TRANSLATING IMMUNE CHARACTERISTICS OF METASTATIC TUMORS INTO THERAPEUTIC TARGETS FOR CERVICAL CANCER
Translating immune characteristics of metastatic tumors into therapeutic targets for cervical cancer

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Translating immune characteristics of metastatic tumors into therapeutic targets for cervical cancer

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Cover: Ilse Modder, www.ilsemodder.nl
Cover image: My first beautiful 7-color multiplex IHC staining, December 2015  “However, cancer still sucks in every color: [Primary Cervical Cancer, CD3/CD8/FoxP3/keratin/PD-L1/CD163/DAPI]
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CHAPTER 1

General introduction and outline of this thesis
Despite preventive measures like early detection screening programs and prophylactic human papillomavirus (HPV) vaccination, cervical cancer is worldwide still a major clinical problem. This disease is the fourth most common cause of cancer-related death among women worldwide and is induced by a persistent infection of the cervix epithelium by various high-risk HPV strains. This sexually-transmitted, double-stranded DNA virus is capable of transforming healthy cells of the basal epithelial cell layer into cervical intraepithelial neoplastic lesions that can further progress to invasive tumor cells. The high-risk types HPV16 and -18 are mainly involved and they are responsible for approximately 70% of all cervical tumors worldwide. The two most common cervical tumor histology types are squamous cell carcinoma (SCC) and adenocarcinoma (AC) which differ substantially in clinical outcome, HPV status, gene expression signature, and immunological characteristics.

Anti-tumor immune response

Cervical cancer is characterized by (1) continuous expression of the HPV oncoproteins E6 and E7 and (2) high rates of oncogenic mutations, like e.g. PIK3CA and KRAS. Both viral oncoproteins and neoantigens arising from non-synonymous mutations are able to evoke tumor-specific antigens that can be recognized and picked up by immature antigen-presenting cells (APCs), including i.e., dendritic cells (DCs) and macrophages. After maturation and migration to the tumor-draining lymph nodes (TDLNs), these APCs can induce an anti-tumor T-cell response. Indeed, HPV-specific T cells have been detected in precursor lesions and cervical cancer patients’ blood samples and tumors. Also, reactive T cells targeting non-viral tumor antigens have shown to be present in cervical cancer. Therefore, this tumor requires a highly immune-suppressed microenvironment to progress and metastasize to the pelvic catchment area. During cancer development, cervical tumor cells can acquire several immune escape mechanisms to avoid an efficient anti-tumor response. An overview of these immune suppressive mechanisms is provided in the paragraphs below.

Immune characteristics of primary cervical cancer

Primary tumor – Lymphocytes

Cervical tumors can be highly infiltrated with different types of activated and proliferating T cells, including cytotoxic CD8+ T cells and CD4+ T helper (Th) cells, but also with suppressive regulatory T cells (Tregs). CD8+ T cells recognize antigens presented by human leukocyte antigen (HLA) class I and are able to kill virus-infected cells and tumor cells via the first apoptosis signal (Fas):Fas-ligand interaction and the secretion of i.e., interferon (IFN)-γ, Granzyme B, and perforin. CD4+ T cells recognize antigens presented by HLA class II and this population mainly consists of Th cells. They can activate macrophages (through a Th1 response) via CD40:CD40-ligand interaction and the secretion of a.o. IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α. In addition, they can stimulate B cell differentiation (Th2 response) via CD40:CD40-ligand interaction, interleukin (IL)-4, IL-5 and IL-10. An effective T cell response can be inhibited by suppressive
Forκhead box P3 (FoxP3)+CD4+ Tregs through the secretion of suppressive cytokines IL-10 and transforming growth factor (TGF)-β and through direct and indirect cell-to-cell contact via membrane-bound TGF-β and Cytotoxic T Lymphocyte-associated Antigen 4 (CTLA-4), respectively.47,48

Patients manifesting with tumors infiltrated with high numbers of CD8+ T cells, a high CD8+/CD4+ T cell ratio, and a high CD8+ T cell/FoxP3+ Treg ratio have less often lymph node metastases and a better survival outcome.42-43,45 In addition, although only a small fraction is present in tumors, higher numbers of Th cells producing pro-inflammatory cytokine IL-17 were found to be associated with improved survival outcome in SCC patients, while these cells had the opposite effect in AC patients.25 High levels of FoxP3+ T cells in the tumor microenvironment (TME) of cervical SCC is correlated to poor patient outcome,45,46 whereas, remarkably, in cervical AC, an opposite effect was seen with the presence of FoxP3+ cells showing a reverse correlation with survival.25 Tumor-produced TGF-β can recruit these FoxP3+ Tregs.47,50 In addition, TGF-β can promote the formation of extracellular matrix, which in turn inhibits intratumoral T cell infiltration by acting as a physical barrier.47,53. Furthermore, T cell function can be inhibited by various coinhibitory immune checkpoint interactions with tumor cells and other immune cells.24,52, which will be discussed below under the subheading ‘Primary tumor - Immune checkpoint molecule expression’.

Next to T cells, Natural Killer (NK) cells are also potent in exerting rapid cytotoxic tumor cell death.47 They have been found in a lesser extent than T cells to also infiltrate cervical tumors.47,48,54 They can function as effector cells through antibody-dependent cell-mediated cytotoxicity (ADCC), through death ligand/receptor ligation, and through the production of various cytokines like IFN-γ and TNF-α.55 However, also NK cells can be inhibited by the tumor, which will be shortly discussed below under the subheading ‘Human leukocyte antigen (HLA) modulation’.

Primary tumor - Myeloid immune cells

APC subsets are key players in orchestrating and initiating an anti-tumor T cell response.24,56 Most tumors are infiltrated by various APC types, which, due to local microenvironmental conditions, often present with an immature and/or tolerogenic phenotype.56-58 Not only the function, but also the number of APCs can be affected by immune suppressive conditions in tumors. In cervical premalignant lesions, relatively high numbers of activated (DC-LAMP+) DCs are present, whereas the number of Langerhans cells (LCs) decreases in the course of tumor development.59-62 A study in mice has suggested a crucial role for the HPV oncoprotein E7 in the depletion of LCs.63 Interestingly, LCs were found to be located only in the cervical tumor cell nests, whereas mature DCs were located in the stromal compartment.64,65

Infiltration of tumor-associated macrophages into cervical tumors has been reported as well.55,56 Macrophages can be polarized to an M1-like phenotype (i.e., classically activated macrophages) or to an M2-like phenotype (i.e., alternatively activated macrophages).47 Interestingly, CD14+CD33+CD163+M1-macrophages infiltrating tumor fields have been associated with an influx of T cells and improved survival outcome of patients with cervical cancer.65 In contrast, M2-like (CD14+CD163+) cells were shown to be positively correlated with higher numbers of Tregs, thus potentially negatively affecting clinical outcome.66

Another myeloid immune cell subset infiltrating cervical cancer tissue is myeloid-derived suppressor cells (MDSCs).67 MDSCs comprise of a heterogeneous group of immune cells from myeloid origin and are able to inhibit CD8+ T cell function via the production of nitric oxide and arginase.48 Moreover, monocytic MDSCs can differentiate into tumor-associated macrophages capable of producing suppressive cytokines such as IL-1, IL-6, IL-10 and TGF-β.68 In cervical cancer, high numbers of MDSCs in tumors are significantly associated with recurrence and metastasis.67

Primary tumor - Human leukocyte antigen (HLA) modulation

One of the escape mechanisms of tumors is to hide from the immune system by the downregulation of surface HLA class I molecules (HLA-A, -B, -C) that are responsible for tumor antigen-derived epitope presentation to cytotoxic CD8+ T cells and the subsequent induction of cytotoxic tumor cell death.69 The majority of cervical tumors show downregulation of classical HLA class I molecules, which can be caused by loss of heterozygosity at the chromosome 6p21 locus where the HLA genes are located or by allele-specific mutations, β2-microglobulin mutations, and antigen processing machinery-associated mutations.70-71 Interestingly, associations have been found between low HLA-A expression and lower numbers of infiltrating cytotoxic CD8+ T cells and between low HLA-A expression and poor survival,72 suggesting that cervical tumors are highly successful in hiding from immune system cell responses.

Cervical cancer does not only hide, but it can also actively suppress the immune system by the (upregulated) expression of non-classical HLA class I molecules that are able to inhibit NK cells, T cells and myeloid cells via various inhibitory receptors.72,83 Non-classical HLA-E and HLA-G expression by cervical tumor cells has been reported.72,74,75,84 However, clear indications for a negative prognostic impact are lacking, and, surprisingly, HLA-E expression has even been shown to correlate with improved survival in patients with cervical AC.72

Primary tumor - Immune checkpoint molecule expression

T cell infiltration and function can be hindered by the expression of and interaction with checkpoint molecules on tumor cells and/or myeloid cells.24 These coinhibitory checkpoints are crucial for maintaining self-tolerance and modulating the duration and amplitude of immune responses against pathogens at peripheral sites.47 These checkpoint pathways are abused by the TME and serve as an important mechanism to induce T cell ‘exhaustion’.53,87 T
cell exhaustion is characterized by a (reversible) reduced effector function and the sustained expression of multiple coinhibitory molecules through which T cells can be inhibited in their function. Therefore, these checkpoint pathways are highly suitable targets for immunotherapeutic approaches aimed at interrupting the immunosuppressive cycle and inducing anti-tumor immunity.

One of the most studied coinhibitory immune checkpoint pathway is the programmed death (PD)-1 receptor:PD-Ligand-1/-2 interaction. Cervical tumors can express PD-L1 and PD-L2 and can inhibit PD-1-positive T cells, present in the TME.

In addition, indoleamine 2,3-dioxygenase (IDO) can also act as an immunosuppressive factor on T cells. IDO is an intracellular enzyme that catabolizes tryptophan (trp), an essential amino acid necessary for protein synthesis and other metabolic cell functions, along the kynurenine (kyn) pathway. In vitro and in vivo studies have shown that especially activated T and NK cells are negatively affected by trp depletion and that Tregs are induced by the presence of IDO. The majority of studies in patients point to a detrimental effect of IDO expression on cytotoxic T cell infiltration and clinical outcome in various tumor types. However, there are also reports that describe the role of IDO as highly complex with no or even a beneficial effect on T cells and/or patient survival. In cervical cancer, IDO positivity is observed in tumor cells and myeloid immune cells. Diffuse IDO expression by primary cervical cancer cells was found to be correlated with an unfavorable clinical outcome.

Another coinhibitory immune checkpoint involved in immune suppression is T-cell immunoglobulin mucin domain 3 (TIM-3), which may interact with Galectin-9. Galectin-9 has been reported to be expressed by cervical tumor cells and CD163+ M2-macrophages and is responsible for inhibiting the function of T cells via binding to TIM-3, also present in the TME. Of note, TIM-3 expression has been observed also in cervical tumor cells and correlates positively with metastatic potential, advanced disease grades, and a shorter overall survival.

Moreover, cervical tumor cells can express B7-H3 (CD276) and B7-H4, which were both inversely correlated with infiltrating CD8+ T cells. There are not yet defined receptors for B7-H3 and B7-H4, but several studies in mice support a coinhibitory role for both molecules.

Many more immune checkpoint molecules exist and have been demonstrated to play a role in other tumor types, like CTLA-4, lymphocyte-activation gene 3 (LAG-3), V-domain Ig suppressor of T cell activation (VISTA), T cell immunoreceptor with Ig and ITIM domain (TIGIT), and B- and T-lymphocyte attenuator (BTLA), but these have not yet been studied in cervical cancer. See Figure 1 for an overview of coinhibitory immune checkpoints in the TME.

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**Figure 1: Coinhibitory immune checkpoints in the tumor microenvironment.** Various coinhibitory immune checkpoints can be expressed by activated T cells: PD-1, TIM-3, LAG-3, VISTA, CTLA-4, TIGIT and BTLA. Upon binding to their counterparts present on other immune cells and/or tumor cells, T cells can be functionally inhibited. IDO can also be expressed by myeloid cells and tumor cells. IDO catabolizes tryptophan (trp, purple) along the kynurenine (kyn, red) pathway, an essential amino acid necessary for protein synthesis and cellular metabolism. In this way, IDO can indirectly and negatively affect T cell function. These coinhibitory checkpoints are highly interesting targets for immunotherapeutic strategies in order to interrupt the immunosuppressive cycle and to induce an anti-tumor T cell response. Figure based on D.M. Pardoll, *Nature Reviews*, 2012 and created by the use of material available on https://smart.servier.com from LABORATOIRES SERVIER, France.

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**Immune characteristics of cervical tumor-draining lymph nodes and local metastases**

Cervical cancer is mainly a locally invading disease spreading to the parametrium, vagina, bladder and pelvic lymph nodes rather than distant sites of the body. The sentinel lymph node (SLN) is the first lymph node that is under the influence of tumor-derived factors, like e.g. IL-6, TGF-β, prostaglandin-E2 (PGE2; controlled by up-regulated cyclooxygenases), and the pro-angiogenic vascular endothelial growth factor (VEGF). During tumor progression and prior to metastasis, SLNs undergo many profound (immunological) alterations to facilitate invasion by cells derived from the primary tumor. Such alterations include increased lymphangiogenesis, blood vessel remodeling, increased chemokine and cytokine...
secretion and changes in immune cell composition resulting in a ‘tumor-supportive’ micro-environment termed the pre-metastatic niche. Eventually this can lead to the metastatic growth of tumor cells that have reached the lymph node(s) (Fig. 2).

Figure 2: Primary tumor and draining lymph node. A tumor-conditioned immune-suppressed metastatic niche emerges in tumor-draining lymph nodes (TDLNs) in the course of tumor progression. Tumor antigens (HPV- as well as neo-antigens) can be recognized and picked up by immature antigen-presenting cells (APCs), including i.e., dendritic cells (DCs) and macrophages. After maturation and migration to the TDLNs, these APC can induce an anti-tumor T- and B-cell response. However, tumors modulate their microenvironment by secreting lymphangiogenic factors in order to increase the lymph flow and by the recruitment of immunosuppressive immune cells to the TDLNs (like regulatory T cells (Tregs), tolerogenic/immature APC cells and myeloid-derived suppressor cells (MDSCs)) in order to escape from the anti-tumor response. The sentinel lymph node (SLN) is the first TDLN to be under the influence of tumor-derived factors and undergoes many profound (immunological) alterations to favor spread of the primary tumor.

Immune microenvironment in TDLNs
In cervical cancer it has been shown that tumor-negative SLN (SLN-) display active lymph-angiogenesis and higher lymphatic vessel density compared to the tumor-negative distant lymph nodes. This phenomenon is even more pronounced in tumor-positive SLN (SLN+), with metastatic tumor cells situated around enlarged vessels. These changes in lymphatic vasculature facilitates a larger influx of tumor-derived factors and immune cells.

Pre-metastatic niche formation in cervical SLN is accompanied by an increase in cytotoxic CD8+ T cells, PD-1+ cells in germinal centers (including T- and B cells), and suppressive FoxP3+ Tregs expressing the coinhibitory molecule CTLA-4. It was also shown that pelvic TDLNs in the proximity of the primary tumor harbor higher CD4+/CD8+ T cell ratios than distal TDLNs. Both CD4+FoxP3+ and CD8+FoxP3+ Tregs are even further increased in numbers and highly activated (expressing i.e., HLA-DR and neuropilin-1 (Nrp1)) in lymph nodes containing metastatic cervical tumor cells, thus promoting a suppressive TME. Interestingly, in metastatic cervical lymph nodes, Tregs were found in close proximity of IDO-expressing S100+ APCs. Tregs can induce IDO expression via a CTLA-4-dependent manner in APCs thereby indirectly suppressing adaptive T-cell-mediated immunity.

The pre-metastatic changes in T cell frequencies are accompanied by more S100+ DCs and CD1a+ DCs in SLN+ compared to non-SLN- from patients with cervical cancer, while less mature CD83+ DCs were found in SLN+ compared to SLN-. Another study reported on an increased plasmacytoid DC (pDC)/myeloid DC ratio in metastatic lymph nodes and claimed that these pDCs expressed Nrp1 and infiltrated VEGF-producing (i.e., a natural ligand for Nrp1) metastatic cervical tumors. Interestingly, the same group reported that Nrp1+ Tregs were more efficient in T cell suppression than Nrp1- Tregs. The authors hypothesized that metastatic cancer cells use their VEGF production as mediator, via Nrp1 pDCs and Tregs, to promote an immunosuppressive microenvironment in cervical cancer.

