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From Cell Biology to Tissue Engineering

Density of CD8-positive tumor-infiltrating T-lymphocytes is an independent prognostic factor in adenocarcinoma of the esophagogastric junction

Juliana Knief[1], Pamela Lazar-Karsten[2], Ulrich Wellner[3], Richard Hummel[3] and Christoph Thorns[1]

[1]Department of Pathology, Marienkrankenhaus Hamburg, Hamburg, [2]Department of Pathology, Section of Hematopathology and Endocrine Pathology and [3]Department of Surgery, University Hospital of Schleswig-Holstein, Campus Luebeck, Luebeck, Germany

Summary. Tumor-infiltrating lymphocytes (TILs) have commonly been associated with markedly improved prognosis in a variety of human cancers, including carcinomas of the upper and lower gastrointestinal tract. Especially the presence of T-cells (cytotoxic as well as helper cells) seems to define a subgroup of patients with prolonged overall and event-free survival. The density of TILs was assessed via immunohistochemistry for CD8 and CD103 in a population of 228 adenocarcinomas of the esophagogastric junction. Density of CD8+ T-lymphocytes was inversely correlated with depth of tumor infiltration (p=0.013) while no correlation with any of the analyzed clinicopathologic factors could be established for CD103-density. High density of CD8-positive T-cells additionally showed significantly longer overall survival (OS) with a p-value of 0.024 while density of CD103+ cells was associated with prolonged tumor free survival (p-value 0.011). Independence could be demonstrated applying Cox proportional hazard analysis (Hazard Ratio 0.742; 95%-Confidence Interval 0.579-0.951; p=0.019). High density of CD8-positive T-lymphocytes identifies a patient subgroup with significantly prolonged overall survival, is correlated with tumor stage and might open up new therapeutic possibilities via immunomodulating drugs.

Key words: Tumor-infiltrating lymphocytes, CD8, CD103, Adenocarcinoma, Esophagogastric junction

Offprint requests to: Dr. Juliana Knief, Department of Pathology, Marienkrankenhais Hamburg, Alfredstrasse 9, 22087, Hamburg, Germany. e-mail: knief.patho@marienkrankenhaus.org

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Introduction

It is commonly thought that evasion of the host's immune system is an important mechanism in carcinogenesis leading to tumor progression, immortalization of tumor cells and metastasis (Dunn et al., 2004). Various previous studies have shown that high proportions of tumor-infiltrating lymphocytes (TILs) – especially cytotoxic T-lymphocytes with CD8-positivity as an indicator of the hosts' anti-tumoral properties (Sato et al., 2005; Sharma et al., 2007; Suemori et al., 2015) – may define patients with a favourable outcome and better prognosis. CD103, an integrin-subunit, is essential for the localization of a subset of CD8-positive (CD8+) T-lymphocytes with memory phenotype (Agace et al., 2000; Sheridan and Lefrancois, 2011) and is commonly found in a variety of tissues, including the gut (Sathaliyawala et al., 2013). CD103 expression is closely related to E-cadherin as one of its most important ligands (Agace et al., 2000) and has been shown to substantially support the host's anti-tumor properties (French et al., 2002; Quinn et al., 2003; Ling et al., 2007). In fact, some studies suggest that CD103 might be an even better and more robust predictor of clinical outcome (compared with other T-cell markers), especially in concert with CD8-positivity (Webb et al., 2014a).

Improved clinical outcome in association with high levels of CD8- and/or CD103-positive T-cells has been shown for epithelial cancers including high grade serous ovarian carcinoma, distinct subtypes of invasive breast carcinoma, urothelial carcinoma, colorectal or lung carcinoma (Ling et al., 2007; Webb et al., 2014b; Djenidi

et al., 2015; Wang et al., 2015, 2016) as well as gastric cancer and intestinal type carcinoma of the cardia (Haas et al., 2009; Solcia et al., 2009; Haas et al., 2011; Kang et al., 2016). However, there are some studies that suggest opposite effects, showing significantly worse outcome in patients with Hodgkin's lymphoma and nasopharyngeal carcinoma with high CD8+ lymphocyte count (Oudejans et al., 1997, 2002).

Recently, we demonstrated that dense infiltration by plasma cells as well as B-lymphocytes in adenocarcinoma of the esophagogastric junction defines subgroups of patients with significantly prolonged overall survival (Knief et al., 2016a). Following these observations, we now additionally assessed density of tumor-infiltrating T-lymphocytes and their relationship with patients' prognosis.

Table 1. Clinicopathologic characteristics of the whole study population (228 patients) including important demographic information.

