Chapter 7

The prognostic benefit of CD27+ tumor-infiltrating lymphocytes in cervical cancer: implications for treatment regimen

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Abstract

Purpose
Tumor-infiltrating lymphocytes (TIL) undoubtedly play a role in cervical cancer prognosis. The prognostic benefit of particular T cell subsets still requires clarification as studies on TIL differ in the correlation with clinical outcome depending on stage, clinico-pathological parameters and treatment. In this study we aim to analyze the association of TIL expressing CD8, FoxP3 and CD27, as a potentially tumor-reactive T cell subset, in primary tumor tissue from patients across different stages of cervical cancer. We specifically compared cohorts receiving different treatment regimens.

Experimental design
Tumor tissue obtained before treatment from 679 cervical cancer patients with FIGO stage Ib to IVa were collected on tissue microarray (TMA) slides. The number of TIL expressing CD8, CD27 and FoxP3 was determined by immunohistochemistry. TIL expressing CD27 was also characterized using immunofluorescence.

Results
For patients primarily treated with surgery, a high number of CD27+ TIL was an independent prognostic factor for disease-specific survival (DSS). The association with DSS was observed for patients specifically receiving radiation after surgery. The prognostic benefit of high CD27+ TIL was also observed for patients receiving (chemo)radiation and was an independent prognostic factor for DSS and disease-free survival. Low CD27+ TIL was an independent predictor of poor response to (chemo)radiation. Neither CD8+ nor FoxP3+ TIL was of independent prognostic benefit in either cohort.

Conclusions
CD27+ TIL is independently associated with DSS and response to chemoradiation therapy in cervical cancer patients independent of disease stage. Our data implicates CD27+ TIL as a biomarker for cervical cancer and might serve as a potential target for immunotherapeutic regimens.
Introduction

Cervical cancer is the second most common malignancy among women worldwide with approximately half a million new cases per annum.\(^1\) Persistent infection in the genital tract with high-risk human papillomavirus (HPV) is associated with cervical cancer development.\(^2\) The most prevalent HPV types, HPV 16 and HPV 18, account for over 50% and 15% of the cases, respectively.\(^2\) The progression from cervical intraepithelial neoplasia (CIN) to invasive cancer manifests in the minority of women as the remaining cases exhibit spontaneous infection clearance within 2 years.\(^3,4\) Expression of early viral oncoproteins E6 and E7 by HPV are essential for malignant transformation by promoting cell proliferation and inhibiting apoptosis.

Treatment regimens are different for early, locally advanced and advanced stage of disease (staging according to the International Federation of Gynecology and Obstetrics (FIGO)).\(^5\) For early stages, radical hysterectomy is the standard of care.\(^6\) A subset of these patients, at higher risk for recurrence of disease, may also receive adjuvant radiotherapy after surgery.\(^6\) For locally advanced disease, radiation concurrent with chemotherapy is well established as primary treatment as studies have shown survival advantages above patients receiving radiation therapy alone.\(^5\) For the latter category, it has been reported that combination with targeted therapy may be favorable for increasing the response rate.\(^7\) Despite the addition of chemotherapy in the treatment regimen for locally advanced disease, the overall 5-year survival rate is approximately 60-70% as patients relapse or are resistant to treatment.\(^8,9\)

There has been substantial effort undertaken in identifying biomarkers that are predictors for response to treatment and clinical outcome. The prognosis of cervical cancer is governed by markers which include FIGO stage, lymph node status, clinical-pathological features such as tumor size and type.\(^10\) Another predictor is the type and number of immune cells in the complex tumor microenvironment. As cervical cancer occurs more frequently in immune compromised individuals, lymphocytes undoubtedly play an essential role in controlling HPV-induced carcinoma. Cervical carcinoma possesses significant numbers of different tumor infiltrating lymphocytes (TIL) and there is increasing interest in identifying subsets that control or promote tumor progression. As E6 and E7 are stably expressed in malignant tissue, they make excellent targets for therapy aiming at enhancing E6- and E7-specific TIL subsets.

Several studies associate TIL infiltration with prognosis of cervical cancer. For example, a high T lymphocyte infiltration is associated with better prognosis in both early and advanced stages of disease.\(^11\) Infiltration and the presence of tumor-infiltrating lymphocytes have been associated with an improved clinical outcome. In the uterine cervix these immunocompetent cells have been associated with improved prognosis in high stage disease. The current study examines the significance of stromal and tumor T-lymphocyte infiltration together with S-100 positive dendritic cell infiltration in a series of 73 women
with low stage (FIGO 1b). This also applies to a low CD8+ CTL to regulatory T cell (Treg) ratio which is associated with a poor clinical outcome in cervical cancer patients. A high Treg infiltration is also associated with worse prognosis in squamous cell carcinoma patients. Yet recent studies show the reverse prognostic effect or none at all of the aforementioned markers. This may be due to differences in sample size, tumor characteristics and standard of care. To this end, there is increasing interest in identifying other immune cell markers that may be more significant in tumor control and might even serve as a target for therapy. For instance, co-stimulatory receptors such as CD27 are targeted in recent clinical trials using agonistic monoclonal antibodies (mAbs) for enhancing anti-tumor immunity by priming T cells and forming memory responses.