Metastatic cervical tumor cells are able, like primary tumor cells, to escape from an immune attack via the down-regulation of classical HLA class I and class II, which results, especially in the HLA class I-downregulated metastases, in a reduced infiltration by CD8+ T cells. Furthermore, metastatic tumor cells can produce VEGF, cyclooxygenases COX-1 and COX-2 (the latter leading to increased PGE2 release), which promote both immune suppression and angiogenesis, thereby further promoting cervical tumor progression and spread. Despite these immune escape mechanisms, both CD4+ and CD8+ T cells have been found in metastatic cervical lymph nodes to react to different HPV16 and -18 E6/E7-derived peptides. However, they are mainly inactive and waiting for proper stimulation (“dormant”). In addition, HPV-specific Tregs were also found to be present in these TDLNs explaining the lack of a detectable specific anti-tumor response in patients with cervical cancer.

(Immu)notherapy in cervical cancer
Cervical cancer lesions are classified according to the criteria of the International Federation of Gynaecology and Obstetrics (FIGO): Stage I (FIGO IA1, IA2, IB1 and IB2) includes tumors that have grown restrictedly to the cervix, stage II (FIGO IIA1, IIA2 and IIB) includes invasive tumors that have grown beyond the uterus (but not to the pelvic wall or to the lower third of the vagina), stage III (FIGO IIIA and IIIB) includes invasive tumors that have reached the pelvic wall and/or involve the lower third of the vagina, and stage IV (FIGO IVA and IVB) includes invasive tumors that have spread to adjacent and/or distant organs. The standard care of treatment in The Netherlands according to FIGO stage is summarized in Table 1. Notably, lymph node status is the utmost important prognostic factor in cervical cancer. Complete lymphadenectomy has been shown to be more beneficial for patient survival as compared to the removal of solely SLNs indicating the presence of an unfavourable (immune) microen-
vironment in lymph nodes. Unfortunately, patients can suffer from severe side-effects due to treatment and surgical removal of TDLNs, like e.g. lymphedema, bladder dysfunction, and infertility.

<table>
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<tr>
<th>FIGO stage</th>
<th>Primary treatment</th>
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<tr>
<td>IA1</td>
<td>Conisation</td>
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<tr>
<td></td>
<td>Hysterectomy</td>
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<tr>
<td>IA2</td>
<td>Conisation</td>
</tr>
<tr>
<td></td>
<td>Hysterectomy</td>
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<td></td>
<td>Without vaso-invasion:</td>
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<td></td>
<td>Hysterectomy</td>
</tr>
<tr>
<td>IB1 and IA1</td>
<td>Conisation + pelvic lymphadenectomy</td>
</tr>
<tr>
<td>IB2, IIA2-IVA</td>
<td>Conisation + pelvic lymphadenectomy</td>
</tr>
<tr>
<td></td>
<td>For stage IB2 without suspected metastases:</td>
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<tr>
<td></td>
<td>For all others:</td>
</tr>
<tr>
<td>IB3</td>
<td>Individual palliative treatment</td>
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Table 1: Standard care of treatment of cervical cancer in The Netherlands

Guidelines from: https://www.oncoline.nl/cervixcarcinoom

In The Netherlands, the 5-year disease-specific survival of patients with advanced cervical cancer (i.e., Stage III-IV) is 18-37%. Still efforts are made to improve quality of life after standard treatment and to achieve a 100% cure rate. New patient-tailored therapies should improve this since at the moment all patients are treated the same without stratification for histology subtype, mutational or immune status. Immunotherapy might be a promising strategy to halt tumor growth due to the presence of tumor-specific T cells reactive to epitopes derived from HPV oncoproteins or from neo-antigens. Over the past decade, therapeutic vaccines, aimed at eradicating HPV-infected cells by stimulating HPV (E6/E7)-specific T cells, have been tested in patients with HPV-associated premalignant lesions and invasive carcinoma with promising results in patients with premalignant vulvar lesions. Unfortunately disappointing clinical responses were observed in patients with advanced invasive carcinoma, including patients with cervical cancer. Furthermore, one clinical trial using adoptive transfer of tumor-infiltrating T cells in metastatic cervical cancer resulted in objective tumor responses in 3/9 patients with complete remission in 2/9 patients. In addition, mainly based on successes in other types of cancer, some clinical trials have been or are implementing angiostatic drugs (anti-VEGF) and checkpoint inhibitors (i.e., PD-1 and CTLA-4) in patients with cervical cancer. However, no impressive clinical responses are achieved yet, and therefore, the focus should be on the development of improved (immunotherapeutic) therapies. In particular, these immunotherapeutic strategies should focus on the immune modulation of TDLNs (through locally applied therapies) to generate tumor-specific T cell responses and in this way prevent metastatic spread. Instead of surgical removal, immune-suppressed cervical TDLNs should be converted into tumor-combating lymphoid organs.

Outline of this thesis

The overall aim of the research described in this thesis is the characterization of the immune microenvironment of the primary tumor and TDLNs in patients with cervical cancer. This will aid in unravelling the complex process of the tumor-immune interplay and how this drives metastatic behaviour. Understanding the strategies that tumors use to evade an effective immune response in the tumor and its draining lymph nodes may aid in the exploration of less invasive, locally applied, and targeted immunotherapy strategies aiming for complete clinical responses.

The TME can hinder T cell infiltration and function through the expression of checkpoint molecules. In chapter 2, we studied checkpoint molecule PD-L1 in two patient cohorts in order to compare SCC vs. AC tumors (patient cohort I, n=205) and to compare primary tumors vs. paired metastatic tumors (patient cohort II, n=127) in cervical cancer. Additionally, we correlated this data with clinicopathological characteristics and patient survival. In chapter 3, we investigated the expression of checkpoint molecule IDO. We examined the association between IDO expression patterns in the TME (n=71 primary tumors; n=14 paired metastatic lymph nodes) and the concentration of IDO metabolites in patients’ serum. In addition, we correlated IDO expression patterns to clinicopathological features and the presence of proliferating cytotoxic CD8+ T cells and Tregs. Also, we compared the relationships between IDO1 and IFNG gene expression and clinical parameters using RNAseq data from cervical tumor samples (n=144) published by The Cancer Genome Atlas.

Another escape mechanism that tumors can employ is hiding from the immune system by aberrant expression of HLA class I molecules. In chapter 4, we compared the expression of classical (HLA-A,-B, and -C) and non-classical (HLA-E and -G) HLA molecules between the two common histological subtypes SCC and AC in cervical primary tumors and paired metastatic lymph node samples (n=136), and linked these data to clinicopathological characteristics and patient outcome. In addition, we aimed at shedding more light on the involvement of NK cells in overcoming aberrant HLA expression by cervical tumor cells. Therefore, in chapter 5 we explored the ant-tumor efficacy of umbilical cord blood-derived NK cells and healthy peripheral blood NK cells combined with cetuximab against 10 cervical cancer cell lines, in relation to expression of NK activating and inhibitory receptors.

Ideally, an effective anti-tumor response should be generated in the TDLNs. To gain a better understanding of the microenvironment in TDLNs, in chapter 6 we studied various T cell populations, five APC subsets (LCs, Dermal-like DCs, CD14+ lymph node resident DCs, CD14+ APCs, and pDCs), granulocytic and monocytic MDSCs and cytokine release profiles (IL-4, IL-6, IL-10, TNF-α and IFN-γ) in cervical TDLN free of tumor (LN-; n=20) vs. tumor-positive TDLNs.
In order to gain deeper insight into the localization and distribution of immune cell subsets in pelvic lymph nodes with respect to lymphatic drainage patterns and tumor involvement, we analyzed the distribution and localization of CD8+ T cells, FoxP3+ Tregs, HLA-DR and PD-L1 myeloid cells in all surgically removed pelvic lymph nodes (LN+ n=9; LN- n=74) of five patients with metastatic cervical cancer in chapter 7.

Based on our findings, in chapter 8 we present a model of the cellular mechanisms underlying immune suppression and metastatic niche formation in cervical TDLNs, which points to a new therapeutic target, i.e., CD14+/PD-L1+ macrophage-like cells. The general discussion in chapter 9 gives a summary of the findings presented in this thesis and highlights clinical implications and future directions.

REFERENCES

13. Samuels, S., et al. Human Leukocyte Antigen-DR Expression is Significantly Related to an Increased Disease-Free
 CHAPTER I

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS


CHAPTER I

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS


Prognostic Effect of Different PD-L1 Expression Patterns in Squamous Cell Carcinoma and Adenocarcinoma of the Cervix


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ABSTRACT

Programmed death-ligand 1 (PD-L1) is expressed in various immune cells and tumor cells, and is able to bind to PD-1 on T lymphocytes, thereby inhibiting their function. At present, the PD-1/PD-L1 axis is a major immunotherapeutic target for checkpoint inhibition in various cancer types, but information on the clinical significance of PD-L1 expression in cervical cancer is largely lacking.

Here, we studied PD-L1 expression in paraffin-embedded samples from two cohorts of patients with cervical cancer: primary tumor samples from cohort I (squamous cell carcinoma \(n=156\) and adenocarcinoma \(n=49\)) and primary and paired metastatic tumor samples from cohort II (squamous cell carcinoma \(n=96\) and adenocarcinoma \(n=32\)).

Squamous cell carcinomas were more frequently positive for PD-L1 and also contained more PD-L1-positive tumor-associated macrophages as compared with adenocarcinomas (both \(P<0.001\)). PD-L1-positive tumor-associated macrophages were found to express CD163 and/or CD14 by triple fluorescent immunohistochemistry, demonstrating an M2-like phenotype. Interestingly, disease-free survival (\(P=0.022\)) and disease-specific survival (\(P=0.046\)) were significantly poorer in squamous cell carcinoma patients with diffuse PD-L1 expression as compared with patients with marginal PD-L1 expression (i.e., on the interface between tumor and stroma) in primary tumors. Disease-specific survival was significantly worse in adenocarcinoma patients with PD-L1-positive tumor-associated macrophages compared with adenocarcinoma patients without PD-L1-positive tumor-associated macrophages (\(P=0.014\)). No differences in PD-L1 expression between primary tumors and paired metastatic lymph nodes were detected. However, PD-L1-positive immune cells were found in greater abundance around the metastatic tumors as compared with the paired primary tumors (\(P=0.001\) for squamous cell carcinoma and \(P=0.041\) for adenocarcinoma).

These findings point to a key role of PD-L1 in immune escape of cervical cancer, and provide a rationale for therapeutic targeting of the PD-1/PD-L1 pathway.

INTRODUCTION

Cervical cancer is the fourth most common cancer among women worldwide and is induced by a persistent infection with one of the high-risk strains of the human papilloma virus (HPV), most frequently HPV16 and/or HPV18.\(^1\)\(^-\)\(^3\) Several types of cervical tumor histology can be distinguished, but the most commonly applied stratification is squamous cell carcinoma vs adenocarcinoma, both with different oncogenic mutations,\(^4\)\(^-\)\(^6\) and different immunological tumor microenvironment.\(^5\)\(^-\)\(^7\) Despite these substantial differences, current treatment modalities are the same for both squamous cell carcinomas and adenocarcinomas.\(^8\) At present, patients with cervical cancer are treated with radical hysterectomy and pelvic lymphadenectomy or chemoradiation, depending on tumor stage and tumor size.\(^8\)\(^-\)\(^10\) Unfortunately, the number of patients with adenocarcinoma is still rising and these patients seem to have a poorer survival rate than squamous cell carcinoma patients, especially if adenocarcinoma present with tumor-positive lymph nodes.\(^11\)\(^-\)\(^14\) To improve the prognosis of cervical cancer patients, novel immunotherapeutic strategies need to be developed and established. In addition, histological subtype-specific treatment needs to be considered, which requires a detailed investigation of the tumor microenvironment in relation to clinical outcome of these tumor types.

Promising immunotherapeutic therapies targeting immune checkpoint molecules, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) expressed on activated T cells, counteract the immunosuppressive cycle prevailing in the tumor microenvironment and have led to complete and long-lasting clinical responses.\(^15\)\(^,\)\(^16\) Also, anti-programmed cell death ligand 1 (PD-L1) therapy has been associated with improved survival outcome in several types of cancer, including lung cancer, melanoma, renal cell cancer, and bladder cancer.\(^17\)\(^,\)\(^18\) At present, in advanced cervical cancer, clinical Phase II/III trials are ongoing examining the effects of ipilimumab (anti-CTLA-4; NCT01711515), pembrolizumab (anti-PD-1; NCT02054806), and nivolumab (anti-PD-1; NCT02488759); however, no study results have been reported yet.

Recently, we have identified a suppressive myeloid cell subset expressing PD-L1, with high and interrelated rates of regulatory T cells in metastatic lymph nodes of patients with cervical cancer.\(^19\) Currently, information is largely lacking about PD-L1 expression patterns in primary and metastatic cervical tumors. Therefore, we investigated the expression of PD-L1 in primary and metastatic cervical cancer in relation to the two major histological subtypes (squamous cell carcinoma and adenocarcinoma), and studied the correlation with pathological and clinical characteristics in two patient cohorts. This study provides more insight into the role of PD-L1 in cervical cancer, and strengthens the rationale for blocking the PD-L1/PD-1 immunosuppressive axis.
MATERIAL & METHODS

Study group
Formalin-fixed, paraffin-embedded material was collected from two different patient cohorts. Patient cohort I consisted of 156 squamous cell carcinomas and 49 adenocarcinomas primary tumor samples from the Leiden University Medical Center (Leiden, The Netherlands), and patient cohort II consisted of 96 squamous cell carcinomas and 31 adenocarcinomas paired primary and metastatic tumor samples from the Academic Medical Center (Amsterdam, The Netherlands), VU University Medical Center (Amsterdam, The Netherlands), or Leiden University Medical Center (Leiden, The Netherlands). Patients in both cohorts underwent surgery as primary treatment between 1985-2008 and the patient characteristics are shown in Tables 1 and 2, respectively. Patient samples were handled and used in accordance with the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies.

Immunohistochemistry
Immunohistochemical staining was performed with an automated Ventana immunostainer (Ventana Medical Systems, Tucson, AZ, USA) as previously described using Cell Conditioning 1 Solution (Ventana Medical Systems) as antigen retrieval, 1:200 rabbit anti-PD-L1 antibody for 48 minutes at 36 °C (clone E1L3N; Cell Signaling, Danvers, MA, USA), and using the OptiView DAB IHC Detection kit (Ventana Medical Systems).

For triple immunofluorescence staining on four squamous cell carcinoma patients from cohort I, 1:100 rabbit anti-PD-L1 (clone SP142; Spring Bioscience, Pleasanton, CA, USA), 1:25 mouse IgG2a anti-CD14 (clone 7; Abcam, Cambridge, UK), and 1:100 mouse IgG1 anti-CD163 (clone 10D6; Novocastra, Milton Keynes, UK) were used and detected with Alexa Fluor 647 goat anti-rabbit, Alexa Fluor 546 goat anti-mouse IgG2a, and Alexa Fluor 488 goat anti-mouse IgG1 (all from Life Technologies, Grand Island, NY, USA), as previously described.

Imaging, scoring & analysis
The immunohistochemically PD-L1 stained slides were analyzed and imaged using a bright-field microscope (Olympus BX50; Olympus, Center Valley, PA, USA). Tumor fields were distinguished from normal tissue by the use of nuclear staining with hematoxylin. Primary and metastatic tumor cells were designated PD-L1 positive, when ≥5% of the tumor cells were positive for PD-L1. Moreover, in both primary and metastatic tumor samples, a distinction was made between diffuse (throughout the whole tumor) or marginal (peripheral staining, on the interface between tumor and stroma) expression of PD-L1 by the tumor cells; scores were given for the presence of PD-L1-positive tumor.

