High and Interrelated Rates of PD-L1⁺ CD14⁺ Antigen-Presenting Cells and Regulatory T Cells Mark the Microenvironment of Metastatic Lymph Nodes from Patients with Cervical Cancer

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Abstract

A better understanding of the microenvironment in relation to lymph node metastasis is essential for the development of effective immunotherapeutic strategies against cervical cancer. In the present study, we investigated the microenvironment of tumor-draining lymph nodes of patients with cervical cancer by comprehensive flow cytometry-based phenotyping and enumeration of immune-cell subsets in tumor-negative (LN⁻, n = 20) versus tumor-positive lymph nodes (LN⁺, n = 8), and by the study of cytokine release profiles (n = 4 for both LN⁻ and LN⁺). We found significantly lower CD4⁺ and higher CD8⁺ T-cell frequencies in LN⁺ samples, accompanied by increased surface levels of activation markers (HLA-DR; ICOS; PD-1; CTLA-4) and the memory marker CD45RO. Furthermore, in LN⁺, we found increased rates of a potentially regulatory antigen-presenting cell (APC) subset (CD11c⁺CD14⁺PD-L1⁺) and of myeloid-derived suppressor cell subsets; the LN⁺ APC subset correlated with significantly elevated frequencies of FoxP3⁺ regulatory T cells (Treg). After in vitro stimulation with different Toll-like receptor (TLR) ligands (PGN; Poly-IC; R848), we observed higher production levels of IL6, IL10, and TNFα but lower levels of IFNγ in LN⁺ samples. We conclude that, despite increased T-cell differentiation and activation, a switch to a profound immune-suppressive microenvironment in LN⁺ of patients with cervical cancer will enable immune escape. Our data indicate that the CD14⁺PD-L1⁺ APC/Treg axis is a particularly attractive and relevant therapeutic target to specifically tackle microenvironmental immune suppression and thus enhances the efficacy of immunotherapy in patients with metastasized cervical cancer. Cancer Immunol Res; 3(1); 48–58. ©2014 AACR.

Introduction

Cervical cancer is the fourth leading cause of cancer-related death among women worldwide and is caused by a persistent infection of the basal layer of the cervical epithelium by sexually transmitted, oncogenic human papillomavirus (HPV; refs. 1, 2).

Cervical cancer tumor cells are able to escape the immune system by provoking an immunosuppressive state of their microenvironment (3–6). Indeed, we and others have shown that various immunosuppressive cells, including regulatory T cells (Treg), regulatory dendritic cells (DC), myeloid-derived suppressor cells (MDSC), N2 neutrophils, and tumor-associated macrophage (TAM) subsets, are recruited to, and expanded and activated at, the site of the primary cervical tumor (5–8). These immune cells are able to inhibit and suppress normal activation of the immune system by their cell surface receptors and the cytokines they release, and may thus promote a tolerogenic microenvironment in the tumor-draining lymph nodes (TDLN), allowing tumors to grow and metastasize (9, 10).

TDLNs are the first lymph nodes (LN) that are under the influence of tumor-derived factors and in which an immune response can be generated by the activation of naïve T and B cells (9, 10). Thus, the state of the TDLN microenvironment is critical in the initial decision between activation and suppression of the immune system by the primary tumor. A better understanding of the microenvironment of the cervical TDLNs is therefore critical for the development of new immunotherapeutic strategies. Very few reports have been published on the phenotyping and enumeration by flow cytometry of immune-cell subsets in the TDLNs of patients with cervical cancer (11, 12). We have therefore undertaken a comprehensive flow cytometry-based study, analyzing various T-cell populations (i.e., activated T cells, effector-memory T cells, Tregs), five antigen-presenting cell (APC) subsets, and two MDSC subsets in tumor-free (LN⁻, tumor-negative lymph node) and metastatic TDLNs (LN⁺, tumor-positive lymph node) of patients with cervical cancer. In addition, we have studied the cytokine release profile (IL4, IL6, IL10, TNFα, and...
IFNγ) after in vitro stimulation of single-cell suspensions of LN− and LN+ samples by different Toll-like receptor (TLR) ligands (PGN, Poly-IC, and R848).

Our data reveal an immunosuppressive microenvironment in the LN+ of patients with cervical cancer, indicated by the accumulation of immunosuppressive immune effector cells and elevated levels of IL10. This study provides crucial information informing the future development of immunotherapeutic interventions aimed at breaking microenvironmental immune suppression in cervical cancer.