In the present study, we aimed to clarify the predictive role of TIL in a large series of cervical cancer patients across all stages of disease. More specifically, TIL expressing CD27, CD8 and FoxP3 were examined in pre-treatment tissue that was dichotomized based on primary treatment to reveal whether the pre-existing immune response enhanced the effects of treatment.

**Methods**

**Patient selection**

In this study, cervical cancer patients primarily treated with surgery or radiotherapy +/- chemotherapy at the University Medical Center Groningen were selected between January 1980 and December 2006. The patients were dichotomized based on primary treatment. Patients with stage IVb disease were excluded, as their treatment was individualized. Staging was conducted according to the FIGO guidelines. The chemotherapy and radiotherapy regimen provided has been previously described. Paraffin-embedded, formalin-fixed primary tumor tissues were collected from each patient. The patients included in the analysis were those from whom tumor tissue was available for construction of tissue microarray (TMA).

**Institutional Review Board Approval**

For the present study, all relevant data were obtained from our computerized database into a separate anonymous database. Patient identity was protected by study-specific, unique patient numbers. Two data managers, who have responsibility for the larger database on a daily basis, were known of the patients numbers. The larger databases could only be checked by the data managers in case of uncertainties due to clinicopathologic and follow-up data. This reassures the protection of patients’ identity. Identified by unique patient numbers, all tissue specimens were retrieved from the archives of the Department of Pathology using the registration database. Therefore, according to Dutch law, no further Institutional Review Board approval was needed.
**Tissue micro arrays**

Tissue micro arrays (TMA) were constructed as described in previous studies. In brief, areas of representative tumor tissue were marked on H&E-stained slides of the paraffin-embedded tissue. TMAs were made using a precision instrument (Beecher Instruments) with three cores of 0.6 mm in diameter punched from a marked area of the paraffin-embedded tissue and transferred to a blank paraffin block. This block was then incubated in an oven at 37°C for 2 min to allow attachment of the cores to the paraffin. Four μm thick sections were cut from the TMAs.

**Immunohistochemistry**

The TMA slides were stained with mouse monoclonal antibodies recognizing CD8 (C8/144B; DAKO, Heverlee, Belgium; 1:25), FoxP3 (m22509; Abcam, Cambridge, UK; 1:100) and CD27 (EPR8569, Abcam, Cambridge, UK; 1:150). The immunohistochemistry (IHC) procedure for CD8 and Foxp3 was performed as previously described. The same procedure was applied to stainings for CD27 with the exception of the antigen retrieval step, which was performed in citrate buffer (pH 6.0), for 20 min. After endogenous peroxidase was blocked in a 0.3% H₂O₂ solution for 30 minutes. The primary antibody stainings were incubated overnight at 4°C. For visualization, Envision secondary antibodies were used (DAKO) with subsequent 3,3’-diaminobenzidine. Slides were then counterstain with hematoxylin.

**Immunofluorescent staining**

Multi-color immunofluorescent staining was performed on a selection of TMA tissue from early stage patients. TMA tissue from 10 patients were selected on the basis of high CD8 infiltration, as determined with IHC, and were used for further analysis. The slides were deparaffinized, rehydrated and antigen retrieval was performed with citrate buffer (10mM citrate, pH 6.0). After cooling, the endogenous peroxidase was blocked in a 0.3% H₂O₂ solution for 30 minutes. The primary antibody stainings with mouse anti-human CD8 (DAKO, clone C8/144B) and rabbit anti-human CD27 (Abcam, clone EPR8569) were incubated overnight at 4°C. For visualization, secondary antibodies goat anti-rabbit Cy3 and goat anti-mouse AlexaFluor555 (Life Technologies, Bleiswijk, The Netherlands) were used. Counterstaining was performed with 4’,6-diamidino-2-phenylindole (DAPI). Slides were mounted with Prolong Gold (Life Technologies) and stored in the dark at RT.

