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ORIGINAL RESEARCH

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CD103+ tumor-infiltrating lymphocytes are tumor-reactive intraepithelial CD8+ T cells associated with prognostic benefit and therapy response in cervical cancer

Fenne L. Komdeur[a,†], Thalina M. Prins[a,†], Stephanie van de Wall[b], Annechien Plat[a], G. Bea A. Wisman[a], Harry Hollema[c], Toos Daemen[b], David N. Church[d], Marco de Bruyn[a,††], and Hans W. Nijman[a,††]

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ABSTRACT

Human papilloma virus (HPV)-induced cervical cancer constitutively expresses viral E6/E7 oncoproteins and is an excellent target for T cell-based immunotherapy. However, not all tumor-infiltrating T cells confer equal benefit to patients, with epithelial T cells being superior to stromal T cells.

To assess whether the epithelial T cell biomarker CD103 could specifically discriminate the beneficial antitumor T cells, association of CD103 with clinicopathological variables and outcome was analyzed in the TCGA cervical cancer data set (n = 304) and by immunohistochemistry (IHC) in an independent cohort (n = 460). Localization of CD103+ cells in the tumor was assessed by immunofluorescence. Furthermore, use of CD103 as a response biomarker was assessed in an *in vivo* E6/E7+ tumor model.

Our results show that CD103 gene expression was strongly correlated with cytotoxic T cell markers (e.g. CD8/GZMB/PD1) in the TCGA series. In line with this, CD103+ cells in the IHC series co-expressed CD8 and were preferentially located in cervical tumor epithelium. High CD103+ cell infiltration was strongly associated with an improved prognosis in both series, and appeared to be a better predictor of outcome than CD8. Interestingly, the prognostic benefit of CD103 in both series seemed limited to patients receiving radiotherapy. In a preclinical mouse model, HPV E6/E7-targeted therapeutic vaccination in combination with radiotherapy increased the intratumoral number of CD103+ CD8+ T cells, providing a potential mechanistic basis for our results.

In conclusion, CD103 is a promising marker for rapid assessment of tumor-reactive T cell infiltration of cervical cancers and a promising response biomarker for E6/E7-targeted immunotherapy.

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CD103; cervical cancer; intraepithelial T cells; therapeutic vaccination; tumor-infiltrating lymphocytes

Introduction

Cervical cancer is the most common gynecologic malignancy and the second most common malignancy afflicting women worldwide (globcan). The development of cervical cancer is largely dependent on persistent human papilloma virus (HPV) infections, with HPV16 and 18 being the dominant subtypes.[1,2] As a virally-induced cancer, control of cervical cancer development appears at least partly mediated by the immune system,[3-5] and multiple studies have demonstrated a clear benefit of T cell infiltration on survival in cervical cancer patients.[6-9]

The malignant transformation of cervical epithelial cells by HPVs involves integration of viral oncogenes, such as HPV E6 and E7, into the cellular DNA. Subsequent expression of these HPV E6 and E7 proteins inhibits the tumor suppressors p53 and pRb, respectively, resulting in a loss of cell cycle control, proliferation and malignant transformation. Importantly, sustained expression of E6 and/or E7 is required for maintaining a malignant cellular phenotype in this setting.[10] E6/E7 therefore represent bona fide cancer-specific antigens that can be targeted for cancer immunotherapy. Indeed, T cell-based therapies targeting E6/E7 have met with clinical success in early trials.[11-21] As readout for therapeutic efficacy of these approaches, systemic immune monitoring in the blood is usually used alone, or in combination with monitoring of CD8+ T cell tumor infiltration. Herein, a distinction is frequently made between CD8+ TIL that infiltrate the epithelial cancer nests or TIL that infiltrate the surrounding stroma. This distinction is based on the known need for contact between TIL and cancer cells for efficient induction of cell death, and the observed stronger association of epithelial TIL compared with stromal TIL with regards to patient prognosis.[22] However, this approach relies on distinguishing epithelial from stromal regions, a non-trivial feat in many tumors. The

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identification of a biomarker for identifying tumor-reactive cells would therefore be of substantial benefit.

Recently, we and others have demonstrated that CD103, also known as the αE integrin subunit, delineates prognostically favorable intraepithelial CD8+ tumor-infiltrating lymphocytes (TIL) in endometrial, ovarian, lung and bladder cancer.[23-27] In contrast to the prognostic benefit observed for CD8+ TIL,[28,29] this survival benefit was also evident when quantifying the total number of CD103+ TIL present within the tumor.[23-27] This finding is in line with the proposed restricted expression of CD103 on CD8+ TIL that have infiltrated the tumor epithelium.

The aim of this study was therefore to determine whether expression of CD103 defines the intraepithelial CD8+ TIL in cervical cancer and whether CD103+ TIL are associated with improved prognosis. Further, we explored the mechanistic basis of our findings in a preclinical mouse model and determined whether CD103 infiltration could be used as a response biomarker for therapeutic HPV16 E6/E7-targeted immunotherapy.

Results

Expression of CD103 is an independent prognostic factor in cervical cancer and strongly associated with an immune signature

To investigate the utility of CD103 as a biomarker of an anti-tumor T cell response in cervical cancer, we first analyzed expression of CD103 (ITGAE) mRNA in The Cancer Genome Atlas (TCGA) cervical cancer data set. CD103 gene expression was strongly correlated with the expression of T cell markers (CD3, CD2), exhaustion molecules (PD1, TIGIT), antigen-presenting molecules (HLA-DR, -DQ) and B cell markers (CD19) suggesting that increased CD103 expression defines a group of immunologically "hot" tumors in this cervical cancer cohort (Fig. 1A). High CD103 expression (>median) was associated with younger patient age (49.9 vs. 46.5 years, $P = 0.03$, t-test) and squamous histology ($P = 0.026$, Fisher exact test), though no association with disease stage, tumor differentiation or treatment use was observed (Supplementary Table 1). Notably, CD103 expression greater than the median was associated with significantly improved cancer-specific survival both in univariable analysis (Fig. 1B; HR = 0.56, 95%CI = 0.34–0.92, P = 0.02) and after adjusting for disease stage in multivariable analysis (HR = 0.55, 95%CI = 0.32–0.94, P = 0.03) (Supplementary Table 2). By contrast, increased expression of CD8A was not significantly associated with cancer-specific survival in this population (Supplementary Table 2). Exploratory analysis according to treatment modality (surgery vs. radio(chemo)therapy) suggested that the prognostic benefit of increased CD103 expression was observed in patients treated with radiotherapy, but not in patients treated with surgery alone (Fig. 1C (p = 0.015) and 1D (p = 0.47), respectively).

CD103+ TIL are associated with prolonged disease-specific and disease-free survival in cervical cancer patients