Materials and Methods

Subjects and ethical approval
Women presenting histologically proven cervical cancer that were scheduled for radical hysterectomy or LN debulking at member institutions of the Center for Gynaecologic Oncology Amsterdam (CGOA), i.e., Antoni van Leeuwenhoek (AvL) Hospital, the Academic Medical Center Amsterdam (AMC), and the VU University Medical Center (VUMc), were enrolled in this study. The study design was approved by the Medical Ethical Committees of the AvL, AMC, and VUMc. All included subjects gave written informed consent. None of the patients underwent chemotherapy or radiotherapy before surgery. See Table 1 for all clinical and pathologic characteristics of the study cohort.

LN collection and processing
In total, 29 TDLNs from patients with cervical cancer were collected and used for this study. LNs deemed of sufficient size were used for the collection of lymphoid cells, essentially as described previously (13). One LN per patient was cut into two pieces and viable lymphoid cells were isolated by scraping (circa 10 times) the surface with a surgical blade (size no. 22; Swann Morton Ltd). Between scappings, the cells were rinsed from the blade in 30-ml dissociation medium composed of Iscove Modified Dulbecco Medium (IMDM; Lonza) supplemented with 0.1% DNase I (Roche), 0.14% Collagenase A (Roche), and 5% FCS. Next, imprints were made by gently touching the LN to a microscope slide. These imprints were allowed to dry at room temperature (RT) for 24 hours and stored at −20°C until they were used for immunofluorescence staining.

After the collection of LN material, the LN was processed for routine diagnostic pathology procedures. Definitive diagnosis by the pathologist was used as criterion for the definition of LN− and LN+.

The collected single-cell suspensions were transferred to a sterile flask and were incubated on a magnetic stirrer for 30 to 45 minutes at 37°C. All further steps were performed with IMDM medium containing 25 mmol/L HEPES, 1-glutamine (BE12722F; Lonza), 10% FCS, 50 IU/mL penicillin (Astellas), and streptomycin (X-Gen Pharmaceuticals). After incubation, the cell suspension was run through a 100-μm cell strainer (BD Falcon) and brought to 50 ml with IMDM medium. Then, the cell suspension was centrifuged in a Rotanta 460R (Hettich) at 1,560 rpm for 5 minutes at 4°C. The cells were washed with 10 ml of IMDM medium and resuspended in 3 to 10 ml IMDM medium for viable cell count with trypan blue exclusion. Most samples were used directly for flow cytometry, and a few samples were stored in liquid nitrogen until testing in the cytokine release assay.

Phenotyping of immune cells by flow cytometry
To phenotype and compare the immune-cell composition of TDLNs, 20 LN− and 8 LN+ were used for flow cytometric analysis. Four-color flow cytometry was performed on the single-cell suspensions using antibodies to CD3, CD11c, CD25, HLA-DR (all from BD), PD-1, and CD15 (Pharmingen; all labeled with APC); CD3, CD8, CD14, and CD123 (all from BD; all labeled with PerCp-Cy5.5); CD45RA, CD86, CTLA-4, Foxp3 (eBioscience; all labeled with PE); B7-H4 (AbD serotec); BDCA-2 (Miltenyi Biotec); CD3, CD4, CD16, CD27, CD56, HLA-DR (BD), CD11b (eBioscience), CD19, CD40, CD80, CD86, PD-L1 (all from Pharmingen), and CD83 (Beckman Coulter; all labeled with FITC); or ICOS (eBioscience; labeled with biotin). For antibodies labeled with biotin, an additional incubation step with streptavidin-APC (eBioscience) was performed.

To identify Tregs (see Table 2 for phenotype), a membrane and intracellular staining was combined and performed in a U-bottom 96-well plate with a minimum of 150,000 cells per well. First, the cells were incubated with antibodies against membrane proteins diluted in flow cytometry buffer consisting of PBS supplemented with 0.1% BSA (Sigma-Aldrich) and 0.02% NaN3, for 30 minutes at 4°C. Then the cells were washed with cold PBS and fixed with 4% concentrate in fix-perm diluent (eBioscience) for 30 minutes at 4°C. After fixation, the cells were treated with 1% permeabilization buffer (eBioscience) and

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Table 1. Clinical and pathologic characteristics of the study cohort

<table>
<thead>
<tr>
<th>Clinical and pathologic characteristics</th>
<th>LN− (n = 9)</th>
<th>LN+ (n = 20)</th>
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<td>Age, mean ± SD</td>
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<td>43.6 ± 8.0</td>
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NOTE: The Fisher exact test was used to assess statistically significant differences between LN− and LN+.