**Scoring**

Two independent observers performed the scoring of the TMA for CD27+, CD8+ and FOXP3+ cells in the tumor epithelium. The observers had no prior knowledge of patient characteristics. At least two cores with at least 20% tumor epithelium were counted. An average number of positively stained cells were noted for a 0.6 mm³ tumor area. This represents 100% of the tumor tissue to correct for differences in sample size and to standardize the analysis.
Statistical analysis
All statistical analyses were performed using SPSS software, version 14.0 (SPSS Inc., Chicago, IL, USA). The clinicopathological parameters between the groups were tested for differences using the chi square test. To determine the associated with disease-specific survival and disease-free survival, a Kaplan-Meier analysis was used. Cox regression analysis was used for multivariate survival analysis. Variables with a p value of >0.10 in univariate analysis were excluded stepwise from multivariate analysis. In the final step of the multivariate analysis, only factors with a p value of <0.05 were included. p< 0.05 was considered statistically significant (tested 2-sided). For evaluation of response to therapy we applied the Cox regression analysis as in Noordhuis et al.22 Specifically, the model testing the response to (chemo)radiation was determined in all patients by locoregional disease-free survival as locoregional recurrence after treatment or during treatment, the time from diagnosis to clinical locoregional progression of disease.

Results

Patient and tumor characteristics
Paraffin-embedded tissue for TMA construction was available from a total of 679 patients diagnosed with cervical cancer from January 1980 to December 2006. This cohort was analyzed separately based on primary treatment. In 304 cases, patients were treated with primary surgery (PS). The second cohort, consisting of 375 patients having received primary (chemo)radiation (PR) were further divided into those receiving primary radiation alone or chemoradiation. FIGO staging and the clinicopathologic characteristics of these two cohorts are summarized in Table 7.1. The median follow-up time is 5.6 years (range 0.31-21.31) and 3.9 years (range, 0.14-18.36) for the PS and PR cohort, respectively. From 8 to 10 weeks after completion of primary treatment or hysterectomy, biopsies were taken from these patients.22
### Table 7.1. Clinicopathological characteristics

<table>
<thead>
<tr>
<th>Clinicopathological factor</th>
<th>PS cohort (n = 304)</th>
<th>PR cohort (n = 375)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at diagnosis</strong></td>
<td></td>
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<tr>
<td>Median</td>
<td>42.8</td>
<td>54.41</td>
</tr>
<tr>
<td>Range</td>
<td>17.5-86.1</td>
<td>20.61-91.95</td>
</tr>
<tr>
<td><strong>Figo stage</strong></td>
<td></td>
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</tr>
<tr>
<td>1b1</td>
<td>195 (64.1%)</td>
<td>37 (10%)</td>
</tr>
<tr>
<td>1b2</td>
<td>61 (20.1%)</td>
<td>32 (9%)</td>
</tr>
<tr>
<td>1a</td>
<td>48 (15.8%)</td>
<td>48 (13%)</td>
</tr>
<tr>
<td>1b2</td>
<td>181 (61%)</td>
<td>48 (13%)</td>
</tr>
<tr>
<td>1la</td>
<td>9 (3%)</td>
<td>2 (1%)</td>
</tr>
<tr>
<td>1lb</td>
<td>56 (18%)</td>
<td>14 (4%)</td>
</tr>
<tr>
<td>IVa</td>
<td>12 (4%)</td>
<td>3 (1%)</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous</td>
<td>196 (64.5%)</td>
<td>311 (82.9%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>86 (28.0%)</td>
<td>52 (14%)</td>
</tr>
<tr>
<td>Other</td>
<td>23 (7.6%)</td>
<td>12 (3%)</td>
</tr>
<tr>
<td><strong>Differentiation grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good/moderate</td>
<td>178 (58.6%)</td>
<td>223 (59%)</td>
</tr>
<tr>
<td>Poor/Undifferentiated</td>
<td>120 (39.5%)</td>
<td>128 (34%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (2.0%)</td>
<td>24 (6%)</td>
</tr>
<tr>
<td><strong>Lymph node status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>218 (71.7%)</td>
<td>248 (66%)</td>
</tr>
<tr>
<td>Positive</td>
<td>86 (28.3%)</td>
<td>54 (14%)</td>
</tr>
<tr>
<td><strong>Lymphangioinvasion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not present</td>
<td>147 (48.4%)</td>
<td>248 (66%)</td>
</tr>
<tr>
<td>Present</td>
<td>156 (51.3%)</td>
<td>54 (14%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (0.3%)</td>
<td>73 (19%)</td>
</tr>
</tbody>
</table>

**Abbreviation:** FIGO, International Federation of Gynecologists and Obstetrics. PS, primary surgery. PR, primary (chemo)radiation. CI, confidence interval.