To validate our findings from the TCGA data set, we analyzed infiltration of CD103+ cells by immunohistochemistry (IHC) in an independent cohort of 630 cervical cancer patients. Patients were included for quantification of CD103+ TIL if the tissue microarray (TMA) used contained at least 2 cores with a minimum of 20% tumor. Representative tumor cores were available from 460 patients. Patient and tumor characteristics did not differ between analyzed and excluded patients (data not shown). Table 1 shows the patient and tumor characteristics of the patients eligible for CD103 quantification. Of the 460 included patients, 123 were treated with surgery alone and 337 were treated with radio(chemo)therapy (R(C)T) (alone or in combination with surgery). The surgery cohort consisted of patients diagnosed with Fédération Internationale de Gynécologie Obstétrique (FIGO) stages IB1-IIA. The R(C)T cohort consisted of patients diagnosed with FIGO stages IB1-IVA. The majority of patients in the surgery cohort were diagnosed with FIGO IB1 (n = 86; 69.9%) and the majority of patients in the R(C)T cohort were diagnosed with FIGO stage IIB (n = 112; 33.2%). Of the surgery and R(C)T cohort, 64.2% (n = 79) and 78.9% (n = 266) of tumors were squamous cell carcinomas (SCC) and 17.9% (n = 22) and 13.1% (n = 44) were adenocarcinomas (AC), respectively. The median follow-up time was 5.12 y with a maximum of 21.31 years. Positive staining for CD103+ TIL was equally present in SCC, AC and other subtypes (Supplementary Figure S1A). Interestingly, the median infiltration of CD103+ cells in patients that received radio(chemo)therapy was significantly lower than for patients that received surgery alone (Table 1; median surgery 55 vs. 24 R(C)T; $p < 0.0001$). Further, within the R(C)T cohort, patients with a higher FIGO stage were characterized by a lower number of CD103+ cells (Table 1; median 38 in IB1 vs. 20 in IIB and 11 in IIIB; $p < 0.05$ and $p < 0.01$, respectively). Likewise, adenocarcinomas in the R(C)T cohort were infiltrated less than squamous cell carcinomas (Table 1; median 25 vs. 13; $p < 0.05$). To analyze survival, patients were dichotomized based on high or low/no infiltration and the cohorts treated with either surgery or radio(chemo)therapy were analyzed together or separately. The cut-off was determined based on median CD103+TIL infiltration of the total cohort and was 29 cells/mm^2. Disease-specific survival (DSS) analysis based on infiltration of CD103+ cells revealed a significant improved survival in the total cohort (Fig. 2A; $p < 0.0001$), a nonsignificant improvement of survival in the cohort treated with surgery only (Fig. 2B; p = 0.9947) and a significant improvement of survival in the radio(chemo)therapy cohort (Fig. 2C; p = 0.0032). Similar results were obtained when determining disease-free survival (Fig. 2D–E; p = 0.0004 for the total cohort, p = 0.7350 for surgery alone, and p = 0.0072 for R(C)T). In analysis of the total cohort, additional prognostic factors were stage (HR = 4.19, $p < 0.001$), use of radio(chemo)therapy (HR = 1.49, $p < 0.001$) and tumor diameter (HR = 2.9; $p < 0.001$) (Supplementary Table 3). In multivariate analysis, stage (HR = 2.43, $p < 0.006$), use of radio(chemo)therapy (HR = 1.30, $p < 0.001$) and CD103+ cells (HR = 0.67, $p < 0.027$) were independent prognostic factors (Supplementary Table 3).

CD103 demarcates intraepithelial CD8+ TIL in cervical cancer

To investigate the localization and the phenotype of CD103+ TIL in cervical cancer, 18 tumors containing high levels of CD103+ TIL were selected, and tumor sections were stained for

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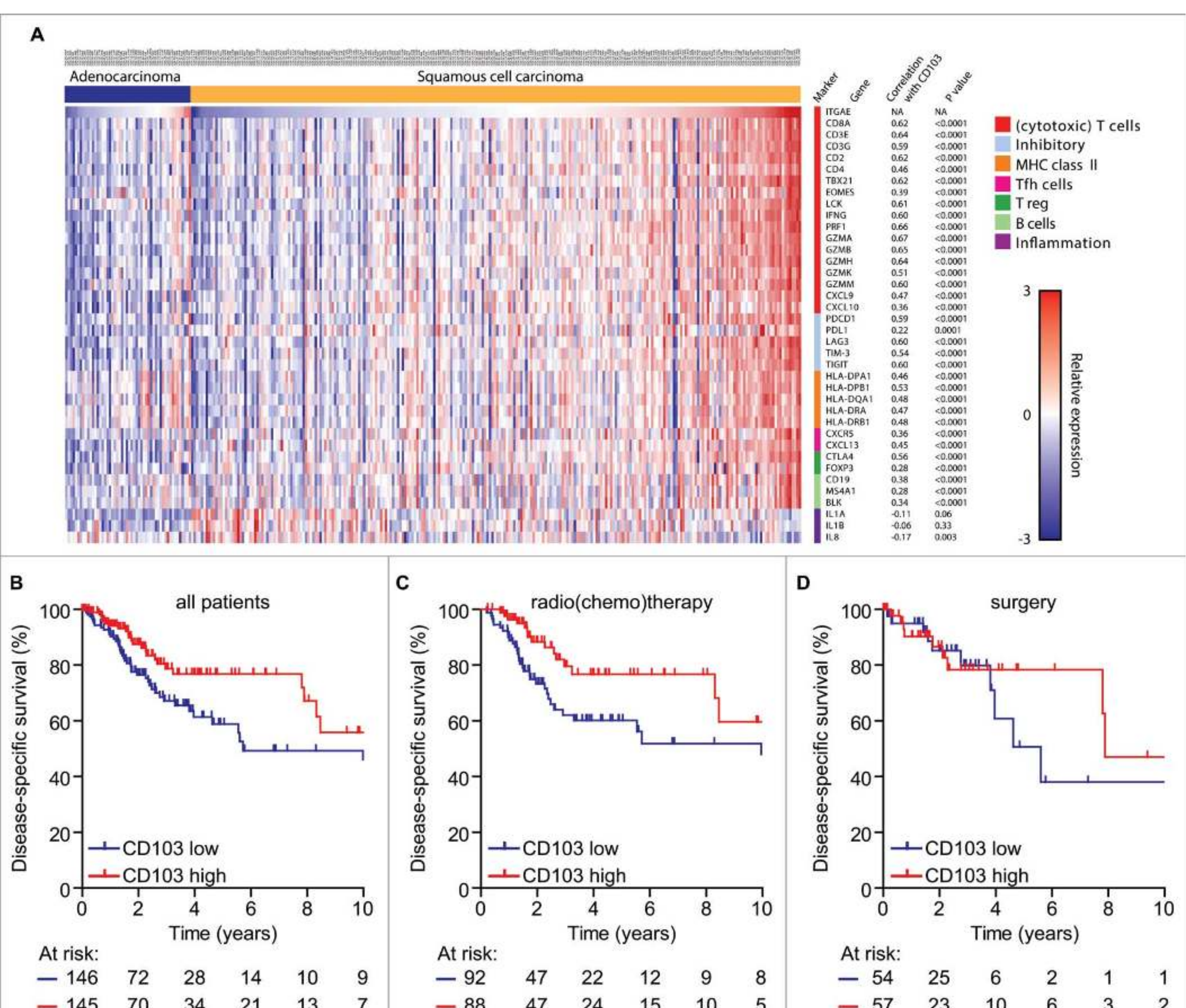

Figure 1. CD103-associated immune responses and clinical outcome in TCGA cervical cancers. A) Heatmap showing expression of immunologic genes according to tumor histology and ordered by CD103 (ITGAE) expression. RSEM-normalized RNAseq expression data were log2 transformed, mean centered and assigned unit variance. For each gene, the correlation with CD103 expression was calculated by spearman rho. B-D) Kaplan–Meier curves demonstrating cancer survival of patients in the TCGA series dichotomized by median CD103 (ITGAE) expression for the total cohort (B) and according to radiotherapy treatment (C, D) (note that survival data were not available for 13 cases). Comparison between groups was made by the 2-sided log-rank test.

CD3, CD8, FoxP3, NKp46, fibronectin, DAPI, and CD103. For each section, cell infiltration was quantified for at least three independent regions. When examining the localization of the TIL we noticed different patterns of stromal infiltration into the epithelial areas previously classified as 'pushing' tumors and 'desmoplastic' tumors.[30] Due to their distinctive nature, both types of tumors were subsequently analyzed separately (Fig. 3A).

Fluorescent staining of the pushing tumor type (n = 12) showed that CD103+ TIL were preferentially localized within the tumor epithelium and not within the tumor stroma (Fig. 3A). Furthermore, these intraepithelial CD103+ TIL largely co-expressed CD8 (Fig. 3B). A subset of CD103+ TIL in the pushing tumor type did not express CD8 (Fig. 3C–D). Further analysis of these CD8- CD103+ TIL showed that these cells did express CD3 and could therefore represent CD4+ regulatory T cells (Treg) or natural Killer T cells (NKT) (Supplementary Fig. 2). Interestingly, the CD3+ CD8- CD103+ TIL did not express NKP46 or FoxP3 (Supplementary Figs. 3 and 4, respectively) suggesting a CD3+ CD4+ non-Treg phenotype.