Table 1: Clinicopathological characteristics of patient cohort I

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>SCC</th>
<th>AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
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<tr>
<td>Age</td>
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<tr>
<td>Mean</td>
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</tr>
<tr>
<td>Min</td>
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<td>26</td>
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<tr>
<td>Max</td>
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<td>72</td>
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<tr>
<td>FIGO stage*</td>
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<tr>
<td>IBII</td>
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<td>41</td>
</tr>
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<tr>
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<td>18</td>
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<tr>
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<td>8</td>
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<tr>
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<tr>
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<tr>
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<td>36</td>
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<tr>
<td>Recurrence within 5 years*</td>
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<td></td>
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<td>Yes</td>
<td>45</td>
<td>15</td>
</tr>
<tr>
<td>No</td>
<td>111</td>
<td>34</td>
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</tbody>
</table>

Abbreviations: AC, adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; SCC, squamous cell carcinoma. *Data shown as n (%).

Table 2: Clinicopathological characteristics of patient cohort II

<table>
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<tr>
<th>Clinicopathological characteristics</th>
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</thead>
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<td>Number of patients</td>
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<td>Max</td>
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<td>FIGO stage*</td>
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<tr>
<td>IBII</td>
<td>37</td>
<td>15</td>
</tr>
<tr>
<td>≥IIIB</td>
<td>1(0)</td>
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</tr>
<tr>
<td>HPV status*</td>
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</tr>
<tr>
<td>HPV 16</td>
<td></td>
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</tr>
<tr>
<td>HPV18</td>
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<tr>
<td>other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
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<td></td>
</tr>
<tr>
<td>Tumor size*</td>
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<td></td>
</tr>
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<td>11</td>
</tr>
<tr>
<td>&gt;4cm</td>
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<td>29</td>
</tr>
<tr>
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<td>1(0)</td>
<td>1(0)</td>
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</table>

Abbreviations: AC, adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; SCC, squamous cell carcinoma. *Data shown as n (%).
We observed PD-L1 positivity in tumor cells, in tumor-infiltrating immune cells and in stromal immune cells. All tumor-infiltrating and the majority of stromal PD-L1-positive immune cells were identified as tumor-associated macrophages, being double positive for CD163 and PD-L1 and/or triple positive for CD163, CD14, and PD-L1 (Fig. 2). PD-L1 positivity was observed in >5% (used as cutoff) of the tumor cells in 54% of the squamous cell carcinomas and in 14% of all adenocarcinomas (P<0.001). In addition, PD-L1-positive tumor-associated macrophages were present in 53% of the squamous cell carcinomas and in 12% of the adenocarcinomas (P<0.001) (Table 3). For stromal PD-L1-positive immune cells and aggregates of PD-L1-positive cells at the tumor stroma interface (termed as PD-L1-positive cordon), no significant differences were found between squamous cell carcinomas and adenocarcinomas.

**RESULTS**

**PD-L1 protein expression in primary cervical cancer**

Representative examples of different PD-L1 expression patterns in primary cervical tumors (patient cohort I, see Table 1) are depicted in Figure 1 and the results are summarized in Table 3.
Table 3: PD-L1 expression in patient cohort I

<table>
<thead>
<tr>
<th></th>
<th>SCC</th>
<th>AC</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD-L1+ (&gt;5%) Tumor cells</td>
<td>83 (54)</td>
<td>7 (14)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PD-L1- Tumor cells</td>
<td>71 (46)</td>
<td>42 (86)</td>
<td></td>
</tr>
<tr>
<td>Diffuse PD-L1</td>
<td>71 (87)</td>
<td>4 (80)</td>
<td></td>
</tr>
<tr>
<td>Margin PD-L1</td>
<td>11 (13)*</td>
<td>1 (20)*</td>
<td></td>
</tr>
<tr>
<td><strong>PD-L1+ TAM</strong></td>
<td></td>
<td></td>
<td>0.533</td>
</tr>
<tr>
<td>Yes</td>
<td>79 (53)</td>
<td>5 (12)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>70 (47)</td>
<td>43 (88)</td>
<td></td>
</tr>
<tr>
<td><strong>Stromal PD-L1+ cells</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High numbers</td>
<td>122 (78)</td>
<td>31 (65)</td>
<td></td>
</tr>
<tr>
<td>Low numbers</td>
<td>34 (22)</td>
<td>17 (35)</td>
<td></td>
</tr>
<tr>
<td><strong>PD-L1+ cordon</strong></td>
<td></td>
<td></td>
<td>0.057</td>
</tr>
<tr>
<td>Yes</td>
<td>38 (25)</td>
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</tr>
<tr>
<td>No</td>
<td>116 (75)</td>
<td>41 (85)</td>
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</table>

Abbreviations: AC, Adenocarcinoma; PD-L1+, PD-L1 positive; PD-L1-, PD-L1 negative; PD-L1+ TAM, PD-L1-positive TAMs in tumor fields; PD-L1+ cordon, PD-L1-positive immune cells accumulated around tumor fields; SCC, squamous cell carcinoma; TAM, tumor-associated macrophage. *P-value was calculated with χ² test, or in case of <5 cases per group with Fisher’s exact test. In some PD-L1+ tumors (n=1 for SCC and n=2 for AC), we found the staining pattern not convincing because of a small tumor field, and excluded those cases for scoring diffuse or marginal expression pattern. Numbers in bold are statistical significant (P<0.05). Numbers in italic are not statistical significant.

PD-L1 expression in relation to clinicopathological characteristics and survival

PD-L1 expression was analyzed in relation to clinicopathological characteristics for patient cohort I. Interestingly, we found the majority of PD-L1-positive squamous cell carcinoma more often to be HPV18-positive than HPV16-positive squamous cell carcinomas (83% HPV18 vs 42% HPV16, P<0.001). In squamous cell carcinoma, tumors with over 15 mm infiltration depth had more often low numbers of PD-L1-positive cells in stroma (P=0.025). In adenocarcinoma, although the group sizes were small, patients with a PD-L1-positive cordon presented with a high FIGO stage (>IBII) (P=0.010). No further significant correlations were found for PD-L1 positivity and clinicopathological characteristics (tumor size, parametrium invasion, vaginal involvement, and lymph node involvement).

In addition, log-rank tests were performed and Kaplan-Meier plots were generated for disease-free survival and disease-specific survival of the two histological subtypes to assess the correlation with PD-L1 positivity. Squamous cell carcinoma patients with diffuse PD-L1 expression had a better disease-free survival compared to those with PD-L1 negative tumors (P=0.002). In adenocarcinoma, patients with PD-L1-positive tumors showed a trend toward a better disease-specific survival (P=0.058).

Figure 2: Identification of programmed death-ligand 1 (PD-L1)-positive tumor-associated macrophages.

A representative triple immunofluorescence staining shows monochromatic (A) PD-L1, (B) CD14, (C) CD163 images, and (D) colocalized PD-L1 (in blue), CD14 (in red), CD163 (in green), and DAPI (4',6-diamidino-2-phenylindole) (in gray) in cervical cancer patients with PD-L1-positive tumor-associated macrophages in primary tumors. NB: Varying intensity of CD163 staining can be observed. Scale bar is 30 μm.
pression or patients with PD-L1-negative tumors had worse disease-free survival ($P=0.022$ and $P=0.029$, respectively) and disease-specific survival ($P=0.046$ and $P=0.096$, respectively) compared with patients with marginal PD-L1 expression in the primary tumor (Fig. 3A and B). In squamous cell carcinoma patients, no significant association was found between PD-L1-positive tumor-associated macrophages and survival (Fig. 3C), whereas adenocarcinoma patients with PD-L1-positive tumor-associated macrophages had a significantly worse disease-specific survival ($P=0.014$) compared with adenocarcinoma patients without PD-L1-positive tumor-associated macrophages (Fig. 3D).

**PD-L1 expression in primary tumor and paired metastatic lymph nodes**

Next, we studied PD-L1 expression by immunohistochemistry in patient cohort II with samples available from primary and paired metastatic lymph nodes from patients with squamous cell carcinoma and adenocarcinoma (Table 2). The results for cohort II are summarized in Table 4 and Supplementary Table 1. In the primary tumor, in correspondence to the results obtained in cohort I, squamous cell carcinomas were more often positive for PD-L1 ($P=0.024$) and had more often PD-L1-positive tumor-associated macrophages ($P=0.012$). In addition, 25% of the squamous cell carcinomas had a strong PD-L1-positive cordon, compared to 3% of the adenocarcinomas ($P=0.012$) (Table 4).

In the metastatic lymph nodes, PD-L1 positivity was detected in tumor cells, tumor-associated macrophages, immune cells surrounding the metastasis in peritumoral areas, immune cells in resident T-cell areas, and in germinal center histiocytes. Representative examples of PD-L1 expression in metastatic cervical lymph nodes are depicted in Fig. 4A and B. No significant difference was found in PD-L1 expression patterns between squamous cell carcinoma and adenocarcinoma metastatic lymph nodes (Table 4). Next, we compared PD-L1 expression between primary tumors and paired metastases. Discordant tumor cell staining of PD-L1 between primary tumor cells and metastatic tumor cells was found for squamous cell carcinomas in 22 of 71 (31%) cases and for adenocarcinomas in 5 of 28 (18%) cases (Supplementary Table 1). Nevertheless, overall in squamous cell carcinoma and adenocarcinoma patients, no significant differences was found between primary tumors and paired metastatic lymph nodes in PD-L1 positivity of tumor cells, diffuse and marginal PD-L1 expression, the presence of PD-L1-positive tumor-associated macrophages, and the presence of a PD-L1-positive cordon (Fig. 4C-E and G). In both squamous cell carcinomas and adenocarcinomas, more dense cordons of PD-L1-positive immune cells were found surrounding the metastases compared with the paired primary tumors ($P=0.001$ for squamous cell carcinoma and $P=0.041$ for adenocarcinoma) (Fig. 4F).

**Figure 3: Survival analysis for PD-L1 positivity.**

Kaplan Meier 5-year survival curves show disease-free survival (DFS) (A) and disease-specific survival (DSS) (B) for patients with diffuse PD-L1 expression by tumor cells, for patients with PD-L1-negative (PD-L1-) tumors, and for patients with marginal PD-L1 expression in squamous cell carcinoma. Kaplan Meier 5-year survival curve shows disease-specific survival (DSS) for patients with (C) squamous cell carcinoma and (D) adenocarcinoma with PD-L1-positive tumor-associated macrophages (TAM+) and for patients without PD-L1-positive tumor-associated macrophages (TAM-). $P$-values were calculated using the log-rank test.
Table 4: PD-L1 expression in patient cohort II

<table>
<thead>
<tr>
<th></th>
<th>Primary tumor</th>
<th>Metastatic tumor</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCC</td>
<td>AC</td>
<td>P-value</td>
<td>SCC</td>
</tr>
<tr>
<td>Tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD-L1+ (&gt;5%)</td>
<td>33 (39)</td>
<td>5 (17)</td>
<td>0.024</td>
<td>21 (26)</td>
</tr>
<tr>
<td>PD-L1-</td>
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<td>25 (83)</td>
<td>0.016</td>
<td>60 (74)</td>
</tr>
<tr>
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<td>27 (82)</td>
<td>4 (100)*</td>
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<td>12 (63)</td>
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<tr>
<td>Margin PD-L1</td>
<td>6 (18)</td>
<td>0 (0)</td>
<td>1000</td>
<td>7 (37)</td>
</tr>
<tr>
<td>PD-L1+ TAM</td>
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<td></td>
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<td></td>
</tr>
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<tr>
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<tr>
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<td>Yes</td>
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<td>28 (93)</td>
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<td>61 (75)</td>
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Abbreviations: AC, adenocarcinoma; PD-L1+, PD-L1 positive; PD-L1-, PD-L1-negative; PD-L1+ TAM, PD-L1-positive TAMs in tumor fields; peritumoral immune cells, PD-L1-positive immune cells in the vicinity of metastatic tumor fields; PD-L1+ cordon, PD-L1-positive immune cells accumulated around tumor fields; SCC, squamous cell carcinoma; TAM, tumor-associated macrophage. P-value was calculated with χ² test, or in case of <5 cases per group with Fisher’s exact test. *In one AC PD-L1+ tumor, we found the staining pattern not convincing because of a small tumor field, and excluded this case for scoring diffuse or marginal expression pattern. Numbers in bold are statistical significant (P<0.05). Numbers in italic are not statistical significant.

Figure 4: PD-L1 expression in metastatic lymph nodes.
PD-L1 (in brown) expression in (A) metastatic squamous cell carcinoma with a PD-L1-positive cordon indicated by the black arrow and high numbers of PD-L1-positive peritumoral immune cells, and (B) metastatic adenocarcinoma lymph node samples. PD-L1 positivity in primary squamous cell carcinoma (SCC) and adenocarcinoma (AC) and metastatic lymph nodes (C) with PD-L1-positive tumor cells, (D) with diffuse PD-L1 expression, (E) with the presence of PD-L1-positive tumor-associated macrophages (TAM), (F) with the presence of peritumoral PD-L1-positive immune cells, and (G) with the presence of a PD-L1-positive cordon. **P=0.001 and *P=0.041 calculated with McNemar test. Scale bar is 50 μm.
DISCUSSION

New immunotherapies targeting the PD-1/PD-L1 axis have been reported to give very promising clinical responses in patients with various types of cancer. However, until recently, little data are available on the clinical efficacy of blocking this checkpoint in cervical cancer. PD-L1 positivity has been reported previously in cervical intraepithelial neoplasia and cervical carcinomas, and, recently, we have reported on the presence of PD-L1-positive immune cells in tumor-draining lymph nodes, including metastasis-free and metastatic lymph nodes. However, extensive studies on PD-L1 expression in a large patient cohort of primary and paired metastatic cervical cancer samples, in relation to histological subtype and clinicopathological patient characteristics, are lacking.

In the present study, we observed diverse, heterogeneous PD-L1 expression patterns among primary tumors from patients with cervical cancer. Although, there are controversies concerning the use of different PD-L1 antibody clones, several studies have shown that the clones used in the present study are specific and validated for immunohistochemical assays. Apart from the tumor cells, we also observed PD-L1 positivity in immune cells present in the tumor fields and in the stromal compartment. In more than 20% of the tumors, we observed a PD-L1-positive cordon which was also described in other tumor types. These PD-L1-positive immune cells might have an immunosuppressive effect by inhibiting T-cell function or might be a sign of immune activation, in conjunction with the co-stimulatory markers CD80 and CD86 as expressed on mature dendritic cells. We identified these PD-L1-positive immune cells as CD63 and/or CD14 tumor-associated macrophages, whereas, remarkably, another study on cervical cancer claimed them to consist mainly of CD8+ T cells. The presence of PD-L1+ T cells was also reported by other studies; however, in these studies compelling evidence in the form of double stainings was lacking. and, therefore, it is more likely that PD-L1-positive tumor-infiltrating cells are from myeloid origin with an M2 macrophage-like phenotype as observed by us, which is in accordance with multiple other studies. Similar M2-like cells, conditioned by tumor-derived soluble factors, have been shown to be poor CD8+ T cell primers, potent inducers of FoxP3 regulatory T cells and pro-angiogenic- and protumor-invasive factor producers facilitating tumor progression. Although different myeloid cell sub-populations and a low CTL/regulatory T-cell ratio have been found to correlate to survival in the cervical tumor microenvironment, the precise role of PD-L1-positive tumor-associated macrophages is yet to be fully elucidated. Nevertheless, in vitro observations by Heusinkveld et al. suggest that cervical cancer-derived IL-6 and prostaglandin-E2 convert monocytes to T-cell-tolerizing macrophages with low levels of costimulatory molecules and IL-12p70, and high levels of IL-10 and PD-L1 expression consistent with a poor ability to prime naïve T cells. In accordance, we have previously shown that high IL-6 in the tumor microenvironment of cervical cancer is associated with poor patient survival. This is the first study to report on the difference in PD-L1 expression between squamous cell carcinoma and adenocarcinoma. Two previous publications on PD-L1 expression in cervical cancer did not include adenocarcinoma patients in the cohorts analyzed. Strikingly, we observed prognostic differences for PD-L1 expression patterns between squamous cell carcinoma and adenocarcinoma patients; we found significantly more PD-L1 expression by tumor cells (cutoff=5%) and higher rates of PD-L1-positive tumor-associated macrophages in squamous cell carcinomas compared to adenocarcinomas. Similarly, differential findings for PD-L1 expression in the two histological subtypes were reported in lung cancer patients. Earlier studies have reported conflicting data on correlations between PD-L1 expression in different solid tumor types with both improved and poor prognosis. However, recent meta-analyses have shown a predominant correlation with poor survival. We were not able to detect an association between PD-L1 expression per se and survival, which is in accordance with an earlier study in patients with cervical cancer, analyzing the whole cohort through the use of tissue microarrays. Of note, we did find an unambiguous survival benefit for squamous cell carcinoma patients with marginal PD-L1 tumor expression (at the tumor-stromal interface) as compared with patients with diffusely positive PD-L1 tumors. In head and neck cancer, diffuse PD-L1 expression was detected in only 1/14 tumors, whereas marginal PD-L1 expression was detected in 13/14 tumors, but no survival analysis was performed. Marginal PD-L1 expression might be induced by extrinsic factors, such as IFN, TNF and IL-18 locally produced by juxtaposed T lymphocytes, whereas diffuse PD-L1 expression is more likely to result from constitutive expression because of underlying tumor-intrinsic molecular mechanisms such as PTEN loss and aberrant JAK/STAT signalling. Importantly, conjunction with infiltrating effector T cells and the release of type-1 effector cytokines might explain the observed association between marginal expression of PD-L1 and a more favorable prognosis (see Fig. 3A and B). Recently, we reported on a survival benefit for cervical cancer patients with high numbers of Tbet-positive T cells, indicative of high IFN production.