### TIL in relation to clinicopathologic factors

TIL was assessed in tumor tissue by immunohistochemistry. Representative stainings for positive and negative infiltration of CD8+, CD27+ and FoxP3+ TIL are provided in Figure 7.1. The number of patients with two representative tissue cores for TIL scoring using immunohistochemistry was 225 in the PS cohort and 189 in the PR cohort and were used for subsequent analysis. The association between the presence of intratumoral lymphocytes and clinicopathological parameters for the PS cohort and PR cohort is shown in Tables 7.2 and 7.3, respectively. In the PS cohort, high CD27+ TIL was less associated with adenocarcinoma than with squamous cell carcinoma [odds ratio (OR), 0.39; 95% confidence interval (95% CI), 0.20-0.73]; P = 0.004; Table 7.2). Intratumoral FoxP3+ cells are less frequently present in stages 1b2 and 2a [OR, 0.48; 95% CI, 0.26-0.88; P = 0.018; Table 7.2). In the PR cohort, high CD27+ TIL was less associated with adenocarcinoma than with squamous cell carcinoma [OR, 0.34; 95% confidence interval (95% CI), 0.14-0.83);
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$P = 0.018$; Table 7.3). FoxP3+ cells are also less frequently observed in adenocarcinoma than in squamous cell carcinoma (OR, 0.11; 95% CI, 0.04-0.30; $P < 0.001$; Table 7.3). In both cohorts, all TIL types were positively associated with each other ($P < 0.001$ (Table 7.2 and 7.3)).

Figure 7.1. Representative images of positive and negative immunohistochemical staining for CD8 (A), CD27 (B) and FoxP3 (C) in cervical cancer tissue at 5x magnification.
Table 7.2. Relationship of tumor-infiltrating lymphocytes in primary surgery cohort to clinicopathologic factors

<table>
<thead>
<tr>
<th></th>
<th>CD8+ lymphocytes</th>
<th>CD27+ lymphocytes</th>
<th>FoxP3+ lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lowest tertile (%)</td>
<td>OR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;54</td>
<td>52 (31.1)</td>
<td>0.86 (0.46-1.62)</td>
<td>0.638</td>
</tr>
<tr>
<td>≥54</td>
<td>20 (34.5)</td>
<td>18 (31.0)</td>
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<tr>
<td><strong>Figo stadium</strong></td>
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<tr>
<td>Stage Ib1</td>
<td>45 (31.3)</td>
<td>0.91 (0.51-1.63)</td>
<td>0.748</td>
</tr>
<tr>
<td>Stage 1b2-IIa</td>
<td>27 (33.3)</td>
<td>27 (33.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous</td>
<td>45 (28.7)</td>
<td>0.61 (0.32-1.17)</td>
<td>0.139</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>21 (39.6)</td>
<td>27 (50.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Differentiation grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good/Moderate</td>
<td>44 (33.1)</td>
<td>1.25 (0.69-2.24)</td>
<td>0.463</td>
</tr>
<tr>
<td>Poor/Undiff.</td>
<td>25 (28.4)</td>
<td>29 (33.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor volume</strong></td>
<td></td>
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</tr>
<tr>
<td>0-4 cm</td>
<td>50 (30.5)</td>
<td>0.78 (0.42-1.44)</td>
<td>0.426</td>
</tr>
<tr>
<td>≥4 cm</td>
<td>22 (36.1)</td>
<td>20 (32.8)</td>
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<td><strong>Lymph node status</strong></td>
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<tr>
<td>Negative</td>
<td>52 (32.9)</td>
<td>1.153 (0.62-2.14)</td>
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<td>Positive</td>
<td>20 (29.9)</td>
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<td><strong>Lymphangioinvasion</strong></td>
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<tr>
<td>No</td>
<td>30 (29.4)</td>
<td>0.823 (0.47-1.45)</td>
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<tr>
<td>Yes</td>
<td>41 (33.6)</td>
<td>47 (38.5)</td>
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<tr>
<td><strong>CD8+ TIL</strong></td>
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</tr>
<tr>
<td>Lowest Tertile</td>
<td>54 (75.0)</td>
<td>18.86 (9.32-38.15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>All others</td>
<td>21 (13.7)</td>
<td>22 (14.4)</td>
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<tr>
<td><strong>CD27+ TIL</strong></td>
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<td></td>
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<tr>
<td>Lowest Tertile</td>
<td>32 (42.7)</td>
<td>3.72 (1.99-6.97)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>All others</td>
<td>25 (16.7)</td>
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</table>
Table 7.3. Relationship of tumor-infiltrating lymphocytes in primary (chemo)radiation cohort to clinicopathologic factors