Characteristic	Total number	Proportion (%)
Sex		
Male	192	84.2
Female	36	15.8
pT (low)		
pT0	5	2.2
pT1a	6	2.6
pT1b	32	14
pT2	41	18
pT (high)		
pT3	120	52.6
pT4a	17	7.5
pT4b	7	3.1
pN		
pN0	88	38.6
pN1	39	17.1
pN2	49	21.5
pN3	52	22.8
Grading		
G1	2	0.9
G2	51	22.4
G3	102	44.7
Not applicable	73	32
Surgical resection		
Complete	204	89.5
Incomplete	23	10.1
Unknown	1	0.4
LVSI		
Present	126	55.3
Absent	102	44.7
Perineural invasion		
Present	54	23.7
Absent	174	76.3
Distant metastases		
Present	50	21.9
Absent	178	78.1
WHO classification		
Tubular	169	74.1
Poorly cohesive	26	11.4
Mucinous	10	4.4
Papillary	2	0.9
Undifferentiated/Other	21	9.2

LVSI, lymphovascular space invasion.

Materials and methods

Selection of cases

Samples of 228 adenocarcinomas of the esophagogastric junction (formalin-fixed, paraffin-embedded tissue of tumors showing their epicentre within 2 cm either side of the junction) which were surgically removed at the University Hospital Schleswig-Holstein, Campus Luebeck during 1992-2014 have been included and analysed in this study. All analyses were approved by the local Ethics Committee beforehand (reference number 14-242A).

Construction of tissue microarrays (TMAs)

In accordance with the manufacturer's instructions, Tissue Microarrays were constructed using the manual QuickRay® kit (Unitma, Seoul, Korea). One representative core per tumor (2 mm diameter) was dissected. Cores were taken from the centre of each tumor. Appropriate positive and negative controls (tonsil, kidney, fat tissue) were included in each slide.

Immunohistochemistry and its evaluation

Immunohistochemical studies were performed using a standard, three-step immunoperoxidase technique and the automated Menarini Bond Max System (Menarini Diagnostics, Berlin, Germany) with the following antibodies: CD8 (Leica Biosystems, Illinois, USA, clone 4B11) and CD103 (abcam, Cambridge, UK, clone EPR4166). Stains were evaluated by two pathologists (JK, CT), in cases where results differed, a consensus was reached. Density of TILs was assessed with the following scoring system for both CD8 and CD103 in analogy to our previous study (Knief et al., 2016a): 0 (no infiltrates); 1+ (scattered cells within tumour tissue); 2+ (dense infiltrates). Examples of the scoring system are shown in Fig. 1. Scoring systems using more complicated methods and specialized equipment, for example manual counting of T-lymphocytes in one or more representative high power fields (Webb et al., 2014b; Wang et al., 2015) or the use of an automated imaging processing software (Haas et al., 2011) were not used as evaluation of tumor-infiltrating lymphocytes – in our study - should reflect tumor evaluation on a daily basis. We therefore sought to use a simple and effective method that is practicable for pathologists around the world and easy to use during routine tumor assessment in analogy to the methods described by Klintrup et al. (2005) and Crumley et al. (2011).

Statistics

Statistical analysis was conducted using SPSS Statistics version 22 (IBM, Ehringen, Germany). Survival differences, outcome, tumor free survival and overall survival were analyzed via Kaplan-Meier estimates (including Log rank-test). Correlation of clinicopathologic parameters and density of T-cell infiltration was determined with χ^2- test. Cox proportional hazard model was applied to test parameters for independence. In multivariate analysis the following parameters were included: age, sex, pT-stage, pN-stage, grading and histologic subtype. Correlation between scoring results for CD8- and CD103-positive cells was determined with Pearson's correlation. A p-value <0.05 was considered to be statistically significant for all tests.

Results

Overall, 228 cases from a previously described patient collective (Knief et al., 2016b) were used for the present study, consisting of 192 males (84.2%) and 36 females (15.8%). For CD8, 225 cases were evaluable (98.7%), for CD103 226 cases could be included (99.1%).

As tumor samples were collected over a longer period of time (1992-2014) some patients received neoadjuvant treatment prior to resection. Neoadjuvant chemotherapy (consisting of a combination therapy using cisplatin and 5-fluoruracil) was administered to 73 patients (32%).