Abbreviations: AC, adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space invasion; SCC, squamous cell carcinoma.
blocked with normal rat serum (eBioscience) for 15 minutes at 4°C. After blocking, the cells were incubated with antibodies against intracellular molecules (FoxP3, CTLA-4) for 30 minutes at 4°C. Then, the cells were washed with PBS and permeabilization buffer, resuspended in flow cytometry buffer, and transferred to Micronics (Micronic) for FACS analysis.

T-cell subsets, DC/APC subsets, and MDSCs were phenotyped by membrane staining (see Table 2 for phenotypes). This staining was performed in flow cytometry tubes (BD Falcon), wherein cells were incubated with antibodies against membrane proteins dilut- 

Table 2. Phenotypes and percentages of immune-cell subsets in TDLNs of patients with cervical cancer

<table>
<thead>
<tr>
<th>Target population</th>
<th>Phenotype</th>
<th>LN</th>
<th>LN</th>
<th>P</th>
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<tr>
<td>T cells</td>
<td>CD3+CD4+</td>
<td>61.07±4.40</td>
<td>79.62±2.14</td>
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<tr>
<td>CD8+ T cells</td>
<td>CD3+CD8+</td>
<td>32.45±4.98</td>
<td>18.31±2.16</td>
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<td>Double-negative T cells</td>
<td>CD3+CD4-CD8-</td>
<td>4.24±0.59</td>
<td>1.51±0.36</td>
<td>&lt;0.001</td>
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<tr>
<td>Double-positive T cells</td>
<td>CD3+CD4+CD8+</td>
<td>2.25±0.95</td>
<td>0.55±0.08</td>
<td>0.002</td>
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<tr>
<td>Activated T cells</td>
<td>CD3+CD4HLA-DR+</td>
<td>37.24±9.45</td>
<td>16.25±1.88</td>
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<tr>
<td>CD3+CD8HLA-DR+</td>
<td>49.47±15.52</td>
<td>20.73±3.80</td>
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<td>CD3+CD4ICOS</td>
<td>28.00±8.78</td>
<td>13.87±2.54</td>
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<td>CD3+CD8ICOS</td>
<td>9.57±4.05</td>
<td>4.58±0.57</td>
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<td>Inhibited T cells</td>
<td>CD3+CD4CTL-A-4</td>
<td>27.01±4.13</td>
<td>15.07±1.19</td>
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<td>CD3+CD8PD-1</td>
<td>22.11±6.09</td>
<td>4.43±0.44</td>
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<td>CD3+CD8PD-1</td>
<td>22.90±8.67</td>
<td>4.10±0.80</td>
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<td>Naive T cells</td>
<td>CD3+CD4CD27CD45RA+</td>
<td>39.32±12.62</td>
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<tr>
<td>CD3+CD8CD27CD45RA+</td>
<td>42.22±22.24</td>
<td>63.74±6.04</td>
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<td>Memory-like T cells</td>
<td>CD3+CD4CD45RO</td>
<td>62.14±12.56</td>
<td>36.64±4.29</td>
<td>0.152</td>
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<tr>
<td>CD3+CD8CD45RO</td>
<td>58.46±17.00</td>
<td>24.21±4.39</td>
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<tr>
<td>CD3+CD4CDRA CD27</td>
<td>54.25±21.39</td>
<td>29.39±5.07</td>
<td>0.099</td>
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<tr>
<td>Effector-like T cells</td>
<td>CD3+CD4CDRA CD27</td>
<td>0.98±0.57</td>
<td>1.33±0.29</td>
<td>0.801</td>
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<tr>
<td>CD4+ Tregs</td>
<td>CD3+CD4CD27Foxp3+</td>
<td>10.08±2.27</td>
<td>2.99±0.36</td>
<td>&lt;0.001</td>
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<td>CD8+ Tregs</td>
<td>CD3+CD8CD25Foxp3+</td>
<td>1.36±0.63</td>
<td>0.42±0.09</td>
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<td>DC subsets</td>
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<td>CD14+CD68</td>
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<td>0.02±0.01</td>
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<td>LCs</td>
<td>CD11c+ICOS</td>
<td>0.01±0.00</td>
<td>0.00±0.00</td>
<td>&lt;0.001</td>
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<tr>
<td>CD4+ (APC)</td>
<td>CD1a+CD1c+</td>
<td>1.08±0.34</td>
<td>0.19±0.04</td>
<td>0.049</td>
</tr>
<tr>
<td>CD14 (LNDC)</td>
<td>CD1a+CD1c+</td>
<td>0.50±0.06</td>
<td>0.34±0.07</td>
<td>0.032</td>
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<td>Plasmacytoid DCs</td>
<td>CD23+BDCA-2</td>
<td>0.59±0.19</td>
<td>0.58±0.18</td>
<td>0.653</td>
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<tr>
<td>MDSCs</td>
<td>Monocytic MDSCs</td>
<td>Lin+CD3+CD4+HLA-DR-/low</td>
<td>0.09±0.03</td>
<td>0.01±0.01</td>
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<td>Granulocytic MDSCs</td>
<td>CD11b+CD3+CD15</td>
<td>0.50±0.41</td>
<td>0.17±0.09</td>
<td>0.660</td>
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</table>