<table>
<thead>
<tr>
<th></th>
<th>CD8+ lymphocytes</th>
<th>CD27+ lymphocytes</th>
<th>FoxP3+ lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lowest tertile (%)</td>
<td>OR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
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<td>&lt;54</td>
<td>32 (32.7)</td>
<td>0.97 (0.53-1.82)</td>
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</tr>
<tr>
<td>≥54</td>
<td>29 (33.0)</td>
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<td><strong>Figo stadium</strong></td>
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<tr>
<td>Stage Ib1-IIa</td>
<td>23 (33.8)</td>
<td>1.08 (0.57-2.03)</td>
<td>0.831</td>
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<tr>
<td>Stage ≥ IIb</td>
<td>38 (32.2)</td>
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<td><strong>Tumor type</strong></td>
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<tr>
<td>Squamous</td>
<td>49 (31.4)</td>
<td>0.50 (0.21-1.21)</td>
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<td>Adenocarcinoma</td>
<td>11 (47.8)</td>
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<td>0.93 (0.48-1.79)</td>
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<td>Poor/Undiff.</td>
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<td>0-4 cm</td>
<td>14 (31.1)</td>
<td>0.94 (0.45-1.96)</td>
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<td>≥4 cm</td>
<td>40 (32.5)</td>
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<td><strong>Lymphangioinvasion</strong></td>
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<td>No</td>
<td>36 (32.4)</td>
<td>1.28 (0.54-3.03)</td>
<td>0.572</td>
</tr>
<tr>
<td>Yes</td>
<td>9 (27.3)</td>
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<tr>
<td><strong>CD8+ TIL</strong></td>
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<tr>
<td>Lowest Tertile</td>
<td>44 (72.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All others</td>
<td>18 (14.3)</td>
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<tr>
<td><strong>CD27+ TIL</strong></td>
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<tr>
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<td>25 (20.2)</td>
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</table>
High CD27+ TIL is associated with improved prognosis depending on treatment type

To determine the prognostic value of TIL infiltration, the patients were dichotomized on the basis of the lowest infiltration of cells (bottom tertile) due to the positively skewed distribution of all TIL subtypes.24 In the PS cohort, 43% of patients received post-operative chemoradiation or radiation alone (approximately 15% and 85%, respectively). As for the PR cohort, 46% of patients received radiotherapy compared to 54% having received chemoradiotherapy. High CD27+ TIL showed a disease-specific survival benefit in contrast to CD8 or FoxP3+ TIL (P = 0.042; Figure 7.2A-C). As this cohort consists of patients also having received post-operative (chemo)radiation, we analyzed the survival benefit of CD27+ separately based on treatment type. In the patient cohort only undergoing surgery the prognostic benefit of a high number of CD27+ cells was lost (P = 0.493; Figure 7.2D), with an association rather observed in patients having received (chemo)radiotherapy after surgery (P = 0.040; Figure 7.2E). In the PR cohort, high CD27+ TIL also showed a disease-specific survival benefit above that of CD8+ and FoxP3+ TIL (P = 0.045; Figure 7.3A-B). As treatment may influence the prognosis of TIL, we analyzed the treatment groups separately within the PR cohort. These consist of patients receiving radiation versus those receiving chemoradiation. Interestingly, the subgroup having received chemoradiation showed a survival advantage with infiltration of high numbers of CD8+ cells (P = 0.016; Figure 7.3D), with high CD27+ TIL showing a trend towards a longer median DSS (P = 0.062; Figure 7.3E). In this subgroup an association was observed with both high CD8+ TIL and CD27+ TIL for locoregional disease-free survival (Supplementary Figure 7.1). No significance was observed in the cohort treated with radiotherapy only (data not shown). Next, a multivariate cox regression analysis was performed and stratified for treatment type for both the PS and PR cohort. In the PS cohort, positive lymph nodes (HR 20.03, 95% CI 6.05-66.33), P = < 0.001) and high CD27+ TIL (HR 0.47, 95% CI 0.24-0.91), P = 0.027) were independent prognostic factors for DSS (Table 7.4). In the PR cohort, stage (HR 0.53, 95% CI 0.30-0.94), P = 0.001) and high CD27+ TIL (HR 0.53, 95% CI 0.30-0.94), P = 0.028) were independently related to survival benefit. This was determined for both DSS (Table 7.5) and DFS (Table 7.6). Locoregional progression during treatment or locoregional recurrence in follow-up was observed in 111 of 365 patients (30%). With this variable we assessed response to (chemo)radiation in the PR cohort with Cox regression analysis as described by Noordhuis et al.22 Stage and high CD27+ TIL (HR 0.53, 95% CI 0.30-0.94), P = 0.028) (Table 7.7) were independent predictors for a beneficial response to (chemo) radiation.
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Figure 7.2. Kaplan-Meier curves for the association between CD8+, CD27+ and FoxP3+ TIL and DSS in the total PS cohort (A-C). Association of CD27+ TIL was determined in the surgery only (D) and the surgery with post (chemo)radiation only (E) cohort.
Prognostic benefit of CD27+ TIL in cervical cancer

Figure 7.3. Kaplan-Meier curves for the association between CD8+, CD27+ and FoxP3+ TIL and DSS in the PR cohort (A-C) and the primary chemoradiation cohort only (D-F).
### Table 7.4. Cox regression analysis of disease-specific death in the primary surgery cohort.