Within the desmoplastic tumor type (n = 6), a distinct selection of stromal versus epithelial areas could not be made (Fig. 3A–B). Nevertheless, the desmoplastic tumors contained an even higher percentage of CD8+ CD103+TIL (Fig. 3D). By contrast, single CD8+ or CD103+ cells could barely be detected in these tumors. In healthy cervical tissue, no CD8+ CD103+ cells were detected (Fig. 3A), but epithelial CD8+ CD103- cells and a small number of stromal CD8- CD103+ cells were found. Untransformed stromal cervical tissue surrounding the pushing tumor types was frequently rich in CD8- CD103- cells that expressed NKp46 (data not shown). Taken together, these data demonstrate that CD103+ cells in cervical cancer tissue are predominantly CD8+ T cells, with a minor fraction of CD4+ non-Treg cells. By contrast, CD103+ T cells are largely absent from

Table 1. Patient characteristics of the IHC cohort.

Variables $N = 460$	Surgery *n* (%)	CD103 median (range)	(chemo-) RT *n* (%)	CD103 median (range)	Total *n* (%)	CD103 median (range)
Patients	123 (26.7)	55 (1–367)	337 (73.3)	24 (0–256)****	460 (100)	29 (0–367)
Age at diagnosis (in years)						
Median	41.2		50.7		47.7	
Range	(24.4–84.7)		(20.6–92.0)		(20.6–92.0)	
FIGO stage						
IA2	0 (0)		0 (0)		0 (0)	
IB1	86 (69.9)	52 (1–367)	77 (22.8)	38 (0–256)	163 (35.4)	50 (0–367)
IB2	20 (16.3)	83 (7–286)	50 (14.8)	23 (2–204)	70 (15.2)	31 (2–286)
IIA	17 (13.8)	80 (10–203)	60 (17.8)	23 (0–215)	77 (16.7)	29 (0–215)
IIB	0 (0)		112 (33.2)	20 (1–150)	121 (24.3)	20 (1–150)
IIIA	0 (0)		4 (1.2)	16 (5–34)	4 (0.9)	16 (5–34)
IIIB	0 (0)		28 (8.3)	11 (0–115)	28 (6.1)	11 (0–115)
IVA	0 (0)		6 (21.8)	16 (5–43)	6 (1.3)	16 (5–43)
Histology						
Squamous cell carcinoma	79 (64.2)	82 (7–367)	266 (78.9)	25 (1–215)	345 (75.0)	30 (1–367)
Adenocarcinoma	22 (17.9)	53 (6–246)	44 (13.1)	13 (0–256)	66 (14.3)	16 (0–256)
Other	22 (17.9)	33 (1–186)	27 (8.0)	36 (4–199)	49 (10.7)	36 (1–286)
Grade of differentiation						
Good/moderate	69 (56.1)	55 (1–367)	190 (56.4)	24 (0–215)	259 (56.3)	29 (0–367)
Poor/undifferentiated	51 (41.5)	83 (3–303)	129 (38.3)	26 (0–256)	180 (39.1)	33 (0–304)
Unknown	3 (2.4)	52 (6–53)	18 (5.3)	14 (3–120)	21 (4.6)	16 (3–120)
Lymphangioinvasion						
No	74 (60.2)	56 (5–367)	173 (51.3)	22 (0–216)	247 (53.7)	28 (3–367)
Yes	49 (39.8)	55 (1–304)	105 (31.2)	35 (2–256)	154 (33.5)	38 (1–304)
Unknown	0 (0)		59 (17.5)	16 (0–128)	59 (12.8)	16 (0–128)
Tumor diameter						
0–4 cm	97 (78.9)	52 (1–367)	118 (35.0)	36 (0–256)	215 (46.7)	40 (0–367)
$\geq$ 4 cm	26 (21.1)	86 (6–286)	203 (60.2)	18 (0–204)	229 (49.9)	22 (0–286)
Unknown	0 (0)		16 (4.7)	33 (3–128)	16 (3.5)	33 (3–128)
Treatment						
WM	123 (100)	55 (1–367)				
WM+ post operative RT			83 (24.6)	42 (2–256)		
WM+ Post operative RCT			14 (4.2))	33 (2–84)		
Primary RT			115 (34.1)	22 (0–198)		
Primary RCT			125 (37.1)	16 (0–133)		
Follow-up (in years)						
Median	5.62		4.81		5.12	
Range	(0.53–16.93)		(0.14–21.31)		(0.14–21.31)	
Result last follow-up						
No evidence of disease	109 (88.6)	77 (3–367)	168 (49.9)	29 (0–216)	227 (60.2)	38 (0–367)
Evidence of disease	2 (1.6)	92 (1–184)	2 (0.6)	52 (3–102)	4 (0.9)	52 (1–184)
Death of other disease	0 (0)		33 (9.8)	24 (0–215)	33 (7.2)	24 (0–215)
Death of disease	12 (9.8)	42 (7–170)	134 (39.8)	15 (0–256)	146 (31.7)	17 (0–256)

Abbreviations: FIGO: International Federation of Gynecologists and Obstetricians
WM: Wertheim Meigs RT: Radiotherapy RCT: Radio-chemotherapy

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untransformed epithelium and stroma. In tumor-adjacent stroma, mainly CD103- NK cells are present.

CD103+ TIL in situ are characterized by ongoing TGFbR1-signaling

We and others have demonstrated that CD103 is upregulated on T cells following concomitant T cell and transforming growth factor (TGF)-β receptor (TGFβR) signaling.[31-35] Indeed, CD103+, but not CD103-, TIL in high-grade serous ovarian cancer are characterized by nuclear phosphorylated mothers against decapentaplegic homolog 2 and 3 (pSMAD2/3) expression, a hallmark of TGF-β signaling. To confirm signs of active TGF-β signaling in CD103+ TIL from SCC, paraffin-embedded tissue was probed by fluorescent microscopy for simultaneous expression of CD8, CD103 and nuclear pSMAD2/3. SCC tumor islets, the surrounding stroma cells, and CD103- and CD103+ TIL were all characterized by a pronounced nuclear expression of pSMAD2/3 (Fig. 4) suggesting TGFβR1-signaling is highly active in the cervical cancer microenvironment, but not restricted to CD103+ TIL. In healthy cervical tissue, pSMAD2/3 signaling was also abundant in epithelial, stromal, CD8+ and CD103+ cells (Supplementary Fig. 5).

Anti-tumor therapeutic efficacy is mediated by recruitment of CD103+ TIL in vivo

Finally, to determine whether CD103 could also be used as a response biomarker for immunotherapy targeting E6 and E7, we used the E6/E7-transformed TC1 mouse model.[36] TC-1 cells are derived from primary epithelial cells of C57BL/6 mice co-transformed with HPV-16 E6 and E7 and c-Ha-ras oncogenes. These cells form tumors composed largely of epithelial cells after subcutaneous injection and should therefore induce CD103 on infiltrating CD8+ T cells. Based on the differential prognostic effects of radiotherapy observed in both the TCGA and IHC series, we also assessed whether radiotherapy synergized with E6/E7-specific antitumor immune responses *in vivo* using our previously published experimental setup[36] (Fig. 5A).