NOTE: Data are expressed as mean ± SEM.

Abbreviations: DDC, dermal-like dendritic cell; LC, Langerhans cell; LNDC, lymph node-resident dendritic cell.

Data were analyzed using CellQuest Pro software (BD), were collected as percentages or as median fluorescence index (MFI; median fluorescence of marker/median fluorescence of isotype), and expressed as mean ± SEM.

Immunofluorescence staining and imaging

Slides with LN imprints (LN+ n = 8, LN- n = 10) were fixed in acetone for 10 minutes at RT. Then, the slides were washed in PBS for 5 minutes and then incubated for 1 hour at RT with directly labeled fluorescent antibodies: mouse IgG2a FITC-conjugated anti-human CD14 (BD), mouse IgG1 PE-conjugated anti-human CD163 (BD Pharmingen), and mouse IgG1 APC-conjugated anti-human CD27 (BD). Afterwards, slides were washed three times in PBS for 5 minutes and incubated with 1:1,000 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 1 minute at RT. Subsequently, slides were washed in PBS and mounted under coverslips with Mowiol. The slides were evaluated using a Fluorescence microscope (Axiovert-200M) at a magnification of ×100 and ×400, and pictures were taken with a sensicam camera (PCO) and Slidebook 6 reader software (Intelligent Imaging Innovations).

Cytokine release assay

To monitor cytokine release in TDLNs, we used 1 × 10^5 viable cells from frozen single-cell suspensions from LN+ (n = 4) and LN- (n = 4). Cryostorage and thawing of LN cells were carried out as previously described (14). Single-cell suspensions were plated directly into a U-bottom 96-well plate in 100 µL IMDM medium with 10% FCS. Cells from each LN were cultured in triplicate per test condition (from one LN+ in
duplicate), i.e., without stimulation (no), or with different TLR ligands: TLR2-L (PGN, 10 μg/mL, InvivoGen), TLR3-L (PolyIC, 20 μg/mL; InvivoGen), and TLR7/8-L (R848, 10 μg/mL; InvivoGen), at 37°C for 24 hours. The next day, supernatants were harvested and stored at –20°C until further analysis. Analysis of IL4, IL6, IL10, TNFα, and IFNγ was conducted using a Cytometric Bead Array (CBA) human Th1/Th2/Th17 cytokine kit (BD), and analyzed on the BD FACScan flow cytometer. Quantity (pg/mL) of the respective cytokines was calculated using FCAP array software (Soft Flow Hungary Ltd.). Values were set at zero when sample intensities did not fit within the limits of the standard curve according to the “limit of detection data table” in the user manual of the CBA Kit (i.e., were below the detection limit). To calculate the IFNγ/IL10 ratio, values under the limit of detection were set at 1 pg/mL.

Statistical analysis

The Fisher exact test was used to assess statistically significant differences in clinical and pathologic patient characteristics between LN− and LN+ in IBM SPSS Statistics 20. Data were tested for normal distribution using the Kolmogorov–Smirnov test. Differences in immune-cell populations and cytokine release levels between LN− and LN+ were analyzed by the two-sided unpaired t test when parameters showed a normal distribution or alternatively analyzed by the Mann–Whitney U test, using Microsoft Excel or GraphPad Prism software. Correlations between two

![Figure 1](https://www.aacrjournals.org/cancerimmunolres/article-pdf/3/1/51/400159/51.pdf)

**A.** T-cell subsets in TDLNs of patients with cervical cancer. A, lower percentages of CD4+ T cells and higher percentages of CD8+ T cells (left) were present in LN+. More double-negative (CD4−CD8−) and double-positive (CD4+CD8+) T cells (right) were found in LN+ compared with LN−. B, higher frequencies of CD4+ (left) and CD8+ T cells (right) expressing the activation markers HLA-DR and ICOS and higher expression of the coinhibitory markers CTLA-4 and PD-1 in LN+ compared with LN−. C, lower frequencies of naive CD4+ (left) and CD8+ T cells (right) in LN+ compared with LN−. Higher frequencies of CD4+ and CD8+ T cells expressing CD45RO in LN+ compared with LN−. There was no statistically significant difference in the frequency of CD45RA−CD27+ or CD45RA−CD27− CD8+ T cells between LN− and LN+. Error bars, SEM. *P = 0.01 to 0.05; **, P = 0.001 to 0.01; and ***, P < 0.001.