<table>
<thead>
<tr>
<th></th>
<th>Univariate</th>
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<th>Multivariate</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p value</td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Age ≥54</td>
<td>0.25 (0.79-2.45)</td>
<td>0.247</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Stage ≥IIb</td>
<td>1.73 (0.98-3.05)</td>
<td>0.060</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1.74 (0.97-3.11)</td>
<td>0.062</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Poor/Undifferentiated</td>
<td>1.70 (1.0-2.88)</td>
<td>0.036</td>
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<td></td>
</tr>
<tr>
<td>Positive lymph nodes</td>
<td>14.84 (5.43-40.55)</td>
<td>&lt;0.001</td>
<td>20.03 (6.05-66.33)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>1.91 (1.04-3.51)</td>
<td>0.037</td>
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<td></td>
</tr>
<tr>
<td>Tumor diameter ≥4 cm</td>
<td>1.77 (1.00-3.12)</td>
<td>0.050</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>High CD8+ lymphocytes</td>
<td>0.80 (0.42-1.56)</td>
<td>0.518</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>High CD27+ lymphocytes</td>
<td>0.50 (0.26-0.94)</td>
<td>0.032</td>
<td>0.47 (0.24-0.91)</td>
<td>0.027</td>
</tr>
<tr>
<td>FoxP3 positive</td>
<td>0.77 (0.39-1.55)</td>
<td>0.471</td>
<td>a</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: HR, hazard ratio. CI, confidence interval. Bold signifies p < 0.05.

*Not included in multivariate analysis.

### Table 7.5. Cox regression analysis of time to clinical locoregional progression of disease during treatment or to locoregional recurrence after treatment in the primary (chemo)radiation cohort

<table>
<thead>
<tr>
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<th>Univariate</th>
<th></th>
<th>Multivariate</th>
<th></th>
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</thead>
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<td>HR (95% CI)</td>
<td>p value</td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Age ≥54</td>
<td>1.02 (0.68-1.53)</td>
<td>0.929</td>
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<tr>
<td>Stage ≥IIb</td>
<td>1.94 (1.22-3.08)</td>
<td>0.005</td>
<td>2.03 (1.08-3.84)</td>
<td>0.029</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1.70 (1.06-2.71)</td>
<td>0.028</td>
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<td></td>
</tr>
<tr>
<td>Poor/Undifferentiated</td>
<td>1.20 (0.81-1.79)</td>
<td>0.366</td>
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</tr>
<tr>
<td>Vascular invasion</td>
<td>1.09 (0.63-1.86)</td>
<td>0.750</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Tumor diameter ≥4 cm</td>
<td>2.19 (1.32-3.61)</td>
<td>0.002</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>High CD8+ lymphocytes</td>
<td>0.77 (0.43-1.38)</td>
<td>0.378</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>High CD27+ lymphocytes</td>
<td>0.56 (0.32-0.98)</td>
<td>0.042</td>
<td>0.53 (0.30-0.94)</td>
<td>0.028</td>
</tr>
<tr>
<td>High FoxP3+ lymphocytes</td>
<td>0.76 (0.43-1.35)</td>
<td>0.349</td>
<td>a</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: HR, hazard ratio. CI, confidence interval. Bold signifies p < 0.05.

*Not included in multivariate analysis.

*Not included in the final step of the multivariate analysis.