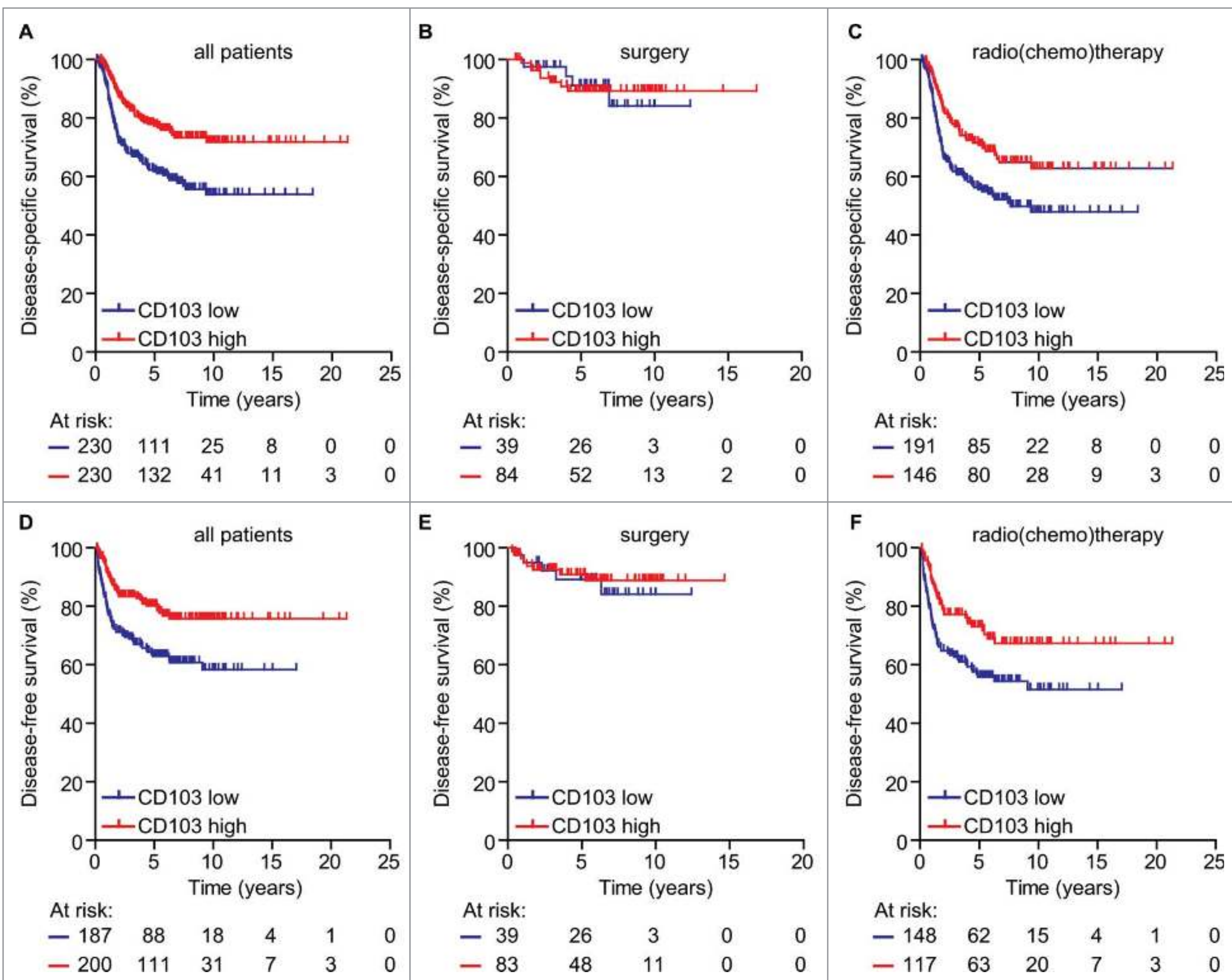

Figure 2. CD103+ TIL are strongly associated with survival in patients with cervical cancer. A) Disease-specific survival (DSS) of patients within the total cohort according to high or low infiltration of CD103+ cells ($p < 0.0001$). B) DSS of patients treated with surgery alone with a high or low infiltration of CD103+ cells. C) DSS of patients treated with radio(chemo)therapy and either a high or low infiltration of CD103+ cells. D) Disease-free survival (DFS) of patients within the total cohort according to high or low infiltration of CD103+ cells ($p = 0.0004$). E) DFS of patients treated with surgery alone with a high or low infiltration of CD103+ cells. F) DFS of patients treated with radio(chemo)therapy and either a high or low infiltration of CD103+ cells. Comparison between groups was made by the 2-sided log-rank test.

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In brief, female C57BL/6 mice were challenged with TC1 tumors and treated with a suboptimal immunization regimen of 5×10^6 i.u. semliki forest virus (SFV)eE6,7 immunization 14 days after tumor inoculation with or without radiation. At this dose, immunization alone is insufficient at inducing tumor eradication and synergizes with ionizing radiation. After 22 days mice were killed, tumors were measured and digested.

For flow cytometric analysis, TC1 tumor digests were gated on lymphocyte singlets and subsequently on DAPI- live cells (Fig. 5B). Within the TC1 tumor digests, untreated mice showed ~10% CD8+ CD103+ cells (Fig. 5C–D). Therapeutic SFVeE6/E7 vaccination increased the intratumoral number of CD8+ CD103+ T cells to ~25%, an effect that further synergized with concomitant irradiation to ~60% (representative plots in Fig. 5C). Irradiation alone resulted in a ~10% CD8+ CD103+ T cell infiltration (Fig 5C–D). Within all treatment groups, and independent of the number of infiltrating cells, CD103+ cells were almost exclusively CD8+ T cells (Fig. 5C). As expected, the percentage of infiltrating CD8+ CD103+ T cells across all treatment groups was negatively correlated with tumor weight (Fig. 5E; $R^2 = 0.53$ $p = 0.008$). Finally, analysis of E7-reactive T cells using E7 H-2Kb dextramer staining revealed E7-specificity to be largely restricted to the CD103+ T cell population (Fig. 5F).

Discussion

In the present study we demonstrate that infiltrating CD103+ T cells are a prognostic factor for survival in cervical cancer patients. By gene expression analysis on tumor samples from cervical cancer patients available within the TCGA data set, we showed that expression of *ITGAE*, the gene encoding for CD103, correlates with significantly improved survival. This prognostic benefit of CD103-expressing T cells was confirmed in an independent cohort of 460 cervical cancer patients by immunohistochemical analysis of CD103+ TIL in FFPE-tumor cores. Furthermore, we show that CD103 is a marker for intraepithelial CD8+ T cells in cervical cancer. Finally, we demonstrate that CD103 holds considerable promise as both a predictive and response biomarker for radiotherapy and/or E6/E7-targeted immunotherapy.

Our results in the cervical cancer cohorts are in line with earlier findings on the localization and prognostic influence of CD103+ TIL in endometrial, ovarian, bladder and lung cancer.