![Figure 2](https://www.aacrjournals.org/cancerimmunolres/article-pdf/3/1/51/400159/51.pdf)

**B.** T-cell activation and inhibition. B, higher frequencies of CD4+ and CD8+ T cells expressing CD45RO in LN+ compared with LN−. There was no statistically significant difference in the frequency of CD45RA−CD27+ or CD45RA−CD27− CD8+ T cells between LN− and LN+. Error bars, SEM. *P = 0.01 to 0.05; **, P = 0.001 to 0.01; and ***, P < 0.001.

![Figure 3](https://www.aacrjournals.org/cancerimmunolres/article-pdf/3/1/51/400159/51.pdf)

**C.** Effector and memory T cells. C, higher frequencies of CD4+ and CD8+ T cells expressing CD45RO in LN+ compared with LN−. There was no statistically significant difference in the frequency of CD45RA−CD27+ or CD45RA−CD27− CD8+ T cells between LN− and LN+. Error bars, SEM. *P = 0.01 to 0.05; **, P = 0.001 to 0.01; and ***, P < 0.001.
Figure 2.
Tregs and APC subsets in TDLNs of patients with cervical cancer. A, more CD4^+ Tregs as well as CD8^+ FoxP3^+ Tregs were present in LN^+ compared with LN^- B, significantly higher percentages of DDCs, LCs, CD14^+ APCs, CD14^- LNDCs and no statistically significant differences for pDCs were found in LN^+ compared with LN^- C, MFI of costimulatory surface molecules (CD40, CD80, CD83, and CD86) and coinhibitory surface molecules (PD-L1 and B7-H4) on CD14^+ APCs in LN^- and LN^+. MFI of PD-L1 on CD14^+ APCs was significantly higher in LN^-. (Legend continued on the following page.)
parameters were examined by linear regression (F-test) using GraphPad Prism software. Differences and correlations were considered statistically significant when P < 0.05.

Results

Phenotype and enumeration of immune-cell subsets in relation to tumor status of TDLNs from patients with cervical cancer

To gain a better understanding of the characteristics of cervical TDLNs in relation to immune escape and metastatic spread to inform the development of new immunotherapeutic strategies against cervical cancer, we set out to identify and compare various immune-cell subsets in the LN- versus LN+ patients with cervical cancer. An overview of the percentages of immune-cell populations detected in LN- and LN+ is given in Table 2.

We studied the T-cell population in LN- and LN+ and found a significantly lower proportion of CD4+ T cells in LN+ (P < 0.001), whereas significantly more CD8+ T cells were present in LN+ than in LN- (P = 0.006; Fig 1A, left). Double-negative (CD4-CD8-) and double-positive (CD4+CD8+) T cells were remarkably more frequent in LN+ (P < 0.001 and P = 0.002, respectively; Fig. 1A, right). Of note, CD4+ T cells in LN+ expressed higher levels of HLA-DR (P = 0.014). ICOS (n.s.), CTLA-4 (P < 0.001), and PD-1 (P < 0.001) on their surface (Fig 1B, left), evidence of an increased activation state. Similarly, expression levels of these markers were elevated on CD8+ T cells in LN+, but only the increase in the expression of PD-1 reached statistical significance (P = 0.001; Fig 1B, right).

Furthermore, we observed a trend for increased rates of memory CD4+ T cells (P = 0.152) and a statistically significant increase in the rates of CD8+ memory T cells (P = 0.011, CD45RO+T cells) in LN+ compared with LN-, whereas there were no statistically significant differences in frequencies of naïve CD4+ and CD8+ T cells (CD45RA+CD27-, or CD45RA+CD27+ (effector) and CD45RA-CD27+ (central memory) CD8+ T-cell populations. Overall, an obvious trend of less naïve T cells and more memory T cells was observed in LN+, suggestive of tumor-associated T-cell activation (Fig. 1C).