In adenocarcinoma, we observed a survival benefit for patients with tumor lacking PD-L1-positive tumor-infiltrating macrophages. These findings point to a difference in immunological microenvironments and tumor escape mechanisms between cervical adenocarcinoma and squamous cell carcinoma in line with previous reports on histology-specific oncogenic mutations, and immunological profiles. Our findings suggest that targeting the PD-1/PD-L1 pathway might be a promising immunotherapy approach in patients with cervical cancer, as PD-L1 is expressed in 54% of the squamous cell carcinomas. In addition, recent studies have shown that even patients with PD-L1-negative primary tumors, including lung cancer, gastric cancer, colorectal cancer, renal cell cancer, and bladder cancer and melanoma, respond to anti-PD-L1 treatment. This might be due to the observed heterogeneous and discordant PD-L1 tumor cell staining between primary tumor cells and metastatic tumor cells with, in some cases, PD-L1-positive metastases originating from PD-L1-negative primary tumors (see Suppl. Table 1), which was also observed in clear-cell renal cell carcinoma. Adenocarcinoma patients with PD-L1-positive tumor-associated macrophages had a poor survival; however, anti-PD-1 or anti-PD-L1 therapy might be successful, since in bladder cancer patients with PD-L1-positive tumor-infiltrating
immune cells objective responses were obtained after anti-PD-L1 therapy. Of note, patient stratification on the basis of PD-L1-positive tumor-associated macrophages is very important in this regard, as patients with adenocarcinoma infiltrated by PD-L1-positive tumor-associated macrophages represented a relatively small minority (see Table 4).

In conclusion, this study showed that PD-L1 was more frequently expressed by squamous cell carcinoma than by adenocarcinoma. Diffuse PD-L1 expression in squamous cell carcinoma patients was correlated with poor disease-free survival and disease-specific survival compared with marginal PD-L1 expression, which was associated with a remarkably favorable prognosis. In adenocarcinoma patients, the presence of PD-L1-positive tumor-associated macrophages was associated with a poor disease-specific survival as compared with patients without PD-L1-positive tumor-associated macrophages. Our data thus suggest that targeting the PD-1PD-L1 pathway may be therapeutically efficacious and should be considered in the treatment of cervical cancer patients.

Acknowledgements
We thank Wim Vos for technical assistance, Jeroen Wierda for assisting with the PD-L1 scoring, Dr Wessel van Wieringen for statistical advice, Dr René Musters for the use of the fluorescence microscopy facility, and Dr Sanne Samuels and Dr Debbie Ferns for patient inclusion.

REFERENCES


## SUPPLEMENTARY TABLE

### Supplementary Table I: PD-L1 expression in primary tumor and metastatic tumor cells.

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CHAPTER 3

Indoleamine 2,3-dioxygenase Expression Pattern in the Tumor Microenvironment predicts Clinical Outcome in Early Stage Cervical Cancer

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ABSTRACT

The indoleamine 2,3-dioxygenase (IDO) enzyme can act as an immunoregulator by inhibiting T cell function via the degradation of the essential amino acid tryptophan (trp) into kynurenine (kyn) and its derivates. The kyn/trp ratio in serum is a prognostic factor for cervical cancer patients, however, information about the relationship between serum levels and IDO expression in the tumor is lacking.

IDO expression was studied in 71 primary and 14 paired metastatic cervical cancer samples by various immunohistochemical (IHC) techniques, including 7-colour fluorescent multiparameter IHC, and the link between the concentration of IDO metabolites in serum, clinicopathological characteristics, and the presence of (proliferating) T cells (CD8, Ki67, and FoxP3) was examined. In addition, we compared the relationships between IDO1 and IFNG gene expression and clinical parameters using RNAseq data from 144 cervical tumor samples published by The Cancer Genome Atlas (TCGA).

Here we demonstrate that patchy tumor IDO expression is associated with an increased systemic kyn/trp ratio in cervical cancer (P=0.009), whereas marginal tumor expression at the interface with the stroma is linked to improved disease-free (DFS, P=0.017) and disease-specific survival (DSS, P=0.043). The latter may be related to T cell infiltration and localized IFNγ release inducing IDO expression. Indeed, TCGA analysis of 144 cervical tumor samples revealed a strong and positive correlation between IDO1 and IFNG mRNA expression levels (P<0.001) and a significant association with improved DFS for high IDO1 and IFNG transcript levels (P=0.031). Unexpectedly, IDO+ tumors had higher CD8+Ki67+ T cell rates (P=0.004).

Our data thus indicate that the serum kyn/trp ratio and IDO expression in primary tumor samples are not clear-cut biomarkers for prognosis and stratification of patients with early stage cervical cancer for clinical trials implementing IDO inhibitors. Rather, a marginal IDO expression pattern in the tumor dominantly predicts favorable outcome, which might be related to IFNγ release in the cervical tumor microenvironment.

INTRODUCTION

In cervical cancer, a persistent infection with high-risk human papillomavirus strains (mainly types 16 and -18) is responsible for initiating carcinogenesis. Expression of the viral E6 and E7 oncoproteins is instrumental in this process, and hereby cervical cancer is a relatively immunogenic disease, employing various escape mechanisms to avoid the host’s immune attack.

One of these putative tumor escape mechanisms is the expression of indoleamine 2,3-dioxygenase (IDO), which might be induced by IFNγ secretion by cytotoxic CD8+ T cells in the tumor microenvironment. IDO is an intracellular enzyme that is able to catabolize tryptophan along the kynurenine pathway. Tryptophan is an essential amino acid, necessary for protein synthesis and other metabolic cell functions. Contradictory results have been reported about the actual effect of tryptophan depletion. Mostly, in vitro and in vivo mice studies have shown that particularly activated, not resting, T- and natural killer (NK) cells seem to be sensitive to tryptophan-depletion and the presence of kynurenine and its derivates in the microenvironment.

Upon tryptophan depletion, arrest of the cell cycle takes place in the G-phase, which in turn renders T cells more sensitive to apoptosis. In addition, it has been shown that IDO-expressing tumors promote differentiation and activation of regulatory T cells (Tregs) which in turn can induce IDO expression in myeloid cells via cytotoxic T-lymphocyte-associated protein-4 (CTLA-4)-CD80/86 interactions and recruit myeloid-derived suppressor cells (MDSCs) to the tumor site. Whereas the majority of reports point to a detrimental effect of IDO expression and activity on patient outcome in various tumor types, others have shown IDO to be associated with favorable outcome.

In cervical cancer, IDO expression has been observed in primary and metastatic tumor cells and in immune cells, like macrophages, dendritic cells and NK cells. In addition, IDO activity, measured by the kynurenine/tryptophan (kyn/trp) ratio, in cervical cancer patients’ pre-treatment sera has been reported by us and by others to be linked to disease stage and poor prognosis. Currently, clinical trials in various tumor types are performed to explore the implementation of IDO inhibitors for cancer therapy, but to our knowledge, this does not yet include cervical cancer patients.

Here, for the first time, we searched for a link between IDO expression patterns in the tumor microenvironment and the presence of systemic IDO metabolites in early stage squamous cervical cancer. To this end, we have examined the association between IDO expression patterns in formalin-fixed, paraffin-embedded (FFPE) tumor tissue and the concentrations of IDO metabolites in serum. In addition, we studied the association of IDO expression patterns with clinicopathological features and the presence of proliferating cytotoxic CD8+ T cells and Tregs. Also, we compared the relationships between IDO1 and IFNG gene expression and linked this to survival outcome using RNAseq data from cervical tumor samples published by The Cancer Genome Atlas (TCGA).

Our findings may contribute to the development of predictive biomarkers for clinical trials using IDO inhibitors and to the development of new and more effective immunotherapy.
strategies for cervical cancer.

**MATERIAL & METHODS**

**Patient cohort**

Previously, we reported on the measurement of serum levels of IDO metabolites (tryptophan, kynurenic, and 3-hydroxykynurenine) in 251 cervical cancer patients. From this cohort, we selected all squamous cell carcinoma patients, diagnosed between 2003 and 2008, with surgery as primary treatment and with sufficient FFPE material available for our study. FFPE tissue blocks with 71 primary tumors and 14 metastatic lymph nodes were obtained from the archives of the Department of Pathology at the Academic Medical Center (AMC) Amsterdam, The Netherlands. The main clinicopathological features of these patients are summarized in Table 1. None of the patients underwent chemotherapy or radiotherapy before surgery. The specimens were anonymously processed and selection of blocks was guided by initial diagnosis and review by the pathologist. Ethical approval was waived according to the regulations in The Netherlands.

**Immunohistochemistry**

Immunohistochemical staining of 71 primary tumors and 14 metastatic lymph nodes was performed as previously described using Tris/EDTA buffer pH 9.0 for antigen retrieval, mouse-IgG1 anti-IDO antibody (1F8.2, Millipore), and ready to use Poly-HRP-GAM/R/R IgG (ImmunoLogic, The Netherlands). Complexes were visualized using 3,3'-diaminobenzidine tetrahydrochloride (Sigma, USA). Slides were counterstained with Hematoxylin.

**Multiplex immunohistochemistry**

On a representative subset of patients, quadruple immunofluorescence staining was performed as previously described using Tris/EDTA buffer pH 9.0 for antigen retrieval. Primary antibodies mouse-IgG1 anti-FoxP3 (236AE7; Abcam, UK), rabbit anti-Ki67 (SP6; ThermoFisher, USA), mouse-IgG2b anti-CD8 (4B11; Novocastra, UK), and secondary antibodies goat anti-mouse IgG1 Alexa Fluor 488, goat anti-Rabbit IgG Alexa Fluor 546, and goat anti-mouse IgG2b Alexa Fluor 647 (all from Thermo Scientific, USA) were used for T cell phenotyping (n=35). Primary antibodies mouse-IgG1 anti-IDO (IF8.2, Millipore), mouse-IgG2a CD14 (clone 7, Abcam), rabbit anti-HLA-DR (ab137832, Abcam), and secondary antibodies goat anti-mouse IgG1 Alexa Fluor 488, goat anti-mouse IgG2a Alexa Fluor 546, and goat anti-Rabbit IgG Alexa Fluor 647 (all from Thermo Scientific, USA) were used for IDO-positive myeloid cell identification (n=6). 4',6-diamidino-2'-phenylindole, dityridochrome (DAPI, Thermo Scientific, USA) was used as a counterstain, slides were enclosed with mounting medium and coverslips.

Multiplexed immunofluorescence staining was performed on eight patients in order to identify the type of tumor-assocated vessels expressing IDO, using the OPAL 7-color fluores-

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**Table 1: Patient distribution according to IDO expression in relation to clinicopathological characteristics**

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>Total n (%)</th>
<th>Tumor cells</th>
<th>P</th>
<th>Tumor cells</th>
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<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>IDO- n (%)</td>
<td>IDO+ n (%)</td>
<td>Patchy- n (%)</td>
<td>Patchy+ n (%)</td>
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<tr>
<td>FIGO stage</td>
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<td>15 (21)</td>
<td>56 (79)</td>
<td>10 (14)</td>
<td>45 (63)</td>
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<tr>
<td>&gt;IBI</td>
<td>16 (23)</td>
<td>5 (7)</td>
<td>11 (16)</td>
<td>8 (13)</td>
<td>3 (6)</td>
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<tr>
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<td>28 (45)</td>
<td>13 (25)</td>
</tr>
<tr>
<td>&lt;4cm</td>
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<td>5 (7)</td>
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<td>1 (2)</td>
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<td>44 (62)</td>
<td>26 (49)</td>
<td>10 (19)</td>
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<tr>
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<td>2 (3)</td>
<td>12 (17)</td>
<td>7 (13)</td>
<td>4 (8)</td>
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<td>Vaginal involvement</td>
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<td>4 (6)</td>
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<td>5 (7)</td>
<td>17 (24)</td>
<td>11 (21)</td>
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FIGO: International Federation of Gynecology and Obstetrics. Of one case we do not have information on tumor size. P-value measured with Asymptotic Pearson's and Fisher's exact test was used when counts were <5. IDO+: IDO-positive; IDO-: IDO-negative. NB: In three cases, we found it difficult to score IDO+ tumors for their expression pattern (patchy/margin) due to small tumor fields. In some cases (n=10) we found it difficult to distinguish between IDO-positive immune cells and IDO-negative. To avoid this bias, we excluded those cases for scoring the expression of IDO in vessels. In some cases (n=2) we found it difficult to score IDO expression in vessels and excluded those cases.
cence immunohistochemistry (IHC) Kit (Perkin Elmer, USA). A blocking step for endogenous peroxidase was introduced with 0.3% H$_2$O$_2$/methanol for 20 min and an extra fixation step was included for 20 min with 10% neutral buffered formalin (Leica Biosystems, Germany), followed by 2 min in Milli-Q water and 2 min in 0.05% Tween 20 in 1x Tris-buffered saline (TBST). The following primary antibodies were used: mouse-IgG1 anti-CD34 (QBEND-10, Cell Sciences), mouse-lgG2a anti-a-smooth muscle (a-sm) actin (tA4, DAKO), mouse-lgG1 anti-CD31 (JC70A, DAKO), mouse-lgG1 anti-IDO (1F8.2, Millipore), mouse-lgG1 anti-podoplanin (D2-40, BIO-RAD), and rabbit anti-galectin-1 (500-P210, PeproTech). Steps were repeated for each primary antibody; microwave treatments were carried out by placing the slides in a plastic tray, after which they were heated in 0.05% ProClin300/Tris-EDTA buffer at pH 9.0 in a 800W standard microwave at 100% power until boiling point, followed by 15 minutes at 30% power. Slides were cooled down in ice water, washed in Milli-Q water and in 1x TBST and were blocked with Normal Antibody Diluent (Immunologic, The Netherlands) for 10 minutes at room temperature (RT). After that, slides were incubated with primary antibody diluted in Normal Antibody Diluent for 30 min at RT and 30 rounds per minute (rpm) on a shaker. Next, slides were washed 3x2 min in 1x TBST at RT and 30 rpm and were subsequently incubated with SuperPicture Polymer Detection Kit - HRP - broad spectrum (Life Technologies, USA) for 20 min at RT and 30 rpm. Afterwards, slides were washed 3x2 min in 1x TBST and were incubated with Opal fluorochromes (Opal520, Opal570, Opal650, Opal690 Opal540, and Opal620) diluted 1:150 in amplification buffer (all provided by the OPAL 7-color fluorescence IHC Kit) for 10 min at RT and 30 rpm. Slides were then washed 3x2 min in TBST. Finally, microwave treatment with AR6 buffer was performed and slides were washed for 2 min in Milli-Q water and for 2 min in TSBT. DAPI working solution (provided by the OPAL 7-color fluorescence IHC Kit) was applied for 5 min at RT and the slides were washed again in Milli-Q water and in 1x TSBT, and then mounted under coverslips with ProLong Diamond antifade mounting medium (Life Technologies, USA).