Patient demographics were as follows: Age range 27-83 years with a mean age of 61.82 years (standard deviation 10.59 years). Complete surgical resection could be achieved in 204 patients (89.5%), in 23 patients (10.1%) surgical resection was incomplete. For 1 patient no such information could be obtained (0.4%). Overall, 5 patients (2.2%) showed no vital tumor after neoadjuvant therapy (pT0 stage), 79 patients (34.7%) had localized disease/low tumor stages (pT1 and pT2) and the remaining 144 patients (63.1%) showed

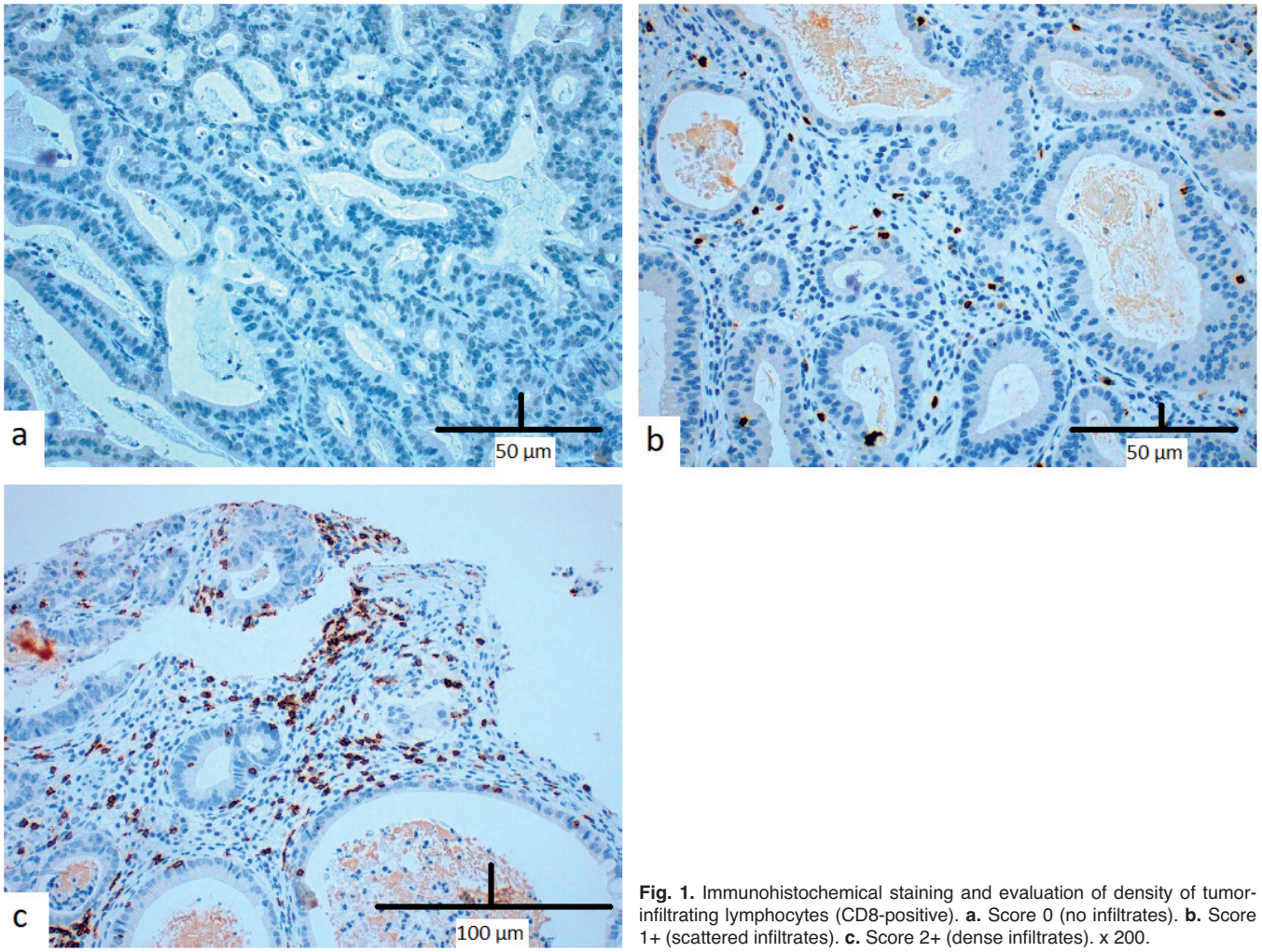

Fig. 1. Immunohistochemical staining and evaluation of density of tumor-infiltrating lymphocytes (CD8-positive). **a.** Score 0 (no infiltrates). **b.** Score 1+ (scattered infiltrates). **c.** Score 2+ (dense infiltrates). x 200.

advanced tumor stages with deep infilttration (pT3 and pT4). Table 1 gives a detailed overview of patient demographics.