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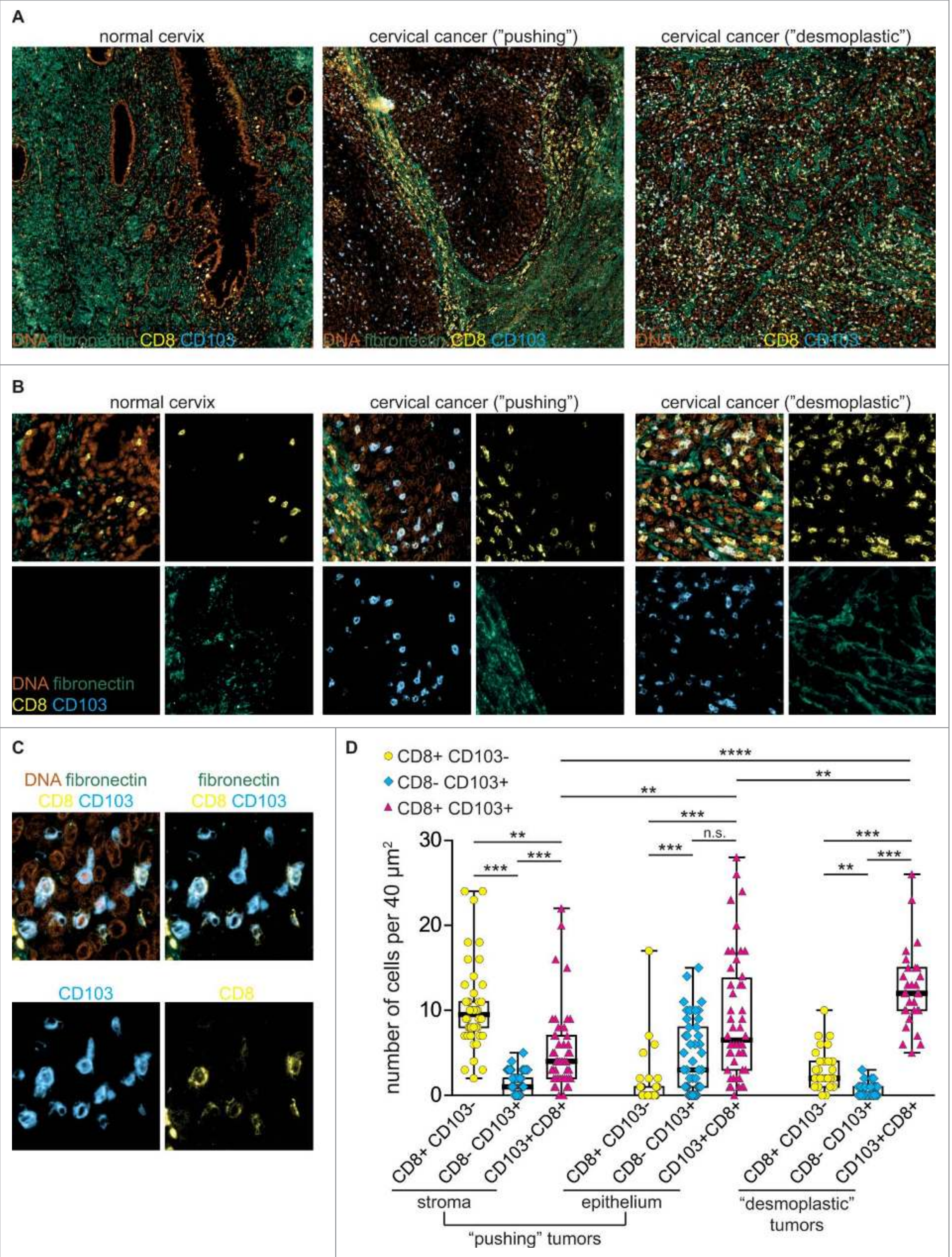

Figure 3. CD103 demarcates intraepithelial CD8+ TIL in cervical cancer tissue. A) Representative image of tissue from a normal cervix, from a patient with cervical cancer of the "pushing" type and of a patient with cervical cancer of the "desmoplastic" type stained with DAPI (DNA, orange), anti-CD8 (yellow), anti-CD103 (blue) and anti-fibronectin (green) antibodies. B) Representative images of CD8+ and CD103+ cells in the epithelial or stromal areas of 40 μm^2 of tumor tissue. C) Representative single and multichannel images of tumor areas showing co-expression of CD8 and CD103. D) Quantification of single CD8+, single CD103+ or CD8+ CD103+ double-positive cells in the stroma and epithelial areas of the "pushing" tumors or total of the "desmoplastic" tumors. Each data point represents a cell count from a 40μm^2 independent region of 18 independent tumors (3–6 in total per tumor section). Groups were compared by ANOVA using a Dunns post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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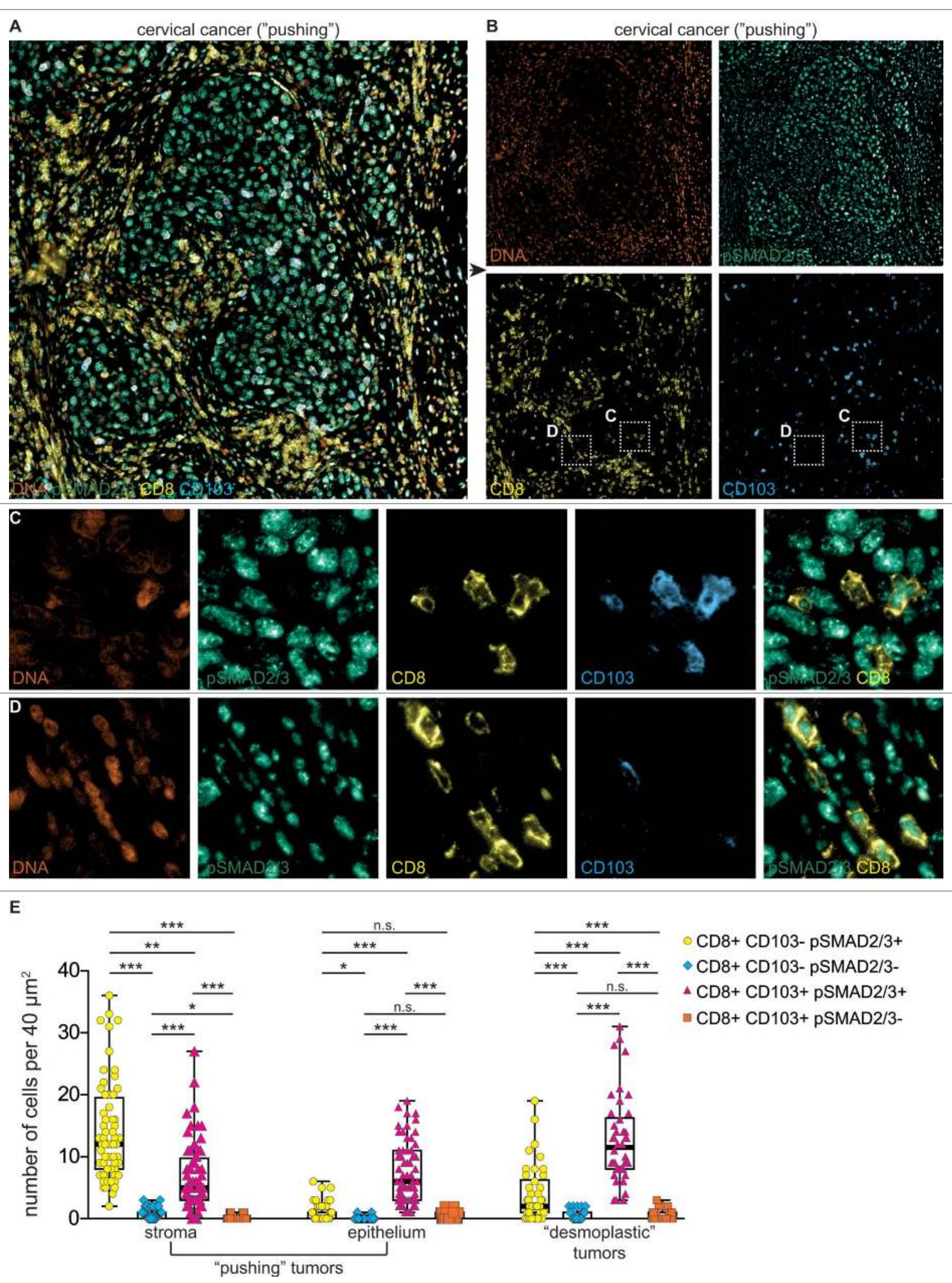

Figure 4. TGF-β signaling is abundant in cervical cancer tissue. A) Representative image of tissue from a patient with cervical cancer of the "pushing" type stained with DAPI (DNA, orange), anti-CD8 (yellow), anti-CD103 (blue) and anti-pSMAD2/3 (green) antibodies. B) Representative single and multichannel images of the tumor area from A showing predominant localization of CD8+ cells in the pSMAD2/3+ stromal region and CD8+ CD103+ cells in the pSMAD2/3+ epithelial region. Insets represent areas magnified in panels C and D. C-D) Representative images of CD8+ and CD103+ cells in magnified epithelial (C) or stromal areas (D) of tumor tissue as indicated by insets in B. E) Quantification of CD8+, CD103+ and/or pSMAD2/3+ cells in the stroma and epithelial areas of the "pushing" tumors or total of the "desmoplastic" tumors. Each data point represents a cell count from a 40μm^2 independent region of 18 independent tumors (3–6 in total per tumor section). Groups were compared by ANOVA using a Dunns post-test. *p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.001.