Imaging and scoring

The standard IHC stained sections were scored for IDO expression by tumor cells, immune cells and tumor-associated vessels using an Olympus BX50 bright-field microscope (Olympus, USA) by two investigators (AMH and ESJ). Primary- and metastatic tumors were designated IDO-negative (< 1% of tumor cells expressed IDO) or IDO-positive (≥ 1% of the tumor cells expressed IDO). Also, tumor cells were divided in different groups: 0%, 1-10%, 10-50%, and >50% positive for IDO, also used by others.22 Furthermore, a distinction was made between patchy (patchy IDO expression throughout the whole tumor field) or marginal (focal staining, on the border between tumor and stroma) expression by tumors. Also, the presence of IDO-expressing tumor-infiltrating immune cells was scored in primary- and metastatic tumor samples either as absent (-) or present (+). Furthermore, IDO expression by stromal immune cells was scored as either present in low numbers (-) or high numbers (+) in stroma of primary tumors. In the metastatic lymph nodes, scores for IDO-positive immune cells were obtained, high (+) or low (-) numbers, for peritumoral area or in resident lymph node tissue distant from the tumor metastases. Finally, IDO expression by tumor-associated vessels was scored as zero/or a few IDO-positive vessels (-) or all vessels positive for IDO (+).

Quadruple immunofluorescence stainings were imaged and analysed using a digital imaging fluorescence microscope (Axiovert-200M, Zeiss, Germany) and SlideBook 6 Reader (Intelligent Imaging Innovations (3i), USA). DAPI staining was used to morphologically distinguish tumor fields from stromal and healthy tissue. From each primary tumor, three to five representative areas, including both tumor and stroma, were randomly selected and imaged with a 20x dry objective with 0.3 Numerical Aperture (NA). CD8-positive, Foxp3-positive, and K67-positive cells from digital images were manually enumerated and results were expressed as number of positive cells per mm$^2$.

Seven-color multiplex stainings were visualized with Leica TCS SP8 microscope (Leica, Germany), tilescan (3x3, 40x oil objective with 1.3 NA) images were generated and viewed using LAS AF Lite software (Leica, Germany). IDO-positive tumor-associated vessels were analyzed for co-localization with the markers CD34, podoplanin, α-sm, galectin-1, and CD31.

TCGA RNAseq patient cohort

Level 3 RSEM normalized, log-transformed RNAseq data, profiled using the Illumina HiSeq RNAseq v2, were retrieved from the TCGA data portal.36 Results of the TCGA RNAseq analysis have been described in detail by the Cancer Genome Atlas Research Network.37 For our analysis, data on 144 cervical SCC patients were used, including downloaded survival data38, IDO1 mRNA and IFNG mRNA expression in primary tumor samples.

Statistical analyses

Statistical analyses were performed using IBM SPSS (IBM, USA) and GraphPad Prism 5 (GraphPad Software, USA) software. Associations between IDO expression patterns in the tumor microenvironment and serum concentrations were performed using the same cutoff ‘low’ (quartiles 1 - 3) and ‘high’ (quartile 4) as previously been ascribed for survival analysis.32 Fisher’s exact test was used to study the association between IDO expression in the tumor microenvironment and serum concentrations of IDO metabolites for (sub)groups with three or more patients. The Mann-Whitney U test, Asymptotic Pearson’s- or Fisher’s exact tests were used for the comparison of IDO expression patterns and clinicopathological characteristics. The log-rank test was performed for survival analyses. Before association analyses between local IDO protein expression and T cells were carried out, normal distribution was tested using the D’Agostino & Pearson omnibus normality test. Then, based on the observed distribution, Mann-Whitney U test or unpaired t test were used or the Kruskal-Wallis or one-way ANOVA, with post hoc Dunn’s Multiple Comparison or Bonferroni’s Multiple comparison tests, respectively. Furthermore, correlation analysis between IDO1 and IFNG mRNA levels, retrieved form the TCGA database, was performed by Pearson’s correlation. Hierarchical cluster analysis was carried out using Euclidean distance and Ward.D2 clustering methods with the function heatmap.plus in RStudio Version 11.423 (RStudio, USA). Survival analysis for
IDO1 mRNA and IFNG mRNA were performed using the two acquired clusters (low and high) or using the median as cutoff (low and high).

Comparisons and associations with P-values below 0.05 were considered statistically significantly different.

RESULTS

IDO expression in primary tumors
IDO protein expression was analyzed by immunohistochemistry. In the primary tumor samples, we observed IDO positivity in tumor cells in a patchy and/or marginal expression pattern. Also, IDO expression was seen in tumor-infiltrating immune cells, stromal immune cells and tumor-associated vessels. Among patients, various heterogeneous IDO expression patterns were observed (Fig. 1A-F for representative images and Supplementary Table 1 for IDO IHC scores per patient).

Next, we aimed to further delineate the specific cell subpopulations and vessel types expressing IDO. We hypothesized that most IDO-positive immune cells were monocytic MDSCs or tumor-associated macrophages and tried to identify these cells using multicolor fluorescent immunohistochemistry for IDO, CD14 and HLA-DR in primary tumor section from six cervical cancer patients. IDO-positive tumor- and stroma-infiltrating cells represented a heterogeneous population of immune cells consisting of HLA-DR-CD14+IDO+ MDSC-like cells, HLA-DR+CD14+IDO+ dendritic/macrophage-like cells, HLA-DR+CD14+IDO+ cells (Fig. 2A). IDO-positive tumor-associated vessels were studied by 7-colour multiplex immunohistochemistry using the markers CD31/CD34 (endothelial cell markers), podoplanin (lymphatic endothelial cell marker), α-sma (perivascular cell marker), galectin-1 (activated endothelial cell marker), and IDO. The IDO-positive tumor-associated vessels were predominantly identified as mature blood vessels since most vessels stained positive for α-sma, a marker of pericytes that cover mature vessels (Fig. 2B). In two patients, IDO expression was also observed in lymphatic (podoplanin-positive) vessels.

IDO expression in metastatic lymph nodes
In the 14 metastatic lymph node specimens available, we observed IDO positivity in tumor cells, tumor-infiltrating immune cells, immune cells surrounding metastatic tumor cells and in resident lymph node tissue (Fig. 1G,H). No IDO-positive vessels were observed. See Supplementary Table 2 for an overview of the IDO IHC scores.

We found no evidence for elevated expression of IDO in the metastatic tumors as compared to the corresponding primary lesions. In one out of 14 metastatic lymph nodes, tumor cells were not detectable in the available tissue sections. In 8 out of 14 metastatic samples, IDO-positive tumor cells were detected. Interestingly, 7 out of 8 metastatic tumors showed a patchy IDO expression pattern. Paired analysis showed that six IDO patchy/patchy+margin expressing primary tumors retained patchy expression in the metastatic tumor cells, one marginal IDO-expressing primary tumor had a patchy IDO-expressing metastatic tumor, one patchy IDO-expressing primary tumor had a marginal IDO-expressing metastatic tumor, two IDO-negative primary tumors remained negative for IDO in the metastatic tumor cells and for three IDO-positive (patchy) primary tumors, corresponding metastatic tumors were negative for IDO (data not shown).

Figure 1: IDO expression in primary cervical cancer and metastatic lymph nodes.
Different patterns for IDO expression (in brown) were detected in primary- and metastatic cervical squamous cell carcinoma. (A) Patchy pattern with cytoplasmic IDO expression by primary tumor cells. (B) Marginal IDO expression by primary tumor cells. (C) High numbers of IDO-expressing stromal immune cells in a marginal IDO-expressing tumor. (D) IDO-negative tumors, with low IDO expression in primary tumor cells and stromal immune cells. (E) IDO-positive tumor-associated vessels (indicated by black arrows). (F) IDO-negative tumor-associated vessels (indicated by black arrows). (G) Metastatic lymph node sample showing metastatic tumor cells negative for IDO and IDO positive immune cells surrounding the tumor fields. (H) Metastatic lymph node sample showing nuclear and cytoplasmic IDO expression by metastatic tumor cells and IDO-positive immune cells. Magnification for A-D is 100x (scale bar in D is 100 μm) and for E-H (scale bar in H is 100 μm) is 200x.
Association between IDO expression at the tumor site and kyn/trp ratio in serum
To determine, in our patient cohort (n=71), whether IDO-positivity in the primary tumor microenvironment correlates with serum levels of IDO metabolites tryptophan, kynurenine and 3-hydroxykynurenine, we used previously measured serum levels from a cohort of 251 cervical cancer patients where a high kyn/trp ratio was shown to be detrimental for survival. The interquartile concentrations of tryptophan, kynurenine and 3-hydroxykynurenine and the kyn/trp ratio for the current patient cohort are summarized in Table 2 and were used for analysis.

We analyzed whether IDO expression in the local tumor microenvironment influenced the levels of IDO metabolites in serum. Notably, we found a significant association between IDO positivity in the primary tumor and a high kyn/trp ratio in serum (P=0.008, Fisher’s exact test) (Fig. 3A), independent of IDO expression by immune cells (infiltrate and stroma) (Fig. 3B). Furthermore, patients with both IDO-positive tumors and vessels had significantly more often a high kyn/trp ratio in serum compared to patients with both IDO-negative tumors and vessels (P=0.001, pairwise Fisher’s exact test) and patients with IDO-positive tumors and IDO-negative vessels (P=0.025, pairwise Fisher’s exact test) (Fig. 3C). Interestingly, we found that the dominance of tumor cell expression on systemic serum levels was independent of the percentage IDO-positive tumor cells (Fig. 3D), but that the serum kyn/trp ratio was apparently determined by the different IDO expression patterns of the primary tumor. All patients with IDO-negative tumors and marginal IDO-expressing tumors had a low kyn/trp ratio, whereas patients with patchy/marginal IDO expression had more often a high kyn/trp ratio in serum which was significantly elevated when compared to patients with IDO-negative tumors (P=0.009 and P=0.017 respectively, pairwise Fisher’s exact test) (Fig. 3E).

No associations were found for IDO positivity in the primary tumor microenvironment and the individual IDO metabolites tryptophan, kynurenine and 3-hydroxykynurenine in serum. Of note, the number of metastatic lymph nodes analyzed was too small for association analysis of IDO expression with serum kyn/trp levels.

IDO expression in relation to clinicopathological characteristics and survival
In Table 1, the associations between IDO expression patterns and clinicopathological characteristics of the patient cohort are shown.

Interestingly, patients with IDO-positive tumors were older (46.5 years vs. 39 years old) (P=0.010, Mann-Whitney U test) and manifested more often with smaller tumors (≤ 4 cm) (P=0.034, Asymptotic Pearson’s χ2-test). However, no difference was observed in survival outcome between patients with IDO-negative and IDO-positive tumors per se (Fig. 4A,B). Remarkably, all patients with marginal, including combined patchy+marginal, IDO expression were disease free and still alive after a median follow-up of 60 months. These patients had significantly more often a high kyn/trp ratio in serum compared to patients with both IDO-negative tumors and vessels (P=0.009 and P=0.017 respectively, pairwise Fisher’s exact test) (Fig. 3E).

Table 2: Concentration of IDO metabolites in serum (n=71)

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<td>28.63-34.91</td>
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Table 2 shows the interquartile concentrations of tryptophan, kynurenine and 3-hydroxykynurenine and the kyn/trp ratio for the current patient cohort. We found a significant association between IDO positivity in the primary tumor and a high kyn/trp ratio in serum (P=0.008, Fisher’s exact test) (Fig. 3A), independent of IDO expression by immune cells (infiltrate and stroma) (Fig. 3B). Furthermore, patients with both IDO-positive tumors and vessels had significantly more often a high kyn/trp ratio in serum compared to patients with both IDO-negative tumors and vessels (P=0.001, pairwise Fisher’s exact test) and patients with IDO-positive tumors and IDO-negative vessels (P=0.025, pairwise Fisher’s exact test) (Fig. 3C). Interestingly, we found that the dominance of tumor cell expression on systemic serum levels was independent of the percentage IDO-positive tumor cells (Fig. 3D), but that the serum kyn/trp ratio was apparently determined by the different IDO expression patterns of the primary tumor. All patients with IDO-negative tumors and marginal IDO-expressing tumors had a low kyn/trp ratio, whereas patients with patchy/marginal IDO expression had more often a high kyn/trp ratio in serum which was significantly elevated when compared to patients with IDO-negative tumors (P=0.009 and P=0.017 respectively, pairwise Fisher’s exact test) (Fig. 3E).

No associations were found for IDO positivity in the primary tumor microenvironment and the individual IDO metabolites tryptophan, kynurenine and 3-hydroxykynurenine in serum. Of note, the number of metastatic lymph nodes analyzed was too small for association analysis of IDO expression with serum kyn/trp levels.

IDO expression in relation to the distribution and localization of T cells
Next, in order to study the effect of IDO expression on tumor-infiltrating T cell numbers, we quantified cytotoxic CD8+ T cells, FoxP3+CD8- Tregs, proliferating CD8+Ki67+ T cells, proliferating Fox-
P3^+Ki67^+ (CD8^+) T cells (proliferative Tregs), and FoxP3^+CD8^+ T cells per mm² in a representative subset of patients (n=35) (Fig. 5A). Nuclear DAPI stain was used to distinguish tumor tissue from stromal tissue. Unexpectedly, we observed higher counts of intratumoral CD8^+Ki67^+ T cells in IDO-positive tumors as compared to IDO-negative tumors (P=0.004, Mann-Whitney U test; Fig. 5B). No significant differences were found between IDO-negative- and IDO-positive tumors for any of the other T cell subtypes (Figure 5C and data not shown). Also, the IDO expression patterns, marginal, patchy+marginal, or patchy did not affect infiltrating T cell numbers (Fig. 5D,E and data not shown).

Figure 3: The influence of IDO expression in the local tumor microenvironment on the kyn/trp ratio in serum. Graphs show low (16.78-34.91, white) and high (34.92-52.37, grey) serum kynurenine/tryptophan (kyn/trp) ratio for (A) patients with IDO expression (IDO- / IDO+) in primary tumor (PT). Further stratification for expression patterns in (B) stroma and infiltrating immune cells (stroma and infiltrate), and (C) tumor-associated vessels (vessels). (D) Patients with IDO-negative tumors (0%) and patients with IDO-positive tumors divided into groups of 1-10%, 10-50%, and more than 50% of IDO positivity in tumor cells, and for (E) patient groups with different IDO expression patterns including IDO-negative (IDO-).

Figure 4: Survival analysis according to IDO expression in cervical cancer. Kaplan Meier 5-year survival curve shows (A) disease-free survival (DFS) and (B) disease-specific survival (DSS) for patients with IDO-positive primary tumors (PT IDO+, black line) and IDO-negative primary tumors (PT IDO-, grey line). Kaplan Meier 5-year survival curves show (C) DFS and (D) DSS for patients with patchy IDO expression (black dotted line), for patients with marginal, including patchy+marginal, IDO expression (grey dotted line) by primary tumor cells. P values were calculated between the different groups using the log-rank test. NB: for some patients DFS and DSS data is missing due to loss of follow up.

Furthermore, we observed higher rates of intratumoral cytotoxic CD8^+ T cells (P=0.041, Mann-Whitney U test), a higher intratumoral CD8^+ T cell/FoxP3^+ Treg ratio (P=0.012, unpaired t test), higher rates of CD8^+Ki67^+ T cells both in the stromal (P=0.004, Mann-Whitney U test) and intratumoral (P<0.001, Mann-Whitney U test) compartment, in tumors with IDO-positive tumor-infiltrating immune cells (Supplementary Fig. 1A-C). Significantly higher rates of intratumoral cytotoxic CD8^+ T cells (P=0.041, Mann-Whitney U test) and higher intratumoral CD8^+ T cell/FoxP3^+ Treg ratio (P=0.012, unpaired t test) were observed in IDO-negative tumors compared to IDO-positive tumors. P values were calculated using the Mann-Whitney U test and log-rank test.
moral CD8+Ki67+ T cells were observed in total IDO-positive primary tumors (PT+stroma+infiltrate+) versus partly IDO-positive primary tumors (PT+stroma+infiltrate-) (Supplementary Fig. 1D, both \( P < 0.01 \)). No further significant associations were found.