CD8 and CD103 cell density

For CD8, 15 cases (6.7%) showed no infiltrates (score 0), 101 cases (44.9%) scattered (score 1+) and 109 cases (48.4%) dense infiltrates (score 2+). Concerning CD103, 29 cases (12.8%) showed no infiltrates (score 0), 121 cases (53.5%) scattered (score 1+) and 76 cases (33.6%) dense infiltrates (score 2+). Significant (inverse) correlation of CD8-density with depth of infiltration (pT-stage; p=0.013) could be shown, while no association with other features (presence or absence of lymphovascular space invasion, perineural infiltration, distant metastases, nodal stage (pN-stage) or histologic tumor type) was present. Concerning CD103+ cells, no significance could be demonstrated for any of the above-mentioned features. Additionally, tumor grading (well-differentiated tumors G1, moderately differentiated tumors G2, poorly differentiated tumors G3) was correlated with density of lymphocytic infiltration. Again, no significant correlation could be shown (p=0.410 for CD8+ T-lymphocytes and p=0.754 for CD103+ lymphocytes, respectively). Results are depicted in Tables 2 and 3, respectively. Correlation between CD8- and CD103-density by Pearson's test revealed only a moderate effect with a correlation coefficient of 0.489.

Correlation with overall survival

Median survival in all patients was 28.14 months, 1-year, 3-year snd 5-year survival 69%, 44% and 37%.

Table 2. Clinicopathologic characteristics of adenocarcinoma of the esophagogastric junction according to density of CD8+ tumor-infiltrating T-lymphocytes.

Characteristic	Score 0	Score 1+	Score 2+	p-value
Total n	15	101	109	
pT (low)				
pT0	0	2	3	**0.013**
pT1a	0	5	1	
pT1b	2	11	19	
pT2	0	13	28	
pT (high)				
pT3	13	60	45	
pT4a	0	5	11	
pT4b	0	5	2	
pN				
pN0	4	38	46	0.495
pN1	4	14	21	
pN2	2	22	22	
pN3	5	27	20	
Grading				
G1	0	1	1	0.410
G2	2	24	25	
G3	12	46	42	
LVSI				
Present	11	58	54	0.156
Absent	4	42	55	
Perineural invasion				
Present	5	23	25	0.659
Absent	10	77	84	
Distant metastases				
Present	5	19	29	0.270
Absent	10	82	80	
WHO classification				
Tubular	12	80	76	0.664
Poorly cohesive1	11	13		
Mucinous	0	3	6	
Papillary	0	0	2	
Undifferentiated/Other	2	7	12	

Bold lettering in p-values indicates a statistically significant difference. LVSI, lymphovascular space invasion.

Table 3. Clinicopathologic characteristics of adenocarcinoma of the esophagogastric junction according to density of CD103+ tumor-infiltrating T-lymphocytes.

Characteristic	Score 0	Score 1+	Score 2+	p-value
Total n	29	121	76	
pT (low)				
pT0	1	3	1	0.666
pT1a	1	5	0	
pT1b	2	19	11	
pT2	4	20	16	
pT (high)				
pT3	17	63	39	
pT4a	3	6	8	
pT4b	1	5	1	
pN				
pN0	12	41	34	0.735
pN1	5	20	13	
pN2	5	31	13	
pN3	7	29	16	
Grading				
G1	0	1	1	0.754
G2	6	26	19	
G3	15	53	34	
LVSI				
Present	16	72	37	0.379
Absent	13	49	38	
Perineural invasion				
Present	7	28	19	0.941
Absent	22	93	56	
Distant metastases				
Present	11	30	13	0.077
Absent	18	91	63	
WHO classification				
Tubular	21	91	55	0.879
Poorly cohesive	3	14	19	
Mucinous	1	7	2	
Papillary	0	1	1	
Undifferentiated/Other	4	8	9	

LVSI, lymphovascular space invasion.

Applying Kaplan-Meier estimates revealed that denser infiltration by CD8+ T-lymphocytes was associated with significantly longer overall survival when using all cases, irrespective of surgical resection status (88.32 months ±10.1 months for score 2+ vs. 55.93 months ±17.86 months for score 0; p=0.024). 1-year, 3-year and 5-year survival in patients with dense CD8+ infiltrates was 74%, 53% and 46%; for patients without tumor-infiltrating lymphocytes 53%, 47% and 27%, respectively. In addition, further analysis of patients using only cases with complete surgical resection (R0-status), also showed a strong trend towards prolonged overall survival (p=0.080).