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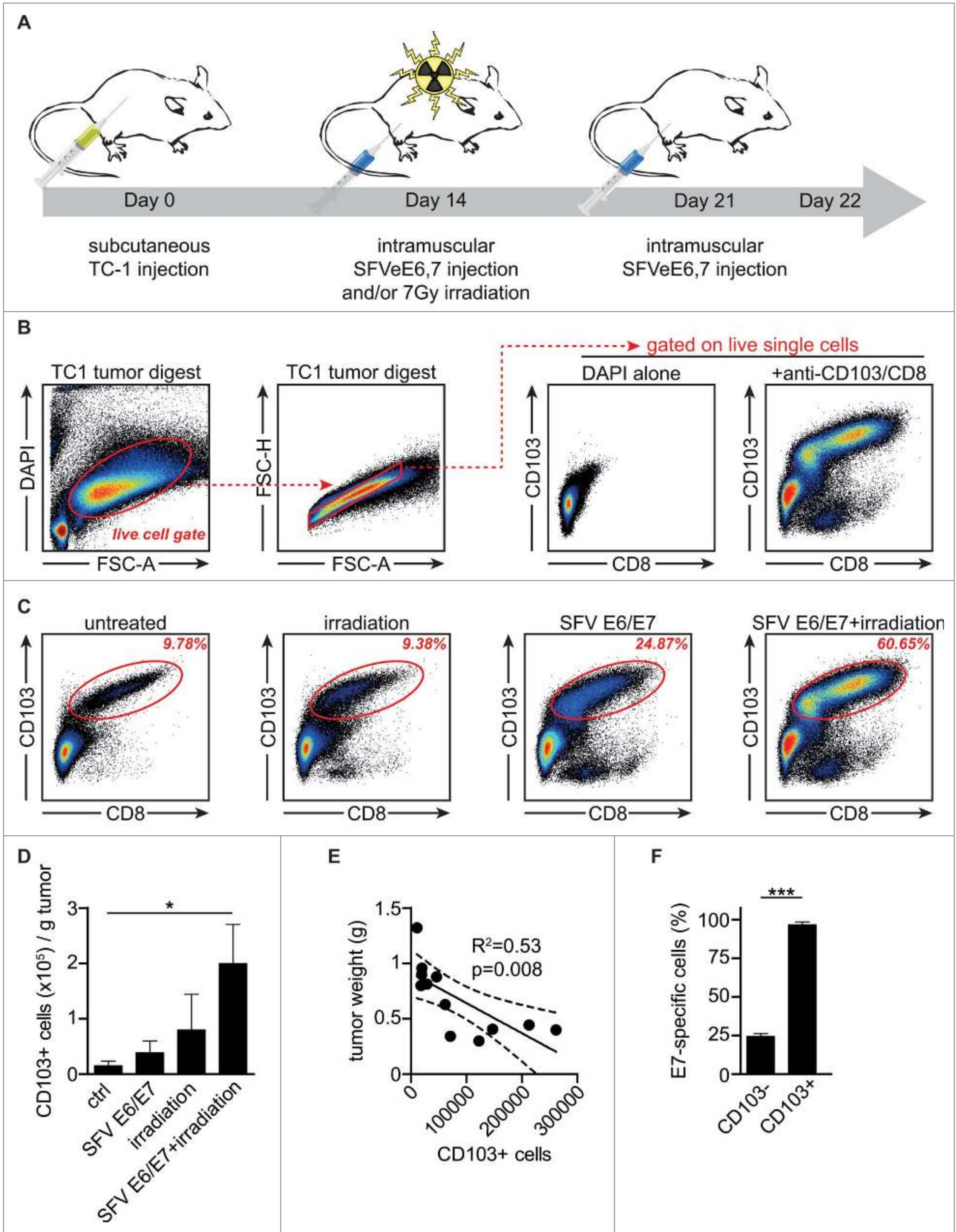

Figure 5. Combination immunotherapy targeting HPV E6 and E7 induces accumulation of CD103+ cells *in vivo*. A) Schematic depiction of the TC1 mouse model. B) Representative flow cytometric plot of a TC1 tumor digest analyzed for expression of CD103 and CD8 within the DAPI-negative live cell population. C) Representative flow cytometric plots of TC1 tumor digests from untreated mice or mice treated with irradiation, a low dose of SFV E6/E7 vaccine, or both analyzed for expression of CD8 and CD103 within the DAPI-negative live cell population. D) Bar graphs representing the absolute number of CD103+ cells per gram of tumor of the experimental groups (n = 3–6). E) Scatter plots representing the number of CD103+ cells per gram of tumor across all groups (n = 3–6). F) Percentage of E7-specific CD8+ T cells across all treatment groups. $^{*}p < 0.05$.

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However, in contrast to other malignancies, infiltration of cervical cancers was related to the type of treatment patients received and the stage of disease. Patients that were treated with radio (chemo)therapy alone or in combination with surgery had fewer infiltrating CD103+ TIL compared with patients that qualified for surgical treatment alone. Moreover, within the radio(chemo)therapy group, patients that presented with a higher stage of disease were characterized by even lower numbers of CD103+ TIL when compared with patients with lower FIGO stages. This strongly suggests an interference of T cells and tumor cells where an equilibrium is reached in the early stages of disease, whereas larger tumors have escaped immune control by T cells and advanced stages of the disease are able to develop.[37] As a result, patients with immunological 'hot' tumors generally present with an early stage of disease, whereas patients with immunological 'cold' tumors show a more aggressive disease with indications for primary (locally advanced disease) or adjuvant radio(chemo)therapy treatment (e.g., positive resection margins after surgery or positive lymph nodes).

In addition to the reduced number of infiltrating cells in clinically more aggressive cancers, analysis of the TCGA cervical cancer data set shows that *ITGAE* expression is not only strongly associated with the common T cell genes such as CD8A, but more importantly also with T cell activation and exhaustion markers such as CD137, CTLA4, PD1, and PDL1. This suggests that these patients may be candidates for additional adjuvant therapy with immune checkpoint inhibitors such as antibodies targeting CTLA-4 (ipilimumab) or PD-1 (nivolumab/pembroluzimab). In patients with melanoma and non-small cell lung cancer in particular, immune checkpoint inhibitors have met with considerable clinical success. In these malignancies, responses to immune checkpoint inhibitors have been strongly linked to the presence of neo-antigens in cancer cells (particularly those expressed across lesions) that provide a true tumor-specific target for T cells for which tolerance has likely not been established.[38,39] In cervical cancer, the constitutive expression and the viral nature of E6/E7 oncoproteins in malignant cells is likely to provide a similar strong and non-tolerant target for T cell recognition that may be exploited with immune checkpoint inhibitors.