**IDO1 vs. IFNG mRNA expression**

To test whether RNAseq data of primary tumor samples could be used for the validation of IDO protein expression and to study a possible link between IDO and IFNγ expression, we retrieved IDO1 and IFNG gene expression data from 144 cervical SCC patients from the Cancer Genome Atlas Research Network database. Hierarchical clustering revealed two groups: patients with both low IDO1 and IFNG mRNA expression (‘Low’ group) and patients with both high IDO1 and IFNG mRNA expression (‘High’ group, Fig. 6A). For DSS analysis, no significant associations were found between the two patient groups (Fig. 6C). However, DFS analysis showed an improved outcome for patients with ‘High’ IDO1 and IFNG as compared to patients with ‘Low’ IDO1 and IFNG mRNA expression (\( P = 0.031 \), log-rank test) (Fig. 6B). Interestingly, IDO1 and IFNG mRNA expression were strongly and significantly correlated (\( P < 0.001 \), Pearson’s correlation; Fig. 6D).

**Figure 5:** T cell numbers in relation to IDO expression by primary cervical tumor cells. (A) Representative immuno-fluorescence images showing monochromatic FoxP3 (in green), Ki67 (in red), CD8 (in blue), and the merged panel with FoxP3, Ki67, CD8 and DAPI. In box 1 a proliferating Ki67+FoxP3+ T cell is depicted, in box 2 a cytotoxic CD8+ T cell is depicted, in box 3 a FoxP3+ Treg is depicted, and in box 4 a proliferating CD8+Ki67+ T cell is depicted. Scale bar is 100 μm. Scatter plots show intratumoral numbers per mm² for (B) CD8+ Ki67+ T cells and (C) CD8+ T cell/FoxP3+ Treg ratio in IDO-negative (n=6, white dots) and IDO-positive (n=29, black squares) primary tumors. Scatter plots show intratumoral numbers per mm² for (D) CD8+Ki67+ T cells and (E) CD8+ T cell/FoxP3+ Treg ratio in primary tumors with marginal IDO (n=4, white triangles), patchy+marginal IDO (n=7, black/white triangles), and patchy IDO expression (n=16, black triangles). \( P \) values were calculated using Mann-Whitney U test. **\( P = 0.004 \).

**Figure 6:** IDO1 and IFNG RNAseq data from TCGA. (A) Hierarchical clustering of IDO1 and IFNG mRNA (rows) measured on primary tumor samples from 144 cervical SCC patients (columns) reveals a ‘High’ (for both IDO1 and IFNG) and a ‘Low’ (for both IDO1 and IFNG) patient group. Kaplan Meier 5-year survival curve shows (B) disease-free survival (DFS) and (C) disease-specific survival (DSS) for patients with both high IDO1 and IFNG (black line) and patients with both low IDO1 and IFNG (grey line), based on hierarchical cluster analysis. (D) Graph shows correlation between IDO1 and IFNG. NB: survival data was missing for two patients. \( P \) values for survival analysis were calculated using the log-rank test. \( P \) value for correlation analysis was calculated using Pearson’s correlation.
Also, when TCGA tumors were divided into two groups based on above- or below-median IDO1 mRNA (9.92) and IFNG mRNA (3.71) expression levels, IDO1 mRNA expression was not linked to survival outcome (Fig. 7A,B), whereas for patients with above median IFNG mRNA expression an improved DFS ($P=0.008$, log-rank test) and DSS ($P=0.039$, log-rank test) was observed (Fig. 7C,D).

**DISCUSSION**

Expression of the metabolic enzyme IDO is one of the many immune escape mechanisms employed by tumor cells. Many clinical trials have investigated, or are currently investigating, the effect of IDO inhibitors (i.e., Epacadostat and Indoximod (1-Methyl-D-Tryptophan)), and IDO peptide vaccination in cancer patients. Currently, patients entering these clinical trials are not stratified for IDO positivity in tumor biopsies and/or systemic kyn/trp levels. Such information could be helpful in order to achieve higher immunotherapy response rates and avoid unnecessary overtreatment. Moreover, it was suggested that IDO activity in serum can be influenced by other factors such as chronic infection, neuropsychiatric diseases, and diet. Regrettably, extensive studies on the systemic effect of local IDO protein expression are lacking, except for a study in patients with diffuse large B cell lymphoma which did not find an association between serum kynurenine level and IDO expression in the tumor and a study in prostate cancer wherein a positive correlation between IDO1 mRNA in primary tumor samples and the kyn/trp ratio in serum was observed.

In this study, for the first time the association between IDO expression in the tumor micro-environment and systemic concentrations of IDO metabolites in cervical cancer patients was comprehensively investigated, using a validated IDO-specific antibody. In the current IHC study, we included a subset of patients from the previously reported serum cohort where association between IDO activity and poor survival was observed. Interestingly, we did find increased systemic kyn/trp ratio levels in cervical cancer patients with IDO expression by primary tumor cells rather than IDO expression by immune cells. Moreover, the dominance of tumoral IDO expression on kyn/trp serum levels was independent of the percentage of IDO-positive tumor cells, but rather related to patchy IDO expression, with or without marginal IDO expression (at the tumor/stroma interphase), in the primary tumor. Remarkably, this did not directly impact patient survival. This can be explained by the fact that the current cohort consists of patients with early stage of disease (FFPE material is not available for patients with advanced disease), while in the previous serum study a patient group comprising various disease stages was analyzed. Interestingly, survival analysis showed that patients with marginal IDO expression in the tumor, including combined patchy+marginal expression, manifested with a significantly improved outcome (DFS: $P=0.017$; DSS: $P=0.043$). These data are in concordance with another IDO study in cervical cancer by Inaba and colleagues. The marginal IDO effect was proposed to be indicative of an effective IFNγ anti-tumor T cell response inducing, among others, immunomodulatory factors like PD-L1 and IDO expression in tumor cells. In line with this hypothesis, we previously reported on an association between marginal PD-L1 expression and improved prognosis in cervical cancer patients. In contrast, and in keeping with our PD-L1 data, patchy IDO expression may result from activation of oncogenic signaling pathways leading to intrinsically elevated expression. Interestingly, 7 out of 8 metastatic tumors exhibited patchy IDO expression suggesting that this oncogenic signaling is more pronounced in tumors with an aggressive phenotype and poor patient survival.

**Figure 7:** IDO1 and IFNG RNAseq data from TCGA.

Kaplan Meier 5-year survival curve shows (A) disease-free survival (DFS) and (B) disease-specific survival (DSS) for patients with low (grey line, median as cutoff, <9.92) and high (black line, median as cutoff, =>9.92) IDO1 mRNA expression.

Kaplan Meier 5-year survival curve shows (C) DFS and (D) DSS for patients with low (grey line, median as cutoff, <3.71) and high (black line, median as cutoff, =>3.71) IFNG mRNA expression.

NB: Survival data was missing for two patients. $P$ values for survival analysis were calculated using the log-rank test.
CHAPTER 3

IDO EXPRESSION PATTERN IN CERVICAL CANCER PREDICTS CLINICAL OUTCOME

outcome. Possibly, as indicated by the high kyn/trp serum ratio, IDO expression relating to a patchy expression pattern and putative oncogenic signaling, occurs at higher levels than the T cell/IFNγ-induced marginal IDO levels. Although, we did not find higher numbers of (proliferating) T cells in tumors with marginal IDO expression, we did confirm a significant correlation between IDO1 and IFNG mRNA expression by analyzing the available TCGA cervical cancer RNA expression data. To draw firm conclusions on the role of IFNγ-producing T cells on IDO expression in the complex tumor microenvironment, more in-depth analysis of the location of these cells and corresponding levels of IFNγ relative to IDO-expressing tumor cells should be performed.

IDO has been designated as one of the major immune escape mechanisms employed by tumors. In the cervical tumor microenvironment, IDO positivity was observed in tumor cells, immune cells, and in tumor-associated vessels making it a potential therapeutic target. Although the first clinical results on IDO inhibitors show that they are safe and well-tolerated by patients with different tumor types, no major responses have been observed yet. IDO inhibitors are not tested yet in cervical cancer. In contrast to other studies that have shown a correlation between IDO expression and lower cytotoxic T cell infiltration rates and higher frequencies of Tregs, as well as an association of IDO levels with poor prognosis in different tumor types, including colorectal cancer, endometrial cancer, ovarian cancer, and breast cancer, our findings did not point to a clear-cut association between IDO protein expression and poor patient outcome.

The finding in the current IHC study rather point to IDO expression in tumor cells and in immune cells as a favorable prognostic factor based on association with disease stage (tumor size and lymph node metastases), survival, and infiltration by actively proliferating cytotoxic T cells. In keeping with this notion, we observed a significant correlation between IDO1 and IFNG mRNA expression, with a survival benefit for patients with high levels of IFNG, whether or not combined with high levels of IDO1 expression. A prognostically favorable association for IDO expression has also previously been observed in breast cancer, ovarian cancer, renal cell cancer, vulvar cancer, and lung cancer. Notably, in literature there are contradictory results about the actual effect of tryptophan depletion on proliferating cells, and proof is yet lacking for an immunoregulatory role in vivo. A recent study using 27 cervical cancer punch biopsies, showed a correlation between IDO1 mRNA levels and a high kyn/trp ratio in primary cervical cancer tissue, suggesting the presence of functionally active IDO. However, tryptophan depletion via IDO might not be efficient enough since tryptophan is able to diffuse rapidly from surrounding tissues into the tumor area, or can directly negatively affect the tumor cells themselves. This might explain why studies investigating IDO inhibitors in combination with other (immunomodulatory) drugs, like chemotherapy, α-PD-1, and α-CTLA-4, are more promising.

This is the first study to apply a multiplex fluorescent immunohistochemistry panel with six different vascular markers for vessel characterization in paraffin-embedded tissue sections. Endothelial IDO expression has previously been reported as an immunoregulatory mechanism in the context of the fetal-maternal interface and of organ transplantation. In tumors, IDO expression has also been observed in vessels in lymphoma, melanoma, prostate cancer, and renal cell cancer. Here, IDO expression was predominantly observed in mature (CD31+/CD34+/α-sm) tumor-associated blood vessels and in two patients in lymphatic (podoplanin-positive) vessels. IDO-positive vessels were associated with parametrium invasion and higher kyn/trp levels in serum. This is in contrast with another study, which showed IDO to be mainly located in neoangiogenic (CEACAM1-positive) micro-vessels and to correlate with lower rates of tumor cell proliferation. However, the number of cases with IDO-positive vessel are small in our study: further analysis on larger cohorts should prove the possible negative effect of IDO-positive vessels on tumor progression.

In conclusion, the effect of IDO in early stage cervical cancer appear to be highly complex. There are several tumor cell expression patterns, many different IDO-positive myeloid cell subtypes as well as varying IDO expression in the vasculature in the tumor microenvironment. Despite this complexity, we have found a dominant effect of patchy IDO expression by primary tumor cells on kyn/trp ratio in serum. Remarkably, marginal IDO expression in tumor fields, independent of the presence of simultaneous patchy IDO expression, was associated with 100% 5-year DSS and DFS. In these patients, the ongoing IFNγ T cell response most likely outweighs any putatively detrimental effect of tryptophan depletion and resulting IDO metabolites. In conclusion, the kyn/trp ratio in serum and IDO1 mRNA and protein expression per se in primary tumors cannot be used as a clear-cut biomarker for prognosis or to identify early-stage cervical cancer patients eligible for clinical trials targeting IDO. Rather, the IDO protein expression patterns in the primary tumor seem vital in this regard.

Acknowledgements

We would like to thank Dr. Sanne Samuels for patient inclusion.
REFERENCES


44. clinicaltrials.gov (2017).


59. Yu, J., et al. Upregulated expression of indoleamine 2,3-dioxygenase in primary breast cancer correlates with
Supplementary Figure 1: T cell numbers in relation to IDO expression patterns in primary cervical cancer. Scatter plots show stromal and intratumoral numbers per mm² for (A) CD8⁺ T cells, (B) CD8⁺ T cell/Foxp3⁺ Treg ratio, and (C) CD8⁺Ki67⁺ T cells in tumors without IDO-positive tumor-infiltrating immune cells (IDO- infiltrate, white dots, n=14) and with IDO-positive tumor-infiltrating immune cells (IDO+ infiltrate, black squares, n=17). Scatter plot shows (D) intratumoral numbers of CD8⁺Ki67⁺ T cells in different groups of primary tumor expression patterns. P values were calculated in A-C using Mann-Whitney U test and unpaired t test in case of normal distribution of data. P values were calculated excluding subgroups with n=2 or smaller (*) in D using Kruskal-Wallis test. * P=0.01–0.05, ** P=0.01–0.001, and *** P<0.001.

Supplementary Table 1: IDO IHC scores for patient cohort.

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### Supplementary Table 1: IDO HIC scores for patient cohort.

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<th>PT IDO</th>
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PT: primary tumor, n/a: not analyzed, difficult to score; *for three IDO-positive tumors it was difficult to score for patchy and/or margin expression pattern due to small tumor fields.

### Supplementary Table 2: IDO protein expression in metastatic lymph nodes.

<table>
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<tr>
<th>IDO expression pattern</th>
<th>Tumor cells</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>IDO-</td>
<td>5 (36)</td>
<td></td>
</tr>
<tr>
<td>IDO+</td>
<td>8 (57)</td>
<td></td>
</tr>
<tr>
<td>Missing*</td>
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<table>
<thead>
<tr>
<th>Tumor-infiltrating immune cells</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>IDO+</td>
<td>9 (64)</td>
</tr>
<tr>
<td>IDO-</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Missing**</td>
<td>3 (22)</td>
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<table>
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<th>IDO+ immune cells in peritumoral area</th>
<th>n (%)</th>
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<tr>
<td>High numbers</td>
<td>12 (86)</td>
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<tr>
<td>Low numbers</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Missing***</td>
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<table>
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<tr>
<th>IDO+ immune cells in resident lymph node tissue</th>
<th>n (%)</th>
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</thead>
<tbody>
<tr>
<td>High numbers</td>
<td>9 (64)</td>
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<td>Low numbers</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Missing***</td>
<td>3 (22)</td>
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</table>

<table>
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<th>Tumor-associated vessels</th>
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<td>IDO-</td>
<td>14 (100)</td>
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<td>IDO+</td>
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IDO+: IDO-positive; IDO-: IDO-negative. *No metastatic tumor cells were present anymore in one stained tissue section. **In three cases we found it difficult to distinguish between IDO+ tumor-infiltrating immune cells and IDO+ tumor cells and excluded those cases for scoring IDO+ infiltrating immune cells. ***In some cases the metastatic tumor involved the whole lymph node and excluded those cases for scoring IDO+ immune cells in peritumoral area and resident lymph node tissue.
Classical and Non-classical HLA Class I Aberrations in Primary Cervical Squamous- and Adenocarcinomas and Paired Lymph Node Metastases


Debbie M. Ferns*
A. Marijne Heeren*
Sanne Samuels
Maaike C.G. Bleecker
Tanja D. de Gruijl
Gemma G. Kenter
Ekatarina S. Jordanova

*Equal contribution to the manuscript.
ABSTRACT

Tumors avoid destruction by cytotoxic T cells (CTL) and natural killer (NK) cells by downregulation of classical human leukocyte antigens (HLA) and overexpression of non-classical HLA. This is the first study to investigate HLA expression in relation to histology (squamous cell carcinoma (SCC) vs. adenocarcinoma (AC)), clinicopathological parameters and survival in a large cervical cancer patient cohort.

Classical (HLA-A and HLA-B/C) and non-classical HLA molecules (HLA-E and HLA-G) were studied on primary tumors and paired lymph node (LN) metastases from cervical cancer patients (n=136) by immunohistochemistry. The Chi² test was used for the comparison of clinicopathological characteristics between SCC and AC patients. The Related-Samples Wilcoxon Signed Rank test was used to compare HLA expression between the primary tumor and metastasis in LN. Patient survival rates were analyzed by Kaplan-Meier curves and Log Rank test. The Mann-Whitney U Test was used to compare the distribution of HLA class I expression between SCC and AC.