We also studied Tregs and found significantly higher proportions of CD4+ (P < 0.001) and CD8+ (P = 0.029) Tregs, gated on CD25+FoxP3+, in LN+ as compared with LN- (Fig. 2A). Moreover, the CD8+ T-cell/Treg ratio was decreased in LN+ compared with LN- (P = 0.048).

Next, we studied four myeloid APC subsets (as previously described in ref. 15), including the migratory DC subsets CD11c+CD1a+CD33-CD123-Langerhans cells (LC), and CD1a+CD11c+CD14- LN-resident DCs (LNDCC) as well as CD11a+CD11c+CD14+ APCs. Our data showed higher frequencies in LN+ for CD11c+CD1a+DDCs (P = 0.019), CD11c-CD1a+LGCs (P < 0.001), CD14+ APCs (P < 0.001), and CD14- LNDCC subsets (P = 0.032). In addition, we investigated the plasmacytid DC (pDC) subset, identified as CD123+BDCA-2+, and found no statistically significant differences in proportions between LN- and LN+ (Fig. 2B). We also studied the expression of costimulatory (CD40, CD80, CD83, and CD86) surface markers on these APC subsets, but found no statistically significant differences in expression levels with the one exception of CD86 on pDCs, which was higher in LN+ (MFI 1.53 ± 0.07) as compared with LN- (MFI 1.02 ± 0.14; P = 0.010; data not shown). We also studied expression of the coinhibitory molecules PD-L1 and B7-H4. Interestingly, only CD14+ APCs in LN+ expressed significantly higher surface levels of the coinhibitory molecule PD-L1 (MFI, 1.56 ± 0.21 vs. 1.13 ± 0.05; P = 0.008; Fig. 2C). As the measured PD-L1 levels were relatively low, we confirmed the elevated PD-L1 expression levels on CD14+ APCs in LN+ by immunocyto logical staining of LN imprints. To check whether these CD14+ cells also expressed the M2-macrophage marker CD163, we established a triple immunofluorescence staining on LN imprints with the markers CD14, CD163, and PD-L1. We confirmed the presence of clearly elevated numbers of CD14+ cells in LN+ that coexpressed PD-L1 and often also CD163. In contrast, lower numbers of CD14+ APCs in LN- generally coexpressed CD163, but only low to undetectable levels of PD-L1 (see Fig. 2D and E and Supplementary. Fig S1 for representative examples). Of note, both the frequency of CD14+ APCs and their expression levels of the coinhibitory molecule PD-L1 showed a significant correlation with increased levels of CD4+ Tregs (P = 0.001 and P < 0.001, respectively; Fig. 2F and G).

We found higher frequencies of two identified MDSC subsets in LN+ as compared with LN-, i.e., monocytic and granulocytic MDSCs, gated as Lin+CD33+CD14+HLA-DRlow (mMDSC) and CD11b+CD33+CD15+ (gMDSC), respectively (Fig. 3A and B). Only for mMDSCs did this difference reach statistical significance (P = 0.011).

Cytokine release in response to different TLR ligands

In vitro stimulation of single-cell suspensions from LN+ and LN- was performed with TLR-2, -3, and -7/8 ligands to study resulting cytokine release profiles. Overall, IL4 release was mostly unaffected, whereas higher release levels of IL6, IL10, and TNF was found in LN+ and higher IFNγ release levels in LN- (Fig. 4A-E). Higher IFNγ/IL10 ratios in LN- than in LN+ under all test conditions signified a more favorable balance between functional type-1 immune activation and immune suppression in LN+ (Fig. 4F).

Association between immunosuppressive immune cells and lymphovascular space invasion of tumor cells

Lymphovascular space invasion (LVI) is a prognostic factor in early-stage cervical cancer and precedes metastasis to cervical TDLNs. In our study cohort, higher rates of LVI were observed in patients with LN+ as compared with patients with LN- (P = 0.015; Table 1). Seven of 20 patients with LN+ manifested with LVI. In these patients with early signs of lymphatic tumor spread,
we found decreased frequencies of CD4$^+$ T cells ($P = 0.023$), increased frequencies of CD8$^+$ T cells ($P = 0.025$), but decreased frequencies of CD8$^+$CD43RA$^{-}$CD27$^-$ effector T cells ($P = 0.025$; Fig. 5).

