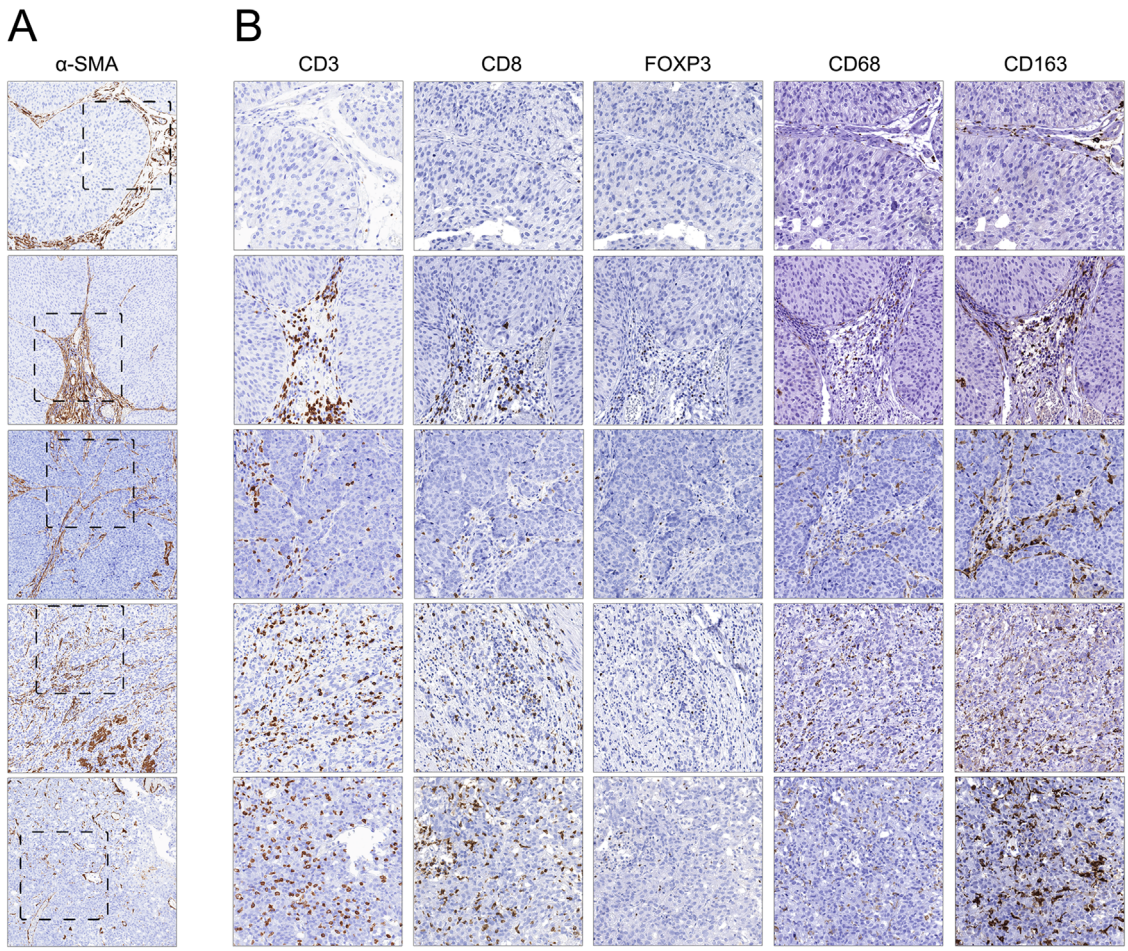

Fig. 1. Representative images of immune cell marker staining in UC. (A) Anti-α-SMA staining was used to facilitate the distinction between stromal (positive) and intratumoral (negative) compartments when present. (B) Representative staining is shown for 5 cases. The areas shown are indicated by the dashed lines in (A). From top down, urobasal tumor without infiltration, urobasal tumor with dense infiltration of the tumor adjacent stroma, genomically unstable tumor with moderate infiltration of both stromal and intratumoral compartments, genomically unstable tumor with dense infiltration and no distinct intratumoral and stromal compartments, and a SCC-like tumor with dense infiltration and no distinct intratumoral and stromal compartments. SMA = smooth muscle actin antibody. (Color version of figure is available online.)