For CD103, no significant correlation with survival could be shown both either using all patients or only those with complete surgical resection (p=0.223 and

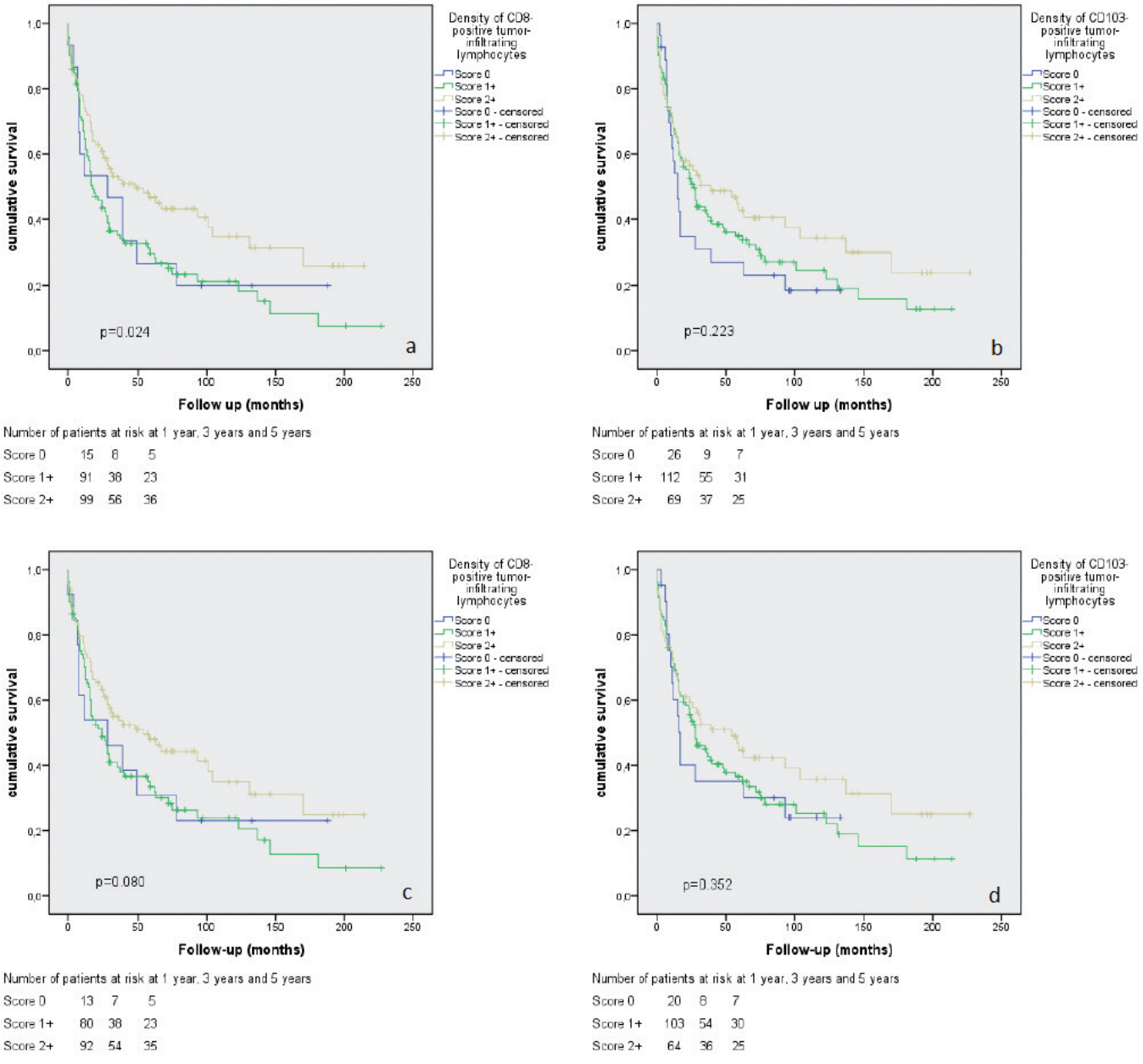

Fig. 2. Kaplan-Meier curves showing overall survival differences in relation to density of tumor-infiltrating T-cells and completeness of surgical resection, including patients at risk at 1 year, 3 years and 5 years. Upper row: Survival curves using all cases irrespective of surgical resection status. Lower row: Survival curves using only cases with complete surgical resection (R0-status). **a.** Assessment for CD8 using scores 0, 1+ and 2+ with a p-value of 0.024. **b.** Assessment for CD103 using scores 0, 1+ and 2+ with a p-value of 0.223. **c.** Assessment for CD8 using scores 0, 1+ and 2+ with a p-value of 0.080. **d.** Assessment for CD103 using scores 0, 1+ and 2+ with a p-value of 0.352.