### Table 7.6. Cox regression analysis of disease-specific survival in the primary (chemo)radiation cohort.

<table>
<thead>
<tr>
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<th>Univariate</th>
<th></th>
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<th></th>
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</thead>
<tbody>
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<td></td>
<td>HR (95% CI)</td>
<td>p value</td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Age ≥54</td>
<td>0.87 (0.63-1.22)</td>
<td>0.425</td>
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</tr>
<tr>
<td>Stage ≥IIb</td>
<td>2.01 (1.37-2.93)</td>
<td>&lt;0.001</td>
<td>2.34 (1.39-3.92)</td>
<td>0.001</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1.40 (0.93-2.11)</td>
<td>0.105</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Poor/Undifferentiated</td>
<td>1.31 (0.95-1.81)</td>
<td>0.098</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>1.35 (0.88-2.05)</td>
<td>0.166</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Tumor diameter ≥4 cm</td>
<td>1.85 (1.25-2.74)</td>
<td>0.002</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>High CD8+ lymphocytes</td>
<td>0.72 (0.46-1.14)</td>
<td>0.163</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>High CD27+ lymphocytes</td>
<td>0.64 (0.41-0.99)</td>
<td>0.047</td>
<td>0.60 (0.38-0.93)</td>
<td>0.023</td>
</tr>
<tr>
<td>High FoxP3+ lymphocytes</td>
<td>0.72 (0.46-1.14)</td>
<td>0.158</td>
<td>a</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: HR, hazard ratio. CI, confidence interval. Bold signifies p < 0.05.

*Not included in multivariate analysis.

*Not included in the final step of the multivariate analysis.
Table 7.7. Cox regression analysis of disease-free survival in the primary (chemo)radiation cohort.

<table>
<thead>
<tr>
<th></th>
<th>Univariate</th>
<th>Multivariate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Age ≥54</td>
<td>0.84 (0.61-1.17)</td>
<td>0.314</td>
</tr>
<tr>
<td>Stage ≥IIb</td>
<td>2.09 (1.43-3.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1.45 (0.96-2.18)</td>
<td>0.077</td>
</tr>
<tr>
<td>Poor/Undifferentiated</td>
<td>1.33 (0.96-1.83)</td>
<td>0.085</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>1.26 (0.83-1.91)</td>
<td>0.285</td>
</tr>
<tr>
<td>Tumor diameter ≥4 cm</td>
<td>1.85 (1.25-2.74)</td>
<td>0.002</td>
</tr>
<tr>
<td>High CD8+ lymphocytes</td>
<td>0.76 (0.48-1.20)</td>
<td>0.244</td>
</tr>
<tr>
<td>High CD27+ lymphocytes</td>
<td>0.64 (0.42-1.01)</td>
<td>0.057</td>
</tr>
<tr>
<td>High FoxP3+ lymphocytes</td>
<td>0.70 (0.45-1.11)</td>
<td>0.128</td>
</tr>
</tbody>
</table>

Abbreviation: HR, hazard ratio. CI, confidence interval. Bold signifies p < 0.05.

αNot included in multivariate analysis.

βNot included in the final step of the multivariate analysis.

Discussion

In the present study, the prognostic value of TIL expressing CD8, FoxP3 and CD27 was assessed in a large cohort of cervical cancer patients. To our knowledge, this is the first study to examine the prognostic significance of these different TIL across all stages of disease among different treatment regimens. No association was found with improved outcome in early stage patients having undergone surgery alone for any marker. However, the approximately 30% of patients who underwent radiation post-surgery with high CD27+ TIL had a significantly longer DSS. Furthermore, high CD27+ TIL is an independent prognostic factor for DSS and DFS in patients primary treated with (chemo)radiation. High CD27+ TIL is also an independent predictor for a beneficial response to treatment.

Various cancer types have demonstrated an association of TIL with improved survival. The infiltration of CD8+ TIL is associated with improved survival in ovarian, colorectal, endometrial carcinoma and melanoma.25 The prognostic benefit of CD8+ TIL is less clear in cervical cancer despite the host immune response is implicated in preventing progression of this cancer type. A positive association of high CD8+ TIL with absence of lymph node metastasis was observed in patients with early stage cervical cancer.14 This was also shown in tissue derived from FIGO stage IIB squamous cell carcinoma patients.26 However, Jordanova et al had not observed an association of high CD8+ TIL with survival but rather the infiltration of FoxP3+ with a high CD8+/Treg ratio being associated with survival.13 Also in locally advanced cervical cancer, one study showed no association of CD8+ TIL with progression-free survival or overall survival.16 In agreement with Jordanova et al, we hadn’t observed a prognostic benefit of CD8+ TIL nor FoxP3+ in a similar cohort (i.e. patients receiving primary surgery). Any indication of the prognostic benefit of CD8+ TIL was in advanced stage patients receiving primary chemoradiation in a univariate analysis for DSS and DFS. This was not the case for the CD8+/Treg ratio (data not shown).
Like CD8+ TIL, the prognostic role of FoxP3+ TIL on clinical outcome in cervical cancer is also subject to uncertainty. A high percentage of CD4+FoxP3+ TIL is associated with a lower survival rate assessed in 35 patients with FIGO stage IIB and IIIB. In contrast, other studies have shown a better prognosis or no association such as in cervical adenocarcinoma, advanced stage ovarian cancer and squamous cell carcinoma. The discrepancy in the results may result from the fact that FoxP3 is not exclusively expressed on regulatory T cells but also on activated CD4+CD25- effector T cell expressing the marker transiently. This may obscure the influence on clinical outcome between studies. The strength of the present work relies on the fact that a large patient cohort is included as well as performing a separate analysis according to treatment type. In addition, most of the prior studies did not assess the predictive value of TIL in relation to treatment type. The large sample size in our, as well as long follow-up time, may explain the contradictory results with prior studies. According to a meta-analysis study by Gooden et al the sample size and follow-up time influences the outcome of the prognostic value of TIL between different studies. To this end, we believe that skepticism still prevails regarding the prognostic value of CD8+ and FoxP3+ TIL in cervical carcinoma.