Decreased expression of HLA-A (SCC P<0.001), HLA-B/C (SCC P<0.01; AC P<0.01) and total classical HLA (SCC P<0.001; AC P=0.02) was apparent in metastatic tumor cells compared to the primary tumor. In primary SCC, there was a clear trend towards complete loss of HLA-A (P<0.05). SCC metastases showed more complete loss of HLA-A, while AC metastases showed more complete loss of HLA-B/C (P=0.04). In addition, tumor size and parametrium involvement were also related to aberrant HLA class I expression. No significant associations between HLA expression and disease-specific (DSS) or disease-free survival (DFS) were found in this advanced disease cohort. However, it has also been reported that KIR2DL4 can act as a stimulatory molecule. In addition, HLA-E can also bind the stimulatory CD94/NKG2C receptor of NK cells, this might have less impact because of a 6-fold lower affinity as compared to the inhibitory receptors.

In cervical cancer, we and others have described loss of classical HLA class I expression in metastatic cervical cancer. In addition, some studies have compared HLA class I expression between primary cervical carcinoma and paired metastatic LNs. In these studies, the patient groups were small and no comparison was made between the different histological subtypes, squamous cell carcinoma (SCC) and adenocarcinoma (AC), despite substantial differences between these two types regarding clinical outcome, oncogenic mutations, and immunological characteristics as shown by us and others.

The HLA class I status of primary and metastasized cervical tumor cells could be of clinical relevance to predict the response to chemotherapy and to immunotherapy focusing on activating CTLs or NK cells in order to destroy tumor cells. In this study, we compared the expression of classical and non-classical HLA molecules between SCC and AC in primary tumors and synchronous metastatic LNs in a large group of patients, and linked these data with clinicopathological characteristics and outcome.

MATERIAL & METHODS

Patients and ethical approval
From 136 patients with cervical cancer and LN metastasis (SCC n=103 and AC n=33) diagnosed...
between 1986 and 2008, formalin-fixed, paraffin-embedded tissue blocks with primary cervical cancer and paired metastasis-positive LN were obtained from the archives of the Departments of Pathology at the VU University Medical Center (VUMc), Amsterdam (n=42), Academic Medical Center (AMC) Amsterdam (n=86), and Leiden University Medical Center (LUMC) (n=8) in The Netherlands. The main clinicopathological characteristics of the patients were retrieved from the databases available at the Pathology departments and Gynecology departments at the different institutes and are summarized in Table 1. None of the patients underwent chemotherapy or radiotherapy before surgery.

All human tissue samples in this study were coded anonymously, and were used according to the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (http://www.federa.org).40

Immunohistochemistry
Formalin-fixed, paraffin-embedded tissue blocks were sectioned at 4 μm and mounted on StarFrost slides (Waldemar Knittel, Germany). Slides were deparaffinized in 3x xylene and washed in 1x 100 %, 1x 90 % of ethanol. Then, endogenous peroxidase was blocked with 0.3 % H2O2 (MERCK, Germany) in methanol for 20 min. Slides were rehydrated in 1x 70 % of ethanol and 1x demineralized water and heated in a microwave for antigen retrieval for 10 min in boiling 0.01 M citrate buffer pH 6.0 (for classical HLA-A, -B/C and HLA-E) or Tris/EDTA buffer pH 9.0 (for HLA-G). The slides were allowed to cool down for 1 h at room temperature (RT). After antigen retrieval, all slides were washed with 2x demineralized water and 2x phosphate buffered saline (PBS) and incubated overnight at RT with one of the following antibodies diluted in 1 % BSA/PBS, mouse-anti-HCA2 (HLA-A), mouse-anti-HC10 (HLA-B/C) (both antibodies provided by Prof. Neefjes from the Dutch Cancer Institute, NKI-Avl, Amsterdam), mouse-anti-HLA-E (MEM-E/02; AbD Serotec, UK), and mouse-anti-HLA-G (4H84; BD Pharmingen®, USA). The next day, slides were washed 3x in PBS and incubated with BrightVision (ImmunoLogic, The Netherlands) for 30 min at RT. Then, slides were washed 3x in PBS, after which immune complexes were visualized with 0.05 % solution of 3,3’-Diaminobenzidine (DAB) containing 0.001 % H2O2 in a 0.05 M Tris-HCl buffer (pH 7.6) for 10 min in the dark at RT. Finally, the sections were counterstained with Haematoxylin followed by 5 min rinsing with running tap water. Finally, slides were dehydrated and mounted under cover slips with Quick-D mounting medium (Klinipath, The Netherlands).

Imaging and scoring
Sections were scored by the percentage and intensity of the immunostained tumor cells using an Olympus BX50 bright-field microscope (Olympus, USA) by three investigators (D.M.F., A.M.H., and E.S.J.). Stromal cells and infiltrating immune cells served as an internal positive control for HLA-A, -B/C and -G detection, while vascular cells served as an internal positive control for HLA-E detection. The percentage of classical HLA class I positive tumor cells was scored as 0 for absent, 1 for sporadic (1-5 %), 2 for local (6-25 %), 3 for occasional (26-50 %), 4 for majority (51-75 %), and, 5 for large majority (76-100 %). Secondly, the intensity of the staining was scored as 0 (absent), 1 (dull), 2 (clear), or 3 (strong), based on comparison with the normal cells present in the same sample. The sum of both scores (percentage and intensity) were used to identify three categories for classical HLAs (0-2 as complete loss of expression, 3-6 as weak expression, and 7-8 as normal expression) and two categories for non-classical HLAs (0-4 as no expression and 5-8 as expression) as previously described by Ruiter et al.41 For non-classical HLA score a cut off of 5 was used to define groups.24 Total classical HLA scoring was based on the combined scores of HLA-A and -B/C.21

Statistical analysis
All statistical analyses were performed using SPSS 20 statistical software (SPSS 20.0, SPSS Inc. Chicago, IL, USA). The Chi2 tests (Pearson Chi2 and Linear-by-Linear Association, Asymptotic or Exact tests, two-sided) were used for the comparison of clinicopathological characteristics between SCC and AC. The Related-Samples Wilcoxon Signed Rank test was used to compare HLA expression between the primary tumor and metastasis in LN. Patient survival rates were analyzed by Kaplan-Meier curves and the Log Rank (Mantel-Cox) test. The Mann-Whitney U Test was used to compare the distribution of HLA class I expression between SCC and AC. Differences were considered statistically significant when P<0.05.

Table 1: Clinicopathological characteristics of the study cohort

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<td>72</td>
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RESULTS

Clinicopathological characteristics of SCC vs AC

Patients diagnosed with metastatic AC, manifested with a higher recurrence rate ($P=0.014$, Chi$^2$ test) (Table 1). Furthermore, these patients had a worse 5-year disease-specific (DSS) and disease-free survival (DFS) compared to patients with SCC ($P=0.003$ and $P=0.006$, respectively, Log Rank test) (Fig. 1).

Figure 1: Survival analysis stratified for SCC and AC subtypes. 5-year Kaplan Meier survival curves and Log Rank test show a significantly poorer disease specific survival (DSS) (A) and disease free survival (DFS) (B) for patients with metastatic AC compared to patients with metastatic SCC. For SCC, DSS data was missing for 12 and DFS data for 15 cases. For AC, DSS data was missing for 3 cases and DFS data for 2 cases.

Figure 2: Representative examples of HLA expression patterns in the primary tumor and corresponding metastatic tumor sample. (A) Heterogeneous HLA-A expression in SCC on the cell membrane of the primary tumor cells (T). In the corresponding LN, no HLA-A was expressed by the metastatic tumor cells (T) in lower panel, while it is expressed by surrounding immune cells. (B) Primary tumor cells (T) showed membrane expression of HLA-A (indicated by black arrow, upper panel). Metastatic tumor cells (T in lower panel) in corresponding LN showed less expression of HLA-A compared to the primary tumor cells.
tumor. (C) Primary AC shows a high cytoplasmic expression of HLA-A (T in upper panel). In the corresponding LN metastasis, there is loss of HLA-A expression in the tumor cells (T in lower panel), while it is expressed by surrounding immune cells. (D) Low expression levels of HLA-G in primary SCC (T in upper panel) and in the corresponding metastatic tumor cells (T in lower panel). HLA-G is abundantly expressed at the cell surface. (F) Primary AC (T in upper panel) show high expression levels of HLA-G, while HLA-G is not expressed in the corresponding metastatic tumor cells (T in lower panel). Scale bar is 50 μm.

Figure 3: HLA expression distribution in primary tumor samples compared to metastatic tumor samples.

Classical and non-classical HLA class I expression in AC
In AC, most tumors manifested with downregulation (including cases both with complete loss and weak expression) of HLA-A (77 %), HLA-B/C (77 %), and total classical HLA (84 %). At the metastatic LN, tumor cells also showed downregulation of HLA-A (80 %), HLA-B/C (87 %), and total classical HLA (87 %). As was the case for SCC, in AC there was a clear association between loss of HLA in the primary tumor and the metastatic LN (Additional file 1: Table S1). In the paired metastatic AC samples a significantly lower expression of HLA-B/C ($P=0.007$, Wilcoxon Signed Rank test) and total classical HLA ($P=0.021$, Wilcoxon Signed Rank test) was found compared to the primary tumor.

At the site of the primary tumor, we observed 33 % and 31 % expression of HLA-E and HLA-G, respectively. The metastatic tumor cells showed less expression of HLA-E (26 %) and HLA-G (28 %), however, this was not significant.

Comparison of HLA class I expression between SCC and AC
When complete loss and weak expression of HLA-A were compared between the histological subtypes, we found a trend toward more complete loss in primary SCC ($P=0.053$, Chi$^2$ test - pairwise) and SCC metastatic LN ($P=0.005$, Wilcoxon Signed Rank test) (Fig. 4A). In the metastatic LN, HLA-A and HLA-B/C expression patterns were significantly different between SCC and AC (for both $P=0.045$, Chi$^2$ test); in SCC, there were more LNs with complete loss of HLA-A expression (Fig. 4A), while in AC there were more LNs with complete loss of HLA-B/C (Fig. 4B). No significant differences for the remaining HLA molecules were found between SCC and AC for the primary tumor or the LN.

Association of HLA class I expression and clinicopathological parameters
In SCC, larger tumors (≥40 mm) showed more often complete loss as compared to partial loss of HLA-A (40 % vs. 17 %) and total classical HLA (45 % vs. 21 %) expression ($P=0.034$ and $P=0.027$, Chi$^2$ test – pairwise). In addition, parametrium involvement was associated with complete as compared to partial loss of HLA-B/C (54 % vs. 31 %) and total classical HLA (54 % vs. 33 %) in the metastatic tumor cells ($P=0.037$ and $P=0.050$, Chi$^2$ test – pairwise). Surprisingly, expression of HLA-G (in 83 % of the cases) in the metastatic tumor cells was also significantly more often present in cases without parametrium involvement ($P=0.034$, Chi$^2$ test).

In AC, larger tumors (≥40 mm) showed more often complete loss (67 %) compared to
partial loss (14%) of HLA-A expression ($P=0.037$, Chi$^2$ test - pairwise). No other significant associations were found.

Figure 4: Comparison of HLA expression patterns in primary tumor and metastatic tumor samples.

The distribution of (A) HLA-A and (B) HLA-B/C expression in LN is significantly different between patients with SCC and AC (both $P=0.045$, Chi$^2$ test). In SCC, more complete loss of HLA-A expression was observed in primary tumor and LN, while in AC, there was more weak expression of HLA-A in primary ($P=0.053$, pairwise Chi$^2$ test) tumor and LN ($P=0.081$, pairwise Chi$^2$ test). NB: We cannot exclude the possibility of HLA haplotype loss or HLA allelic loss as previously described in the cases defined as having ‘normal HLA expression’ since these were solely based on HCA2 and HC10 staining.

Survival and HLA class I expression

Log rank test was performed and Kaplan-Meier plots were generated for DSS and DFS to assess the correlation with classical and non-classical HLA expression in the primary tumor and metastatic LN. We found no significant associations between classical HLA class I expression and DSS (Additional file 2: Fig. S1) nor DFS (not shown) for both histological types. Noticeably, patients with cervical AC with weak HLA-A expression in the primary tumor cells had the poorest DSS (not significant). Furthermore, AC patients with HLA-E expression in the primary tumor also appeared to have a poor DSS (not significant).

In addition, we analyzed combinations of classical and non-classical HLA expression patterns. In primary SCC two significant findings were made; patients without HLA-A expression but with HLA-G expression had the worst DSS ($P=0.001$) and DFS ($P=0.003$) (Fig. 5). The same was true for the patients with complete total classical HLA loss but with HLA-G expression (DSS $P=0.014$, DFS $P=0.010$) (Fig. 5).

Concerning DSS and DFS of AC patients, no significant correlations were observed and no conclusions could be drawn as the number of AC patients per group was too limited.

Figure 5: Survival plots for SCC patients, combining classical HLA and HLA-G expression in the primary tumor.

5 year disease-specific survival (DSS) (A) and disease-free survival (DFS) (B) curves for SCC patients in relation to HLA-A/HLA-G expression and total classical HLA/HLA-G expression in the primary tumor. Both patient groups with down-regulated HLA-A(-) or total classical HLA(-) in combination with HLA-G expression had the poorest survival (red line). Log Rank test $P$-value combining all groups, *Log Rank test $P$-value between classical HLA(-)/HLA-G(+) (red line) and classical HLA(-)/HLA-G(-) (yellow line).

DISCUSSION

We believe the present study to be the first to include a large cervical cancer patient cohort and to report on the differences between SCC and AC, regarding classical and non-classical HLA expression in paired primary tumors and metastatic LN and to relate the expression patterns to clinicopathological characteristics and patient survival. In the past, only one publication on a small cervical cancer cohort (n=26) has reported analysis on primary tumor and paired metastatic LN and has taken the two major histological subtypes into account.40
Here, the vast majority of SCC and AC manifested with downregulation of classical HLA at the site of the primary tumor and even lower expression in the metastatic tumor cells. This phenomenon has been also observed in other tumor types, like breast-, lung-, and liver carcinomas. This is likely resulting in a decreased sensitivity to T cell lysis, which is supported by the observed significant correlation between HLA class I downregulation and a decrease in tumor-infiltrating CD8 T cells, particularly with lower numbers of CD8 T cells in primary tumors with weak HLA-A expression. Moreover, this outcome fits with the concept that tumor cells are positively selected based on low- or no-expression of classical HLA, caused by genetic alterations like HLA class I allele-specific mutations, β2-microglobulin gene mutations, and antigen processing machinery-associated mutations, and can be linked to invasiveness and metastatic potential. Precedently, Menon et al. reported on the lack of downregulation of HLA class I in liver metastasis compared to primary colorectal cancer samples; however, we and others showed the opposite, probably explained by differences in the microenvironment of the metastatic site; liver metastasis versus lymph node metastasis. Interestingly, we did find a trend towards more HLA-A complete loss in primary SCC tumors compared to primary AC tumors, which was also observed in the LNs and supported by another study in cervical cancer patients. In addition, complete loss of HLA-A was associated with larger tumors in both SCC and AC, which is consistent with a study in breast cancer reporting that larger tumors manifest with low HLA class I expression.

The crucial role of the non-classical HLA molecules was recently demonstrated in large cohorts of breast, ovarian, and colorectal cancer patients. Until now only HLA-E was studied in SCC and AC primary cervical cancer samples and high expression of HLA-E was found in 56%, with significantly higher expression in cervical AC compared to SCC. In contrast, in the present study, we found 30% expression of HLA-E in the primary tumor for both groups. This difference might be explained by the fact that we analyzed an advanced-stage, metastatic patient group, while HLA-E expression might be an earlier event in tumor progression. In fact, in SCC, there was significantly less HLA-E expression in the metastatic tumor cells compared to the primary tumor.

In previous studies, HLA-G expression was found in 27-30% of the primary and 11% of the metastatic tumor samples, but without histological subtyping. Here, comparable results were obtained with approximately 25% of the cases having expression of HLA-G in the primary tumor and 11% in the metastatic LNs. No significant differences were observed between histological subtypes.

Next to the association between primary tumor, metastases and clinical parameters, we analyzed the importance of classical HLA loss and non-classical HLA expression for patient survival. As here we have focused on an advanced, metastatic patient cohort these patients already had a worse prognosis than patients without metastases. We could identify significantly higher progression rates and a significantly poorer outcome for patients with metastatic AC compared to patients with metastatic SCC, which is in accordance with earlier studies showing AC to be the more aggressive subtype. Studies on penile cancer, colorectal cancer, and primary cervical cancer (including a mixed SCC and AC patient cohort with or without metastasis), described a strong association between partial loss of HLA expression and poor prognosis. In contrast, we did not find significant associations between classical HLA expression and survival rates. Possibly, due to the obvious poor survival of this metastatic patient group, contrary to previous findings, we could not confirm survival advantage for AC patients with HLA-E expression in the primary tumor.