p=0.352), although survival curves as well as survival times hint at prolonged overall survival for patients with denser infiltration. 1-year, 3-year and 5-year survival in patients with dense CD103+ infiltrates was 70%, 50% and 43%; for patients without tumor-infiltrating lymphocytes 62%, 31% and 27%, respectively. Appropriate survival curves and times as well as patients at risk are depicted in Fig. 2 and Table 4. In addition, subgroup analysis only including patients with tubular adenocarcinoma and excluding all poorly cohesive cases could show no survival benefit for patients with dense infiltration by CD103+ cells (p=0.257; graph not shown).

Correlation with tumor free survival

Additionally, tumor free survival was analyzed. 78 patients (34.2%) showed tumor recurrence during the observation period, 150 patients (65.8%) remained tumor free. Mean time to tumor recurrence was 16.26 months (median 12 months; range 1 - 96 months).

Denser infiltration by CD103+ T-lymphocytes was associated with significantly longer tumor free survival. This held true both when using all patients irrespective of surgical resection status with a p-value of 0.011 (145.42 months ±11.82 months for score 2+ vs. 47.67 months ±10.62 months for score 0) or when analysing

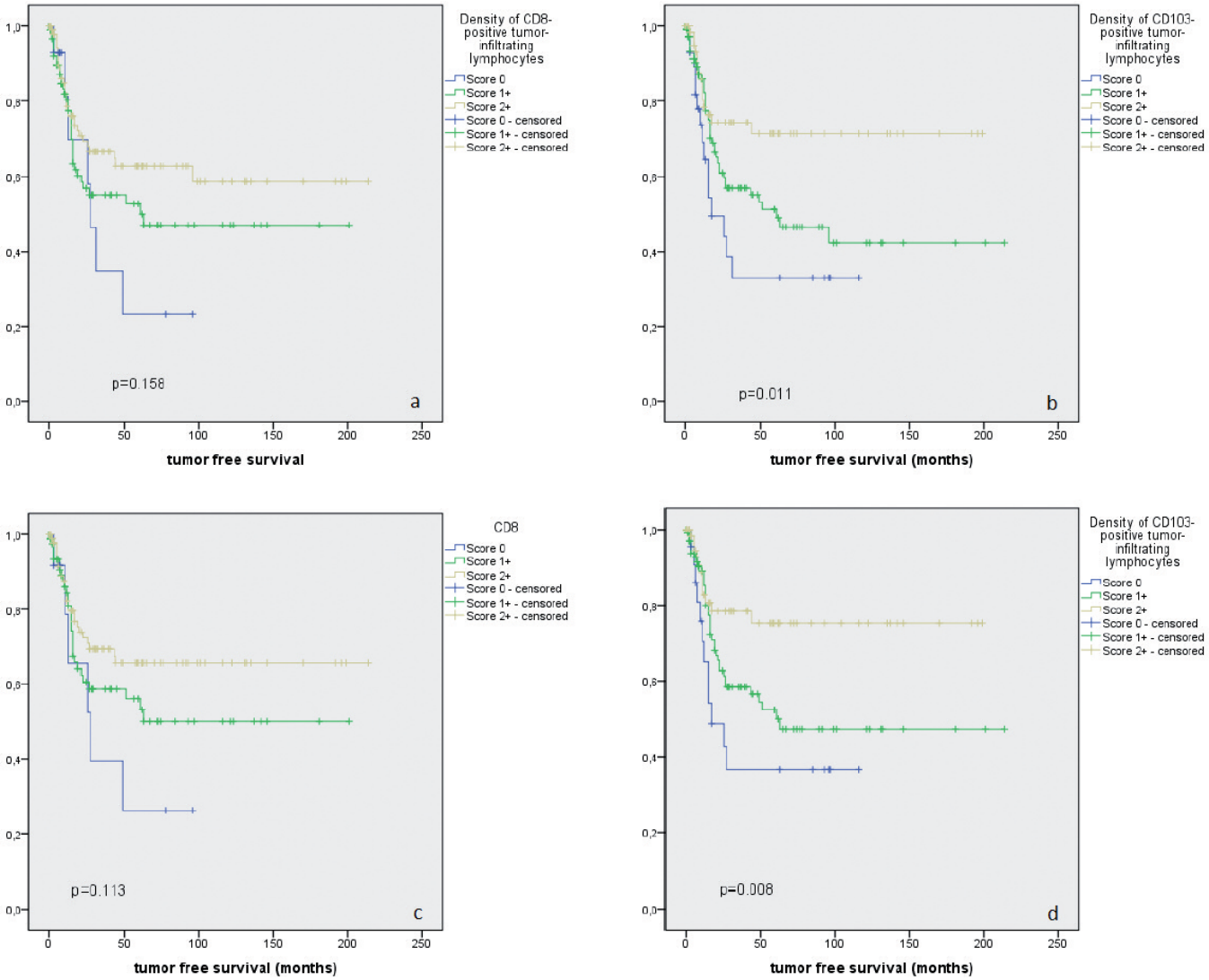

Fig. 3. Kaplan-Meier curves showing tumor free survival in relation to density of tumor-infiltrating T-cells and completeness of surgical resection. Upper row: Survival curves using all cases irrespective of surgical resection status. Lower row: Survival curves using only cases with complete surgical resection (R0-status). **a.** Assessment for CD8 using scores 0, 1+ and 2+ with a p-value of 0.158. **b.** Assessment for CD103 using scores 0, 1+ and 2+ with a p-value of 0.011. **c.** Assessment for CD8 using scores 0, 1+ and 2+ with a p-value of 0.113. **d.** Assessment for CD103 using scores 0, 1+ and 2+ with a p-value of 0.008.

only patients with complete surgical resection (R0-status) with a p-value of 0.008 (153.23 months ±11.63 months for score 2+ vs. 50.82 months ±11.66 months for score 0).

For CD8, no significant correlation between density of infiltrates and tumor free survival could be shown either when using all patients or only those with complete surgical resection (p=0.158 and p=0.113). Appropriate Kaplan Meier curves and tumor free survival times are shown in Fig. 3 and Table 5.

Multivariate analysis

Cox proportional hazard analysis for CD8-positive TILs confirmed CD8 as an independent prognostic factor with a Hazard Ratio of 0.742 (95%-Confidence Interval 0.579-0.951; p=0.019).

Discussion