**Discussion**

In the present study, we have investigated the microenvironment in LN$^-$ and LN$^+$ of patients with cervical cancer by flow cytometric characterization of different immune-cell subsets and their cytokine release profile after *in vitro* TLR stimulation. Our results indicate that high frequencies of immunosuppressive cell subsets and an immunosuppressive cytokine profile are associated with LN metastases in early-stage cervical cancer. This finding is consistent with the study from Battaglia and colleagues (12), who looked at other subsets than in our study, but similarly found an immunosuppressive microenvironment mediated by activated Tregs and VEGF production by metastatic tumor cells in LNs of patients with cervical cancer. In addition, we correlated the clinical patient characteristics with the measured TDLN immune parameters and observed the first signs of an immune-suppressed microenvironment in patients with LN$^+$ manifesting with lymphatic tumor involvement (LVSI).

Despite the finding of activated effector T-cell subsets, which suggests activation of the immune system against the spreading tumor, the microenvironment of LN$^-$ from patients with cervical cancer is predominantly marked by immunosuppressive cell subsets most likely for keeping the effector cells in check. Higher frequencies of CD4$^+$ and CD8$^+$ T cells expressed the immune checkpoint molecules PD-1 and CTLA-4, and, as expected, higher proportions of suppressive CD4$^+$ and CD8$^+$ Tregs were present in LN$^+$ (12). Also, increased levels of Tregs have been reported to be present at the site of the primary tumor (7) and in the peripheral blood of patients with cervical intraepithelial neoplasia (CIN) or cervical cancer (16). Of note, we previously reported that high Treg frequencies in the peripheral blood were associated with high-grade CINIII lesions and persistent HPV16 infection (17). Patients with higher CD8$^+$ T cell/Treg ratios in primary tumor tissue have a prolonged survival time compared with patient with lower CD8$^+$ T cell/Treg ratios (7). Consistent with this notion, we observed significantly lower CD8$^+$ T cell/Treg ratios in LN$^+$ as compared with the ratios in LN$^-$ van der Burg and colleagues (18) showed the presence of HPV-specific CD4$^+$ Tregs in LN$^+$ of patients with cervical cancer, which were able to inhibit proliferation and cytokine production by other T cells. Tregs in cervical cancer draining LNs express Neuropilin-1 (Nrp1) on their surface, which can bind to tumor-derived VEGF, thus further promoting their immunosuppressive activity (12). Moreover, we found two other potentially suppressive subsets, CD4$^+$CD8$^+$ T cells and CD4$^+$CD8$^-$ T cells, to be significantly overrepresented in LN$^+$ (19, 20). Similarly, elevated proportions of CD4$^+$CD8$^+$ T cells were detected in patients with breast cancer (21), Hodgkin lymphoma (22), and melanoma (23). The functional role of CD4$^+$CD8$^-$ T cells, however, remains controversial, as they may represent a regulatory subset, involved in immune regulation and tolerance (20), but they have also been ascribed antitumor activity (24).

Our study is the first to investigate four DC/APC subsets in cervical cancer TDLNs that were identified previously in sentinel LNs from patients with early-stage melanoma (15). Interestingly, compared with LC proportions in skin-draining lymph nodes (15), almost no LCs were present in cervical TDLNs. This phenomenon has also been observed at the site of the primary tumor, where a low density of LCs was reported in CIN and cervical cancer as compared with the steady-state healthy cervix (25–27). Our observation of elevated levels of mature CD1a$^+$ migratory DC subsets (including LCs) in LN$^+$ indicates increased migration of these cells from cervical tumors and, possibly combined with disturbed homeostatic
microenvironment in metastatic cervical cancer lymph nodes

DC development at the tumor site, may account for the reported decreases of these DCs in the primary cervical tumor site. This contrasts with observations of decreased density of mature DCs in metastatic LNs from patients with breast cancer (28), gastric cancer (29), melanoma (30), and endometrial cancer (31). Although we did not observe differences in the maturation state of the migratory and LNDC subsets, we did find significantly higher expression of PD-L1 by the CD14

Figure 4.
Spontaneous or TLR ligand-induced cytokine release by single-cell suspensions from cervical cancer TDLNs. Release in pg/mL of (A) IL4, (B) IL6, (C) TNFα, (D) IL10, and (E) IFNγ after in vitro culture for 24 hours with or without PGN, Poly-IC (PIC), or R848 in LN− (n = 4) and LN+ (n = 4, n = 3 for PIC stimulation) suspensions of patients with cervical cancer. F, IFNγ:IL10 ratio for each condition was calculated and showed a higher ratio in LN− vs. LN+ after PGN, PIC, and R848 stimulation; all ratios went down relative to no stimulation. Error bars, SEM. †, P = 0.01 to 0.05.