One caveat herein may be the poor infiltration of these tumors by immune cells. Indeed, as discussed above, aggressive tumors at higher stages of the disease show relatively poor infiltration by CD103+ TIL that may preclude effective responses to checkpoint inhibition. In one melanoma trial, a high number of preexisting T cells was a determinant for subsequent responses to therapy with anti-PD1 antibody pembroluzimab. In the absence of a strong T cell response, additional therapeutic strategies may therefore be required to pre-condition these patients for therapy with checkpoint inhibitors.[40] One promising approach is the use of therapeutic vaccins targeting the E6/E7 oncoproteins. Indeed, several clinical trials have demonstrated promising results in cervical intraepithelial neoplasia (CIN) and cervical cancer patients treated with therapeutic E6/E7 targeted vaccines.[11,16,18,19,41] In one study using a therapeutic DNA vaccine, 7 out of 9 CIN 3 patients showed complete regression and viral clearance within 36 weeks of follow up.[16] In a randomized, double-blind, placebo-controlled trial in CIN2/3 patients, 49.5% of the DNA vaccine recipients showed regression of the disease vs. 30.6% in the control group.[11] These promising results may eventually lead to a change in treatment strategy for CIN 2/3, in which therapeutic vaccination could represents a non-surgical option.

In this work, we similarly demonstrate that an E6/E7-targeted SFV vaccine can induce accumulation of CD103+ T cells in tumors *in vivo*, an effect that synergized with radiotherapy. SFV E6/E7 vaccination therefore not only promotes systemic immune responses, but T cells induced by this vaccination effectively penetrate the tumor lesion and engage the epithelial cells present, resulting in CD103 upregulation. Importantly, all infiltrating cells in this model were CD8+, in line with the phenotype observed in the human setting.

With regards to this phenotype and ontogeny of CD103+ TIL in human tumors the literature remains diverse. In the gut, CD103 has been described to be expressed on Intra-epithelial Lymphocytes (IEL), characterized by a CD8$\alpha\alpha$+ phenotype.[42,43] In NSCLC the phenotype of CD103+ TIL has been described as tissue-resident memory T cells (characterized by a CD69+ CD62L− CD28− CD27+ CD45RA+ CD45RO+ CCR7- phenotype).[24] In endometrial cancer CD103+ TIL were of heterogenous memory phenotypes,[34] and in HGSC, CD103+TIL were classical CD3+ CD56- TCRab+ CD8ab+ CD4- T cells, also with heterogeneous differentiation status.[35] While we demonstrate dominant CD8 co-expression in cervical cancer, the precise differentiation status has not been investigated. We hypothesize that, as the cervix functions as a barrier against pathogens, CD103 tissue-resident memory T cells may also be present. However, in the context of tumor-specific (E6/E7-directed) immune responses, the majority of CD103+ TIL are likely recruited as a result of an adaptive immune response. Within cervical cancer tumor slides, we also show that CD103+ CD8- TIL expressed CD3 but were negative for NkP46 and FoxP3, suggesting a CD4 but not a NKT cell nor a Treg origin.

Interestingly, when analyzing the fluorescent images of the tumor tissue we noticed different patterns of stromal infiltration into the epithelial areas, namely a pushing and desmoplastic type.[30] The pushing tumor type was characterized by a distinct separation of epithelial and stromal areas. Whereas in the desmoplastic tumor type, a separation between stromal vs. epithelial areas could not be made. The desmoplastic tumor type has been described in literature as a more invasive tumor, but the exact consequences of this remains unclear. Interestingly, we observed that the desmoplastic tumors contained an even higher percentage of CD8+ CD103+TIL when compared with the pushing type. This might suggest that the desmoplastic tumors are more accessible to infiltration of CD8+ T cells which then engage the epithelial tumor cells and upregulate CD103 after T cell receptor (TCR) activation.

In line with this hypothesis, our data strongly suggests that upregulation of CD103 in cervical cancer is mainly the result of TCR signaling upon cancer cell contact. It has been well established that ITGAE (CD103) expression is induced by dual TCR and TGFβR1 activation.[32,33,44] In HGSC, we have further shown that CD103+, but not CD103-, TIL are characterized by nuclear pSMAD2/3 expression, a hallmark of TGF-β signaling.[35] In contrast to HGSC tissue, the total tumor microenvironment in cervical cancer tissue was rich in pSMAD2/3 expression and no differences in expression between epithelial and stromal areas

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were observed. This TGF-β rich microenvironment might be explained by the E6/E7-dependent ontogeny of cervical cancer. HPV-16 E6 and E7 oncoproteins have been shown to directly regulate the TGF-β1 promoter in cervical tumor cells through a specific DNA sequence motif in the TGF-β1 core promoter.[45] It is thought the upregulation of TGF-β facilitates the development of cervical neoplasia after E6/E7 integration by promoting genomic instability in the infected epithelial cells.[46] As a consequence, the immune environment is rich in TGF-β expression likely rendering T cell contact with the cancer cell as the key determinant of CD103 induction. CD103 may therefore represent an excellent biomarker for tumor-reactive T cells in cervical cancers that could be quantified in a rapid manner without having to account for epithelial vs. stromal compartments. It is tempting to speculate that the same may therefore hold true for other types of HPV-mediated cancers, such as head and neck squamous cell carcinoma (HNSCC). Indeed, TGF-β is overexpressed in ~80% of HNSCC cases[47] and likely produced by both cancer cells, stroma, and/or infiltration immune cells (reviewed in Yang et al.[48]) It will be interesting to assess whether differences exist between CD103+ cells infiltration in HPV-positive vs. HPV-negative HNSCC tumors, as has been reported for CD8+ cells.[49]

This use of CD103 as an easy-to-use biomarker for assessing immune responses against cervical cancer is supported by our *in vivo* data that demonstrate increased infiltration of TC1 tumors by CD103+ CD8+ cells upon treatment with a synergizing combination of HPV E6/E7 vaccination and radiotherapy. Indeed, an inverse correlation exists between the number of CD103+ cells in the tumor digest and the size of the tumor. With the clinical advent of therapeutic E6/E7-based vaccination strategies, CD103 may be incorporated both for patient selection and for monitoring early therapy responses in the tumor by biopsy. Of note, clues for synergistic effects on tumor control for radiation and E6/E7-targeted therapy in the human setting were also found in this study. In particular, the prognostic benefit of CD103+ cell infiltration in both the TCGA and IHC data sets were found within the group of patients that received adjuvant radio(chemo)therapy within 6 months of surgical intervention, but not in patients that received surgery alone. Assuming infiltrating T cells in most patients react against E6/E7 proteins to a certain extent, it is tempting to speculate that the pre-existing immune responses are augmented by the radiotherapy, similar to what was observed in the animal model. Future studies on clinical vaccination in combination with radiotherapy therefore appear warranted in this patient population.

Taken together, we demonstrate here for the first time that CD103 is a suitable marker for rapid unbiased assessment of prognostically beneficial CD8+ T cell infiltration of cervical cancers and might be used as a response biomarker for E6/E7-targeted immunotherapy alone or in combination with radiotherapy.

Methods

TCGA data and analysis

TCGA RSEM normalized[50] RNAseq and clinical data were downloaded from FireBrowse (http://firebrowse.org) on August 22nd, 2016. After removal of normal tissue controls and technical duplicates, 304 cervical cancer cases were informative for this study. RNAseq data were log2 transformed before further analysis. The expression of CD103 (*ITGAE*) relative to that of other immune markers[51] was visualized by means of a heatmap using GENE-E (Broad Institute). For analysis of CD103 expression with clinicopathological variables and patient survival, cases were dichotomized according to median CD103 expression. Analyses of clinical outcome excluded 13 patients for whom survival data were not available. For the exploratory analyses of the relationship between CD103 expression, radiotherapy treatment and clinical outcome, we excluded cases in which radiotherapy was given $\geq$ 6 months after diagnosis, to avoid misclassification of patients irradiated after disease recurrence.