While carcinomas of the distal part of the stomach have shown a steady decline in incidence in the past decades, the incidence of adenocarcinomas of the esophagogastric junction has remained stable (Powell and McConkey, 1990; Li-Chang et al., 2015). Novel insights into pathogenetic mechanisms as well as new therapeutic strategies for these patients are desperately needed as the overall prognosis is still poor (Han et al., 2016b). Additionally, studies targeting the microenvironment of tumors might help to find subgroups of patients with favorable pathologic features making the possibility of administration of immunomodulating drugs available in these cases.

In the present study, we assessed the density of T-lymphocytes in the tumor microenvironment and its impact on patients' survival. The presence of TILs and other inflammatory cells in tumor tissue is commonly regarded as evidence of the host's anti-tumor immunity (Haas et al., 2011; Dutta et al., 2012), especially the promotion of cytotoxic (CD8+) T-cells which have been shown to prevent tumor progression via activation of cytokines and various interleukines (Pellegrini et al., 1996) although some studies could not confirm this favourable effect (Zingg et al., 2010). In accordance with previous studies (Haas et al., 2011), our results show that density of TILs (CD8+) decreases with disease progression, and is inversely correlated with depth of tumor infiltration (p=0.013). Further, it has been reported that higher numbers of CD8+ T-cells in the tumor environment lead to improved outcome for patients (Haas et al., 2011; Djenidi et al., 2015; Kang et al., 2016; Noble et al., 2016). This is in line with our results which show statistically longer overall survival in cases with dense infiltrates compared to scattered or no infiltration (average survival times 88.32 months vs. 54.21 months and 55.93 months; p=0.024). This holds true only for CD8+ cells (for which independence could be proven in multivariate analysis) which are often seen as being most strongly associated with patients' prognosis (Sato et al., 2005), while no effect on overall survival could be demonstrated for CD103-positve cells. However, patients with increased numbers of CD103+ T-lymphocytes showed significantly longer tumor free survival with later tumor recurrence (time to recurrence 145.42 months vs. 47.67 months; p=0.011). Using only patients with complete surgical resection for further analysis revealed an even stronger association (p-value 0.008) with a mean time to tumor recurrence of 153.23

Table 4. Relationship between density of TILs and overall survival times. Survival times are given in months with standard deviation (SD), 95% Confidence Interval (95% CI) and corresponding p-values.