patient cohort reduced the statistical power of Cox regression analyses, the macrophage marker CD68 showed borderline significance (hazard ratio [HR] = 1.523; CI (95%): 1.082–2.144; $P = 0.016$). However, owing to the few progression events in this cohort, no further subdivision of BCG-treated and non–BCG-treated patients could be done. Next, we divided the MI cases by infiltration into "high" and "low" scoring cases by applying a threshold at the midpoint score 3 or greater. In the MI cases treated with radical cystectomy, univariate Cox regression with DSS as end point revealed that CD3 scores above this threshold were associated with a better patient survival ($P = 0.009$) (Table 3). The result was not improved by using the full range of scores, suggesting a threshold effect. The subsequent Kaplan-Meier analysis clearly identified a good prognosis group composed of CD3 high cases (Fig. 3A). Pair-wise bivariate tests, again with DSS as the end point, revealed both CD3 and CD68 as significant variables; $CD3^+$ counts associated with good (HR = 0.5; 95% CI: 0.29–0.69; $P = 3 \times 10^{-4}$) and CD68 counts with a bad prognosis (HR = 1.7; 95% CI: 1.14–2.51; $P = 0.0096$). To investigate the opposite actions of $CD3^+$ and $CD68^+$ counts further, we divided the cases into CD68 high/low in analogy with CD3 using a threshold of 3, resulting in 4 groups; CD3 high/CD68 low; CD3 high/CD68 high; CD3 low/CD68 low; and CD3 low/CD68 high, and we performed a 4-group Kaplan-Meier analysis (Fig. 3B). This analysis revealed a best prognosis group of patients with CD3 high and CD68 low and a worst prognosis group with CD3 low and CD68 high. In fact, all patients in the CD3 low/CD68 high group succumbed to their disease within 20 months. This result suggests that CD68 adds information on outcome to both the CD3 high and the CD3 low group but does not necessarily have prognostic value on its own. We, therefore, calculated a CD68/CD3 ratio based on the marker scores for each tumor and used these ratios in subsequent Cox regression (Table 3) and Kaplan-Meier analyses (Fig. 3C). This greatly

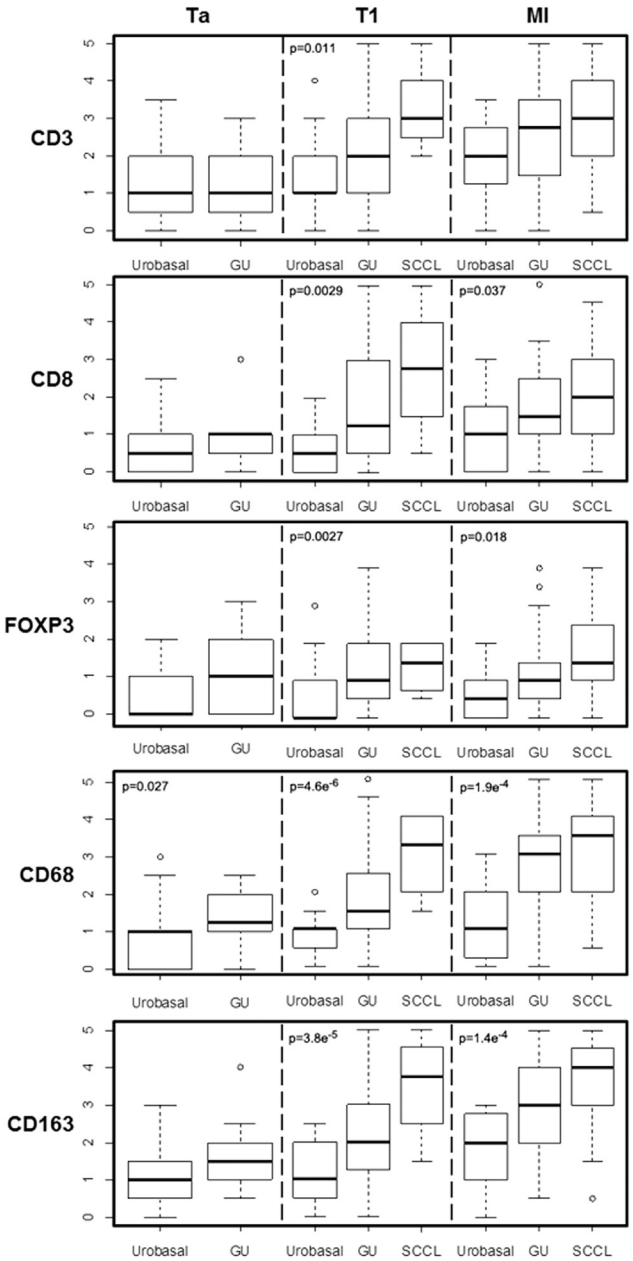

Fig. 2. Box plots of immune cell marker scores in molecular subtypes of UC, stratified by tumor stage. *P*-values shown when significant, $P < 0.05$. Mann-Whitney *U* test for pair-wise comparison, Kruskal-Wallis rank sum test for comparisons across the 3 groups.