Patient selection for the immunohistochemical series

Clinicopathological characteristics of cervical cancer patients treated within the University Medical Center Groningen were prospectively stored in a database since January 1980. As described by Maduro et al,[52] a separate anonymized database was retrieved containing all patients with stage IA2-IVA cervical cancer. Patients were treated between January 1980 and December 2004 with either surgery or radiotherapy depending on stage of disease and/or results of surgical outcome. We categorized patients into two groups based on their treatment modality, namely surgery or radio(chemo)therapy. The treatment modality was considered surgery in those patients in whom a radical hysterectomy combined with pelvic lymph node dissection was performed (first choice of treatment in early stage disease). The treatment modality was considered radio(chemo)therapy (first choice of treatment in locally advanced disease) if patients received radiotherapy or radio-chemotherapy, even if a surgical procedure was performed, as is the case in e.g., patients where positive nodes are detected after primary hysterectomy/lymph node dissection. Patients were selected if sufficient formalin-fixed, paraffin-embedded (FFPE) tissue was available for tissue microarray (TMA) construction. For the construction of the TMA, only pretreatment biopsies were used. Follow up data was collected up to April 2012. According to Dutch law, no approval from our institutional review board was needed.

Tissue microarray (TMA) construction

From the patients meeting the inclusion criteria, a TMA was constructed as described previously.[53] In brief, cancer nests were determined by a gynecologic pathologist based on H&E staining. Triplicate 1mm^2 cores were randomly selected from cancer nests and placed in a recipient paraffin block by a tissue microarrayer (Beecher instruments). After insertion of cores, recipient blocks were placed at 37°C for 15 minutes to maximize tissue adhesion to the wax. The paraffin block was sliced into 4μm sections and placed on APES-coated slides (Starfrost).

Immunohistochemical analysis of CD103± TIL infiltration

TMA sections were dewaxed in xylene and rehydrated using degraded concentrations of ethanol to distilled water. Antigen retrieval was initiated using a preheated 10 mM citrate buffer (pH6), endogenous peroxidase activity was blocked by submerging of sections in a 0.45% H_2O_2 solution. Sections were

incubated in a blocking buffer (1% human AB serum in 1% BSA/PBS solution), followed by an avidin/biotin block. Afterwards, sections were incubated with rabbit-anti human CD103 mAb (anti-$\alpha E\beta 7$-integrin, Abcam, ab129202, 1:200 in blocking buffer) and incubated at 4°C overnight. Slides were incubated with a peroxidase-labeled polymer (Envision+ anti-rabbit Dako) and a Biotin Tyramide working solution according to the manufacturer's instructions (TSA Kit Perkin Elmer, NEL700A001KT). Subsequently, slides were incubated with streptavidin-HRP (dilution: 1:100) (TSA kit, Perkin Elmer) and specific signal visualized by 3,3′diaminobenzidin (DAB). Slides were counterstained with hematoxylin.

The total number of positively stained CD103+ cells was counted per core and the percentage of tumor/stromal surface was estimated. Patients were included if at least 2 cores contained >20% tumor epithelium. All slides were counted manually by 2 individuals that were blinded for clinicopathological data. The 2 individual scores were compared and differences in counts of over 10% were reanalyzed until consensus was reached. Cell count was re-calculated per 1 mm^2 (i.e. the surface of one core).

Immunofluorescent analysis of CD103+ TIL localization and phenotype

Preparation, antigen retrieval and incubation with primary CD103 antibody of full tumor slides was performed as described in immunohistochemistry (IHC). Sections were subsequently incubated with Envision-HRP anti-rabbit followed by fluorophore tyramide stock solution:amplification diluents (TSA KIT Perkin Elmer,1:50) according to the manufacturer's instructions. Slides were incubated overnight at 4°C with either biotinylated rabbit anti-fibronectin (Abcam, ab6584 1:50 in blocking buffer) or rabbit anti-phospho-SMAD2/3 Mab (cell signaling, Ser 465/467 #3101, 1:50 in blocking buffer), and either mouse anti-human CD8 (DAKO, clone C8/144B, M710301–2, 1:25 in blocking buffer), anti-NKp46 (R&D Systems, ab1850, 1:25 in blocking buffer), anti-CD3 (Abcam, ab11089, 1:25 in blocking buffer) or anti-FoxP3 (Abcam, ab20034, 1:50 in blocking buffer) antibodies. Sections were subsequently incubated with goat-anti-mouse Alexa Fluor 555 (Life Technologies, Eugene,1:150) and streptavidin dylight 488 (Life Technologies, 1:150) Nuclei were visualized with DAPI. Sections were embedded in prolong Diamond anti-fade mounting medium (Life Technologies, Eugene) and scanned using a TissueFaxs imaging system (TissueGnostics). Processed channels were merged using Adobe Photoshop. On each slide 3 to 6 representative epithelial and 3 to 6 stromal areas of 40 μm^2 were selected based on DAPI staining. Within each area, single-positive (CD103-CD8+ or CD103+CD8-) cells as well as double-positive (CD103+CD8+) cells were counted manually.

Mice

Specified pathogen-free female C57BL/6 mice were used at 8 to 12 weeks of age at the onset of the experiment. They were purchased from Harlan CPB (Zeist, The Netherlands) and kept according to institute guidelines. All animal experiments were approved by the local Animal Experimentation Ethical Committee.

Tumor inoculation, local tumor irradiation and rSFV immunization

TC-1 cells were a kind gift from Dr. Cornelis J. Melief and Dr. Rienk Offringa (Leiden University Medical Center, The Netherlands). The TC-1 cell line was generated from C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7. All cells were cultured as described before.[54] Mice were inoculated subcutaneously in the neck with 2×10^4 TC-1 cells suspended in 0.2 mL Hank's Balanced Salt Solution (Invitrogen,). Fourteen days after injection of TC-1, mice were locally irradiated with 7 Gy and/or semliki forest virus (SFV) eE6,7 immunization. Radiation was performed using X-RAD 320 Biological Irradiator (Precision X-Ray) with a delivery rate of 1.64 Gy/min. Immunization was performed intramuscularly with a dose of 5×10^6 i.u. of SFVeE6,7 and boosted twice with a one-week interval (day 14 and 21) as a suboptimal immunization regimen. Control mice were injected intramuscularly with PBS. Tumors were isolated 22 days after TC-1 inoculation.

Tumor digestion and flow cytometry analysis

Tumors isolated from mice were processed as previous described(51). In brief, pre-warmed Collagenase A (Roche) solution was used for digestion and tumors were homogenized using the gentle MACStm Dissociator (Miltenyi Biotec). Tumors cells were further stained with PeCy7-anti-CD8 (eBioscience, clone 53–6.7, 25–5273–41) and FITC-anti-CD103 (BD Biosciences, clone 2E7, 333155). For the dextramer staining, cell suspensions were first washed twice in FACS buffer (PBS containing 0.5% bovine serum albumin) and stained with PE-H-$2D^b$ $E7_{49-57}$ dextramers (Immudex, Copenhagen, Denmark) for 10 min at room temperature. Subsequently, the cells were stained with PE-Cy7-anti-CD8a Ab and FITC-anti-CD103 Ab. To exclude dead cells, cells were stained with Zombie Violet™ (Biolegend, 423113).

Statistics

Differences in cell count were determined by a Mann-Whitney U, Kruskall-Wallis, ANOVA or t-test. Disease-specific survival (DSS) and disease-free survival (DFS) were analyzed using a Kaplan-Meier function; differences in survival were assessed by log Rank test. DSS was defined as date of diagnosis to date of death due to disease. DFS was defined as date of diagnosis to date of recurrence or date of death of disease, in case no recurrence was reported previously. Differences in DSS and DFS according to clinicopathologic characteristics and to infiltration of CD103+ TIL were analyzed using the Cox regression analyses. Variables with a p-value <0.05 in the univariate analyses were included in the multivariate analyses. Significance was defined as a p-value of <0.05, all tests were performed 2-sided. Statistics were performed using SPSS software version 22.0 (SPSS inc.), Stata (StataCorp,) or GraphPad Prism (GraphPad Software inc.).

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Disclosure of potential conflicts of interest

HW Nijman and T Daemen have a financial ownership interest in Vicini-Vax and may financially benefit if the company is successful in marketing its product related to this research. The terms of this arrangement have been reviewed and approved by the University of Groningen.

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