Figure 5.
Immune subsets associated with LVSI in patients with cervical cancer with LN−. The graph shows significantly (A) less CD4+ T cells, (B) increased frequencies of CD8+ T cells, (C) less CD8+CD45RA−CD27+ effector T cells when patients with cervical cancer with LN− manifested with LVSI compared with patients with LN+ without LVSI (n = 6-7 for LVSI (no); n = 8 for LVSI (yes)). Error bars, SEM. †, P = 0.01 to 0.05.
frequencies of these CD14+ APCs and their expression levels of PD-L1 on the one hand with Treg rates on the other indicates an important role of these cells in the creation of an immunosuppressive microenvironment in tumor-containing LNs and to a possible involvement of suppressive feedback through PD-1+ T cells. Interestingly, in HPV-associated head and neck cancer, a link has been found between PD-L1 and PD-1 in lymphoid tissue and possible immune escape (41). Our study is the first to report the presence of MDSCs in human LNs (42), with a specific enrichment in LN2. Through cellular cross-talk, these MDSCs may further amplify the immunosuppressive effects of the CD14+ APCs and Tregs (42–44). Thus, blocking of PD-1 or PD-L1 could serve as a potential therapeutic target to interrupt this immunosuppressive cycle mediated by CD14+ PD-L1+ APCs, PD-1+ T cells, Tregs, and further reinforced by MDSCs.

Spontaneous and TLR-L–induced ex vivo cytokine release confirmed the functionally immunosuppressive microenvironment in LN2 by concerted elevated levels over LN1 of IL6, IL10, and TNFα, the combined effects of which may be expected to result in T-cell and DC suppression, TAM and MDSC activation, as well as enhanced tumor invasion and angiogenesis (44–46). In contrast, higher IFNγ release levels specifically upon R848/TLR-7/8 stimulation in LN2 point to type-1 immune activation with antitumor potential. Nevertheless, concomitant TLR-induced increases in IL10 release (leading to reduced IFNγ/IL10 ratios relative to spontaneous release), as well as IL6 production with potentially tumor-promoting properties (5, 18, 47, 48), calls for caution and indicates that the therapeutic use of TLR-L in the context of vaccination or immune potentiation should be combined with agents targeting immune suppression, including JAK2/STAT3, IDO, or checkpoint inhibitors. TLR stimulation might be able to “awaken” the tumor-specific T cells present in the TDLNs (49), whereas blocking of immune inhibitors can further alleviate immune suppression.

The distance of the lymph nodes relative to the primary tumor or other tumor-containing lymph nodes is likely to influence the immune state in LN2 and is important to take into account. This was supported by studying LVSIs, which is a prognostic factor in early-stage cervical cancer and precedes, and is significantly associated with, the risk of pelvic node metastases in cervical cancer (50). As expected, we found that all patients with LN2+ manifested with LVSIs. In addition, we found decreased frequencies of CD8+ T cells to be significantly associated with the presence of LVSIs in patients with LN+. Of note, despite the overall increase in CD8+ T cells, we found a selective and significant decrease in CD8+ CD45RA−/CD27− effector T cells in these LNs. We observed the same changes in CD4/CD8 ratios in LN2, suggesting that this marks the first sign of a switch to an immunosuppressive microenvironment in which the tumor cells are able to escape and invade the lymphovascular space, with a particular role of CD8+ effector T cells in control of this early tumor spread.

In conclusion, our findings demonstrate substantial differences in the frequencies of immune effector cell subsets and cytokine production between LN1 and LN2 of patients with cervical cancer. These changes may be related to the metastatic tumor cells in the LNs, resulting in T-cell activation, which may be overruled by suppression perpetrated by regulatory subsets, including Tregs, CD14+ APCs (41), and MDSCs. This immunosuppressive microenvironment is most likely able to negate a successful antitumor response and thus facilitates metastatic spread. In patients with cancer, the presence of suppressive factors and regulatory immune subsets can hinder vaccination efficacy. We found that high and interrelated rates of PD-L1+CD14+ APCs and Tregs mark the microenvironment of LN2. Therefore, a combinatorial immunotherapy with PD-1/PD-L1 checkpoint inhibition and immune potentiation, for example via TLR stimulation, might be considered to interrupt this immunosuppressive cycle and induce effective antitumor immunity to halt metastatic spread in patients with cervical cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


High and Interrelated Rates of PD-L1<sup>+</sup>CD14<sup>+</sup> Antigen-Presenting Cells and Regulatory T Cells Mark the Microenvironment of Metastatic Lymph Nodes from Patients with Cervical Cancer

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