	Total (n)	average survival	SD	95% CI	p-value
CD8					
Score 0	15	55.93	17.86	20.93-90.93	**0.024**
Score 1+	101	54.21	8.22	38.11-70.32	
Score 2+	109	88.32	10.10	68.52-108.12	
CD103					
Score 0	29	40.73	9.52	22.07-59.40	0.223
Score 1+	121	63.37	8.02	47.66-79.09	
Score 2+	76	87.15	12.36	62.93-111.38	

Bold lettering indicates a statistically significant difference.

Table 5. Tumor free survival and correlation with density of TILs. Survival times are given in months with standard deviation (SD), 95% Confidence Interval (95% CI) and corresponding p-values.

	Total (n)	average survival	SD	95% CI	p-value
Cases irrespective of surgical resection status:					
CD8					
Score 0	15	40.61	10.93	19.17-62.04	0.158
Score 1+	101	104.71	11.88	81.42-128	
Score 2+	109	135.07	11.66	112.21-157.92	
CD103					
Score 0	29	47.67	10.62	26.86-68.49	**0.011**
Score 1+	121	106.02	11.87	82.76-129.28	
Score 2+	76	145.42	11.85	122.19-168.65	
Cases with complete surgical resection only (R0-status):					
CD8					
Score 0	13	41.76	12.27	17.71-65.82	0.113
Score 1+	82	110.89	12.43	86.54-135.24	
Score 2+	94	145.73	11.24	123.70-167.71	
CD103					
Score 0	22	50.82	11.66	27.98-73.67	**0.008**
Score 1+	104	113.16	11.92	89.79-136.53	
Score 2+	65	153.23	11.63	130.43-176.03	

Bold lettering indicates a statistically significant difference.

months (Score 2+) vs. 50.82 months (Score 0). This is in line with previous studies which report prolonged disease free and recurrence free survival in various tumor entities, such as laryngeal and pulmonary squamous cell carcinoma as well as bladder cancer (Wang et al., 2015; Koh et al., 2017; Mann et al., 2019).

Overall, the implications of the subset of CD103-positive lymphocytes are not as well understood as those concerning CD3- or CD8-positive T-cells but a number of studies suggest a similar role in anticancer immunity, especially concerning tumors derived from epithelium (Quinn et al., 2003; Ling et al., 2007; Webb et al., 2014b; Djenidi et al., 2015; Wang et al., 2015). CD103+ lymphocytes possess a so-called "cytolytic" phenotype and express higher levels of the programmed cell death protein 1 (PD-1), a cell surface receptor acting as T-cell costimulator and promoter of apoptosis (Webb et al., 2014b). Additionally, as E-cadherin is one of the most important ligands for CD103 (Gorfu et al., 2009), we investigated whether there was a correlation between CD103-positivity and survival in tubular adenocarcinomas while excluding cases with histologically confirmed poorly cohesive phenotype, as those cases traditionally show loss of intercellular bridges and lower E-cadherin expression (Han et al., 2016a). Interestingly, no such relationship could be found in our study (p=0.257). It has been discovered that CD103 infiltration shows a great inter-patient variability and that the induction of CD103+ cells inside tumor tissue takes a certain time of residency inside the tumor (Djenidi et al., 2015). Therefore, CD103 infiltration could be seen as a delayed anti-tumor reaction of the host's immune system which might not be discernible in all tumor specimen analyzed. To verify these observations, further analyses of similar patient collectives might be helpful.

In conclusion, our data confirms that TILs (CD8+) are of prognostic value in adenocarcinomas of the esophagogastric junction and define a subgroup with significantly longer overall survival, additionally, we could confirm an association with prolonged tumor free survival concerning CD103+ lymphocytes. However, as the specific underlying mechanisms that contribute to this positive effect are still not completely, further studies targeting these molecules that might provide more profound insights are needed. In the future, new possibilities for application of immunomodulating drugs and therapeutic approaches might open up for a subset of patients boosting and using the patient's own immune system to attack carcinoma cells. To date, there are a few promising therapeutic approaches that have been used in advanced tumor stages, mainly consisting of immune checkpoint inhibitors and chimeric antigen receptor T-cell therapy, that have been reported to lead to prolonged progression free survival and prolonged survival even in patients with widespread disease. However, data concerning adenocarcinomas of the esophagogastric junction are still scarce as there are only few studies addressing this issue (Cohen et al., 2018).

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Author contributions. JK and CT performed morphological and immunohistochemical studies, analyzed the data and wrote the manuscript. PLK, UW and RH contributed cases and clinical data and revised the manuscript. All authors read and approved the final version of the manuscript.

Conflict of Interest. The authors declare that they have no conflict of interest.

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