improved the identification of a bad prognosis group. Of 17 cases with CD68/CD3 ratios above 1, 14 patients (82%) had succumbed to their disease within 3 years. In multivariate analysis, we included clinical stage (T2 vs. >T2) and the CD68/CD3 ratio. Only the CD68/CD3 ratio was used in this analysis, as the CD3 total had a smaller prognostic effect in univariate analysis and because the ratio and CD3 total are not independent variables. Too few patients had a positive clinical node status ($n = 5$) or received neoadjuvant chemotherapy ($n = 4$) to be included in multivariate analyses. Both clinical stage and CD68/CD3 ratio were independently associated to DSS (Table 3). No significant associations with outcome were observed for CD163, CD8, or FOXP3 in any analysis.

Table 3
Univariate and multivariate Cox regression analyses: Disease-specific survival of muscle-invasive cases where the patient underwent cystectomy ($n = 52$)

	HR	CI (95%)	*P*-value
Univariate			
CD3 total	0.60	0.41–0.89	**0.009**
CD8 total	0.82	0.57–1.16	0.26
FOXP3 total	0.83	0.56–1.22	0.34
CD68 total	1.16	0.82–1.62	0.40
CD163 total	0.99	0.69–1.41	0.95
Ratio (univariate)			
CD68/CD3 >1	1.67	1.26–2.21	**6.9×10^{-5}**
Multivariate			
Clinical stage >T2	5.37	2.09–13.81	**4.9×10^{-4}**
CD68/CD3 >1	7.73	3.13–19.10	**9.5×10^{-6}**

P-values < 0.05 are indicated in bold.

4. Discussion

We performed IHC using 3 T-cell markers, CD3 for T cells in general, CD8 for CTLs, and FOXP3 for Tregs [10], and 2 macrophage-specific markers, CD68 for macrophages in general [11], and CD163 for M2-like macrophages [12] to evaluate infiltrating immunological cells in UC. In general, T-cell and macrophage infiltration was highly correlated, albeit with the possible exception for Tregs (FOXP3). This indicates that in most cases infiltration by immunological cells encompass a complex set of cells, including both T cells and macrophages. The level of infiltration varied considerably from a few isolated immunological cells to massive infiltration. In fact, in a previous whole-genome gene expression analysis of UC, we identified cases that showed a gene expression signature dominated by an immunological profile to such an extent that they formed a separate group of "infiltrated" tumors [3]. As expected, the level of infiltration varied across the tumor stages. This is most likely due to differences in invasion depth and level of damage to the surrounding tissue. It has also been shown that the cytokine profiles of MI tumors differ radically from that of NMI tumors [13]. Furthermore, it has been shown that bladder TILs are in an activated state [14]. Even though the level of immune infiltration was highly stage dependent, we observed significant differences between the molecular subtypes Uro, GU, and SCCL within the same stage group. Among T1 cases, Uro showed the lowest, GU showed intermediate, and SCCL showed the highest TIL and TAM counts. This was particularly evident with respect to infiltrating macrophages. It could be argued that the differential response seen among molecular subtypes for T1 cases is associated with the pathological grade, and not with molecular subtype, as GU tumors are of grade