We extended our analysis to investigate CD27+ TIL as a novel target in cervical cancer. In patients receiving surgery as primary treatment with subsequent radiotherapy, high CD27+ TIL is associated with better survival. It is important to note that the lack of prognostic significance of CD27+ TIL in patients treated with primary surgery only may not be a reflection of the immune-mediated control but in the fact that the cohort has a higher survival rate in itself. Nevertheless, we show that irrespective of treatment, high CD27+ TIL is an independent positive factor for DSS in the primary surgery cohort. This also applies to patients receiving radiation or chemoradiation as primary treatment. CD27+ TIL is also associated with a beneficial response to chemoradiation. The prognostic value of CD27+ TIL has only been demonstrated so far in one study in patients with high grade serious ovarian cancer. Like our study, CD27+ TIL is suggested to exert potentially better tumor control than CD8+ TIL given the significance over CD8+ TIL. The authors also claim that it may be likely to represent an activated, tumor-reactive subset of cells given a high percentage co-expressing CD137. In this study, we also confirmed with immunofluorescence that most lymphocytes that are positive for CD27+ are also positive for CD8 with variability between patients in relation to CD27-CD8+ TIL (Supplementary Figure 7.1).

As a member of the tumor necrosis receptor family, CD27 has an important role in CD8+ effector T cell priming, survival and memory formation in the tumor setting upon costimulation with CD70. CD27+ TIL is currently being explored as a potential tumor-reactive subset for targeting in cancer immunotherapy. For instance, in a xenograft ovarian cancer mouse model, the antitumor efficacy was enhanced upon administration of T cells transduced with an antigen-specific chimeric antigen receptor (CAR-T) with a
CD27 costimulatory positive phenotype. The antitumor immunity in melanoma and lymphoma mouse models was also improved with agonistic mAb targeting CD27. A humanized mAb antibody (CDX-1127) is now under evaluation in phase I clinical trials for the treatment of renal cell carcinoma, melanoma and B-cell malignancies. There is profound interest in combining this strategy with other therapies for improving tumor responses in patients. There are numerous clinical trials focusing on vaccination against cervical cancer where the frequency of HPV-reactive T cells were correlated with response. Immunotherapy includes therapy with check point inhibitors which may affect the therapeutic efficacy of vaccination in cervical cancer, for instance, with programmed death-ligand-1 significantly upregulated in cervical carcinoma. Possible combination of inhibitors with immune checkpoint proteins with CD27 agonism may increase the success of vaccine strategies. For instance, a synergistic effect of CD27 agonism with programmed cell death protein 1 blockade was observed with an enhanced anti-tumor effect of an HPV-specific DNA vaccine in a preclinical study of cervical cancer. As high CD27+ TIL predicts the poor response to (chemo)radiation in this study, it may be of interest in combining CD27 agonism with conventional therapies to further enhance antitumor immunity. It may also be of value to assess whether the phenotype and function of CD27+ TIL are affected by (chemo)radiotherapy by comparing paired tumour biopsies pre- and post-treatment.

In conclusion, our study indicates that CD27+ TIL is an independent prognostic marker for DSS and predicts poor response to (chemo)radiation. Hence, CD27 may be a promising selective marker for stratification of patients depending on standard or novel treatment regimens.
Supplementary Figure 7.1. Representative image of part of a cervical carcinoma tissue core stained with immunofluorescence for CD8 and CD27 shown as a single or double staining (A-B). Bar graph representing double or single positive cells for CD8 and CD27 counted in the tumor epithelium in a TMA core from tissue selected on the basis of high TIL infiltration. Each bar represents one patient (C).
Supplementary Figure 7.2. Kaplan-Meier curves for the association between CD8+, CD27+ and FoxP3+ TIL and locoregional DFS in the primary chemoradiation cohort only (A-C).
Chapter 7

References