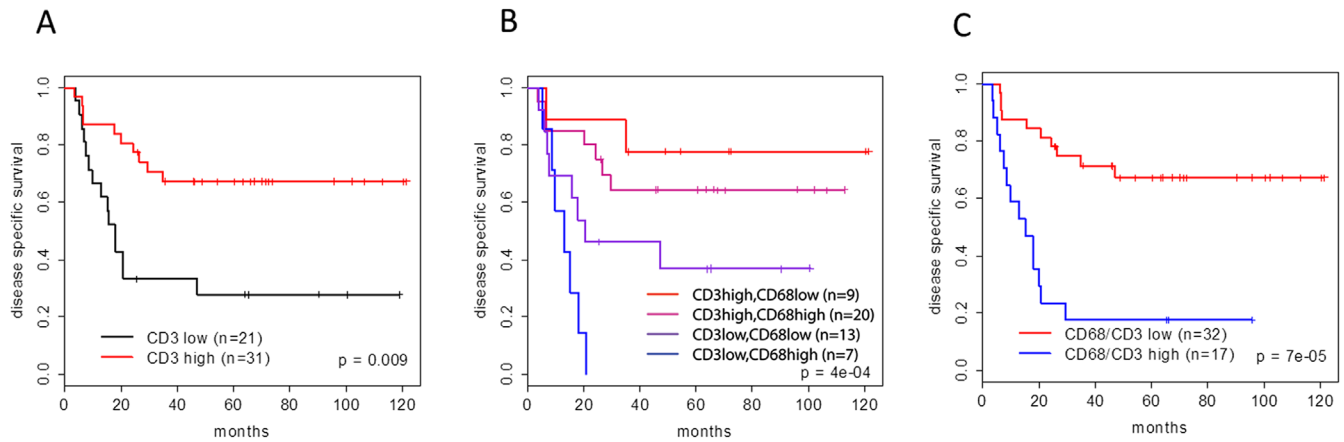

Fig. 3. Kaplan-Meier estimates of disease-specific survival in cystectomized muscle-invasive cases. (A) $CD3^+$ high vs. $CD3^+$ low cases. CD3 high, score $\geq$ 2.5; (B) cases grouped into 4 risk groups based on $CD3^+$ and $CD68^+$ scores; CD3 high, score $\geq$ 2.5; and CD68 high, score $\geq$ 2.5; and (C) tumors partitioned according to high vs. low CD68/CD3 ratio and CD68/CD3 high, score >1. (A) A total of 52 samples, whereas (B) and (C) includes 49 samples owing to 3 samples that could not be evaluated for CD68. (Color version of figure is available online.)

3 to a greater extent than Uro tumors [3]. However, the same pattern of differential response and ranking of subtypes based on levels of TIL and TAM counts were seen in MI cases, that are almost exclusively of grade 3. Thus, our data indicate that the strength of the induced immunological response is an intrinsic character of the UC molecular subtypes. Apart from the molecular differences existing between Uro, GU, and SCCL cases, the subtypes also show radically different growth patterns [2], both of which may influence the level of immunological responses induced by the host. In addition, the antitumor activity of TILs and TAMs may also be dependent on costimulatory signals from the microenvironment, which were not investigated in this study.

The immunological response in NMI cases is lower than in MI tumors, particularly, evident for Ta cases. The presence of TILs did not have a detectable effect on tumor progression, a finding also noted by Sharma et al. [4]. High levels of $CD68^+$ TAMs have been shown to be associated with recurrence of NMI tumors [15]. In this cohort, the presence of TAMs were associated to tumor progression. Owing to the limited number of progression events in this study, the association between $CD68^+$ TAMs and progression needs to be investigated further.

High $CD3^+$ score was found to be a good prognostic factor in MI cases. This strengthens the conclusion that high levels of infiltrating T cells have a protective effect [4,5,16,17]. In contrast to previous reports [4,5], no significant association with patient outcome was observed for CTLs ($CD8^+$) or Tregs ($FOXP3^+$). Investigating a possible combined effect of T-cell and macrophage infiltration, we identified an interaction between CD3 and CD68 in a Cox regression model. The subsequent Kaplan-Meier analysis indicated that the levels of $CD68^+$ TAMs modulated the effect of $CD3^+$ TILs and, in particular, that the ratio of $CD68^+$ relative to $CD3^+$ cells was the dominating factor, not the absolute $CD68^+$ counts. In multivariate analysis, both clinical stage and CD68/CD3 ratio were independent significant predictors of survival with the ratio having the stronger effect. Intriguingly, also in preoperative peripheral blood samples, a high neutrophil/lymphocyte ratio has been reported to be an indicator of poor prognosis after cystectomy [18]. The findings reported here are similar to findings in breast cancer, where a TAM/TIL ratio predicts outcome as well as chemotherapy response for tumors of the basal-like or $HER2^+$ subtypes [19]. In colon cancer, levels of TILs are strongly related to prognosis and the location of infiltrating cells is critical [20]. In the current study, separate analysis of stromal and intratumoral compartments did not improve outcome prediction, although it should be noted that tumors intermixed with stroma in the TMA cores were not analyzed. The effect of localization and the effect of more detailed subsets of TILs and TAMs merit additional studies. In summary, our results indicate that for MI UC treated with radical cystectomy clinical stage combined with a tumor CD68/CD3 ratio is a valuable tool for prognostication. The prognostic value of a CD68/CD3 ratio needs to be independently validated, preferably using a larger cohort of MI samples.

5. Conclusions

The molecular subtypes of UC display immunological responses at different levels. A high CD68/CD3 ratio identifies a bad prognosis group among MI UC cases.

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