Although very low numbers of NK cells are present in cervical tumors report downregulation of classical HLA may lead to decreased sensitivity of T cell lysis, but simultaneously may enhance sensitivity to NK cell lysis, we studied classical HLA expression in combination with non-classical HLA expression. Interestingly, SCC patients with HLA-G expression in combination with downregulation of HLA-A or total classical HLA in the primary tumor had a significantly poorer DSS and DFS, which is in accordance with a study in breast cancer. Previous studies, including cervical cancer, also reported on HLA-G upregulation in case of classical HLA loss. However, our findings should be interpreted with caution, because of the limited number of patients per group and the limited number of patients with normal expression of classical HLA in the present patient group (eight for SCC and five for AC). The same holds true for the metastases group; there were only two SCC metastases and four AC metastases with normal HLA class I expression.

CONCLUSION

In conclusion, in both histological tumor types, SCC and AC, our results show evidence of aberrant patterns of HLA expression with even lower classical HLA class I expression in corresponding LN metastases. Our study is likely to be an under-representation of the actual HLA aberrations due to specific allele mutations, which cannot be identified by immunohistochemistry. In patients with primary SCC, complete loss of classical HLA was found more frequently than in patients with AC (P=0.05), suggesting that this is one of the important mechanisms for tumor progression in SCC. Based on these findings, we speculate that there could be a more pronounced immunological pressure on SCC cells to completely downregulate classical HLA expression thereby becoming less sensitive for T cell lysis. Moreover, this immunological pressure is prominent in SCC tumors, in which downregulation of HLA-A or total classical HLA in combination with HLA-G expression is related to poor prognosis, suggesting escape from both T cell and NK cell antitumor activity.

It would be of great interest to study the differences in quality and quantity of HPV-specific immune responses between SCC and AC, since AC is more frequently associated with HPV18. Recently, Safaeian et al. found an association between HLA allele sub-types and HPV type-specific peptides, suggesting that HLA recognition is HPV type-specific. In addition, reduced levels of TNFα and IFNγ were found in HPV18-infected patients compared to HPV16-infected patients, and less IL-6 and IL-12 was present in the supernatant of the SiHa (HPV16+) cell.
line compared to the suprantum of the HeLa (HPV18+) cell line, while both cell lines were able to induce immunosuppressive M2 type marker CD163 expression on macrophages.67 However, still not much is known about the immunological differences between SCC and AC. It is puzzling that AC retain higher HLA class I expression levels as compared to SCC. We have recently published on substantial differences between the SCC and AC histological subtypes regarding oncogenic mutations, with e.g., KRAS mutation exclusively found in AC.31 In contrast, we found EGFR upregulation exclusively in SCC.38 Currently, we are focusing on understanding the immunological differences between SCC and AC, and we are investigating the presence of different immune cell subsets which may give us more information on the different role of HLA expression in these histological subtypes.32-36 In addition, there was more complete HLA-A loss in SCC metastasis, while there was more complete loss of HLA-B/C in AC metastasis, again emphasizing the need for better immune-characterization of the two major cervical cancer histological subtypes.

The translational relevance of these findings is potentially high with the exponential rise of T cell based immunotherapeutic approaches in the past few years,43,48 as it is conceivable that tumor cells with low or absent classical HLA will not respond, or will respond differently, to these therapies due to reversible or irreversible HLA class I alterations.49,50 It will be of great interest to dissect the effect of classical and non-classical HLA expression in (metastatic) cervical cancer on the clinical effect of therapeutic vaccination and other immunotherapies, which will potentially lead to the selection of a patient group that is most likely to respond to this type of intervention.

Acknowledgements

We thank all the patients who participated in this study. We would like to thank Prof. Dr. Folkert van Kemenade from the Pathology Department of the VUmc, Amsterdam for additional help with identifying metastases in H&E stained LNs. We would like to thank Prof. Dr. Vincent Smith and Dr. Tjalling Bosse from the Pathology Department of the LUMC, Leiden for providing the HC10 and HCA2 antibodies for the immunohistochemistry studies.

REFERENCES

20. Vermeulen, C.F. et al. Frequent HLA class I loss is an early event in cervical carcinogenesis. Hum. Immunol 66,
CHAPTER 4
HLA CLASS I ABERRATIONS IN CERVICAL CANCER


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### ADDITIONAL FILES

**Additional file 1, Table S1: HLA expression pattern comparison between primary tumor and metastatic tumor samples.**

<table>
<thead>
<tr>
<th>Lymph node metastasis</th>
<th>Complete loss</th>
<th>Weak expression</th>
<th>Normal expression**</th>
<th>P-value</th>
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<tr>
<td><strong>HLA-A</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Complete loss</td>
<td>34 (100.0)</td>
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<td>0 (0.0)</td>
<td>&lt; 0.001</td>
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<tr>
<td>Weak expression</td>
<td>14 (39.0)</td>
<td>20 (55.5)</td>
<td>2 (5.5)</td>
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<tr>
<td>Normal expression</td>
<td>4 (11.1)</td>
<td>10 (28.3)</td>
<td>5 (13.8)</td>
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<td><strong>HLA-B/C</strong></td>
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<td></td>
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<tr>
<td>Complete loss</td>
<td>26 (81.9)</td>
<td>5 (16.1)</td>
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<td>&lt; 0.001</td>
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<tr>
<td>Weak expression</td>
<td>14 (39.8)</td>
<td>29 (65.9)</td>
<td>1 (2.3)</td>
<td></td>
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<tr>
<td>Normal expression</td>
<td>1 (2.8)</td>
<td>5 (12.2)</td>
<td>2 (5.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Total classical HLA</strong></td>
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<td></td>
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<tr>
<td>Complete loss</td>
<td>16 (88.9)</td>
<td>2 (11.1)</td>
<td>0 (0.0)</td>
<td>&lt; 0.001</td>
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<tr>
<td>Weak expression</td>
<td>20 (32.3)</td>
<td>41 (66.1)</td>
<td>1 (1.6)</td>
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<tr>
<td>Normal expression</td>
<td>0 (0.0)</td>
<td>4 (8.0)</td>
<td>1 (20.0)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lymph node metastasis</th>
<th>Complete loss</th>
<th>Weak expression</th>
<th>Normal expression**</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HLA-E</strong></td>
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<tr>
<td>No expression</td>
<td>45 (95.7)</td>
<td>2 (4.3)</td>
<td>0 (0.0)</td>
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<tr>
<td>Expression</td>
<td>32 (92.0)</td>
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<td>0 (0.0)</td>
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<tr>
<td><strong>HLA-G</strong></td>
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<tr>
<td>No expression</td>
<td>65 (92.9)</td>
<td>5 (7.1)</td>
<td>0 (0.0)</td>
<td></td>
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<tr>
<td>Expression</td>
<td>7 (35.0)</td>
<td>13 (65.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
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</table>

**Data shown as n (%). SCC: squamous cell carcinoma; AC: adenocarcinoma; In total classical HLA group cases were missing, because not interpretable for HLA-A or HLA-B/C. P-value measured with Exact Linear-by-Linear association (counts were less than 5).**

**We cannot exclude the possibility of HLA haplotype loss or HLA allelic loss normal expression as previously described**

**In the cases defined as having ‘normal HLA expression’ since these were solely based on HCA2 and HC10 staining.**
Additional file 2, Figure S1: Survival plots of HLA expression in the primary tumor and paired metastatic tumor samples. 5-years disease specific survival (DSS) rates for patients with cervical SCC (A) and AC (B) in relation to HLA-A, HLA-B/C, total classical HLA, HLA-E and HLA-G expression in primary tumor cells and paired metastases. P-value was calculated by Log rank test.
High-efficiency Lysis of Cervical Cancer by Allogeneic NK cells derived from Umbilical Cord Progenitors is Independent of HLA status


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Tanja D. de Gruijl

*Equal contribution to the manuscript.
ABSTRACT

Down-regulation of HLA in tumor cells, low numbers and dysfunctionality of NK cells are commonly observed in patients with end-stage cervical cancer. Adoptive transfer of high numbers of cytotoxic NK cells might be a promising treatment approach in this setting.

Here, we explored the cytotoxic efficacy on ten cervical cancer cell lines of activated allogeneic NK cells from two sources, i.e., peripheral blood (PBNK) with and without cetuximab (CET), a tumor-specific monoclonal antibody directed against EGFR, or derived from umbilical cord blood (UCB-NK).

Whereas CET monotherapy was ineffective against the panel of cervical cancer cell lines, irrespective of their EGFR expression levels and despite their RASwt status, it significantly enhanced the in vitro cytotoxic efficacy of activated PBNK (P = 0.002). Equally superior cytotoxicity over activated PBNK alone was achieved by UCB-NK (P < 0.001). Both PBNK- and UCB-NK-mediated cytotoxic activity was dependent on the NK-activating receptors natural killer group 2, member D receptor (NKG2D) and DNAX accessory molecule-1 (DNAM-1) (P < 0.05) and unrelated to expression levels of the inhibitory receptors HLA-E and/or HLA-G.

Most strikingly, whereas the PBNK’s cytotoxic activity was inversely correlated with HLA-ABC levels (P = 0.036), PBNK + CET and UCB-NK cytotoxicity were entirely independent of HLA-ABC expression.

In conclusion, this study provides a rationale to initiate a clinical trial for cervical cancer with adoptively transferred allogeneic NK cells, employing either UCB-NK or PBNK + CET for EGFR-expressing tumors. Adoptive transfer of UCB-NK might serve as a generally applicable treatment for cervical cancer, enabled by HLA-, histology- and HPV-independent killing mechanisms.

INTRODUCTION

Persistent infection of the cervical epithelium by high-risk HPV can lead to cervical intraepithelial neoplasia which may progress to invasive cervical cancer, such as squamous cell carcinoma, adenosquamous cell carcinoma or adenocarcinoma.1-3 Treatment for cervical cancer includes conventional surgery, chemotherapy and/or radiation. In addition, in advanced (metastatic) disease, targeted therapies are widely explored. Unfortunately, targeted intervention strategies using small molecules, angiogenesis inhibitors and monoclonal antibodies directed against specific tumor antigens and proliferation pathways have had limited success in restricting cervical tumor growth so far.4,5 In cervical cancer, EGFR is variably expressed in 80 % of the tumor tissues.6 Overexpression of EGFR has been associated with poor prognosis in cervical cancer, making EGFR an obvious candidate for therapeutic targeting.7 However, treatment with cetuximab (CET) (chimeric IgG, anti-EGFR mAb) as monotherapy or CET in combination with chemotherapy was ineffective in patients with cervical cancer, in spite of the apparent absence of activating mutations in the EGFR pathway.8,9 Immunotherapy of cervical cancer has been clinically explored with limited success. Efforts so far have mostly focused on vaccination approaches against HPV-derived oncogenes (E6 and E7) to trigger an efficacious antitumor T-cell response.10 Failure to improve clinical outcome may at least in part be due to extensive HLA down-regulation commonly observed in cervical cancer.10-12 In these cases, NK cell-based therapies may prove more effective than T cell-based approaches. Indeed, the role of the innate immune response in host defense and viral clearance during (early) infection is well recognized.11 NK cells are potent in exerting rapid cytotoxicity by releasing cytotoxic granzyme B and perforin in order to lyse virus-infected cells and tumor cell targets. Functional activity of NK cells is regulated by an equilibrium between inhibitory (e.g., CD94/NKG2A) and activating (e.g., CD16, DNAM-1, CD94/NKG2C, CD94/NKG2D) receptors.13,14

Infiltrating NK cells are observed in low-grade and high-grade cervical intraepithelial neoplasia lesions and to a lesser extent in cervical carcinoma.15-17 In vitro studies have shown that peripheral blood NK cells (PBNK) are able to kill HPV-infected cell lines.15-17 However, NK cells are often dysfunctional and low in number in cervical cancer patients and thereby unable to mount efficient cytotoxicity against tumors.20-24 NK cytotoxic function is also counteracted by several cervical tumor escape mechanisms, including low expression of activating NK cell receptor ligands (e.g., MICA/B, ULBPs, Nectin, PVR) and aberrant expression of suppressive non-classical HLA molecules (e.g., HLA-E and -G) by tumor cells.18-22 Ex vivo expanded autologous NK cells, adoptively transferred for the treatment of solid tumors, in most studies have yielded disappointing results, underscoring the dire need for the development of more powerful therapeutic approaches to overcome tumor-associated NK cell dysfunctionality and the inherent resistance to cytolysis of cancer cells. Clinical studies exploring the use of ex vivo expanded allogeneic PBNK from healthy donors also yielded low antitumor efficacy.23-25
which may have been due to their limitations in terms of cell yield, purity, ability to expand in vivo, and cytotoxic capacity.\textsuperscript{20}

An attractive alternative approach would be the use of umbilical cord blood CD34+ stem cell-derived NK cells (UCB-NK), which are feeder cell-free cultures that can be differentiated and efficiently expanded up to 10,000-fold, resulting in a highly pure product with a high cytolytic capacity.\textsuperscript{21} Yet another alternative might be to enhance PBNK cell-mediated cytolyis of cervical tumor cells by the tumor-targeted IgG monoclonal antibody CET, to invoke antibody-dependent cell-mediated cytotoxicity (ADCC).\textsuperscript{22}

In this comparative study, we explored the antitumor efficacy of two clinically applicable therapeutic strategies, i.e., UCB-NK versus allogeneic PBNK + CET, for cervical cancer. Of note, the combination with CET is not a viable option for UCB-NK as in vitro they do not express sufficient levels of the required Fc receptor CD16 to obtain functional benefit.\textsuperscript{23,24}

A series of in vitro NK cytotoxicity assays was conducted to compare antitumor potency of PBNK from healthy volunteers, with or without co-incubation with CET with that of umbilical cord blood-derived NK cell (UCB-NK) monotherapy against various cervical cancer cell lines. These cell lines \((n = 10)\) were stratified based on infection with different HPV types, histological origins and differential expression levels of NK-activating and inhibitory ligands. The findings from this preclinical study strongly support the use of allogeneic UCB-NK derived from umbilical cord precursor cells and outline the advantages of their use as monotherapy in the treatment of cervical cancer.

MATERIAL & METHODS

Cell lines

Cervical cancer cell lines CSCC7, CC8, CC10A, CC10B, CC11A, and CC11B were generated in the Department of Pathology of Leiden University Medical Center (The Netherlands) from primary tumors as described previously.\textsuperscript{25} These patient-derived cell lines as well as commercially obtained cervical cancer-derived cell lines, HeLa, SiHa, CaSkI and C33A (American Type Culture Collection) were maintained in DMEM (Lonza) medium containing 4.5 g/L glucose, 10 % FCS (HyClone), 10 μg/mL gentamicin and 0.25 μg/mL amphotericin B (Gibco), 100 units penicillin/100 units streptomycin/0.3 mg/mL Glutamine (Thermo Fisher Scientific). Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO\textsubscript{2}. See Table 1 for cell line characteristics.

Phenotyping of cervical cancer cell lines

To phenotype cervical cancer cell lines, cell suspensions in PBS supplemented with 01 % BSA and 0.02 % NaN\textsubscript{3} (FACS buffer) were stained for 30 min at 4 °C using antibodies to HLA-ABC (clone w6/32, Immunotools) (labeled with FITC), HLA-E (clone 3D12H.1A-E, eBioscience), HLA-G (clone 87G, Biolegend), EGFR (clone EGFR.1, BD Biosciences), PVR (clone SK11.4, Biolegend), MICA/B (clone 6D4, Biolegend), ULBP2/5/6 (clone #165903, R&D systems), ULBP1 (clone #170818, R&D systems) and ULBP3 (clone #166510, R&D systems) (all labeled with PE), IgG\textsubscript{1}, IgG\textsubscript{2a} and IgG\textsubscript{2b} isotype antibodies were used as negative controls. After incubation, the cells were washed with FACS buffer and analyzed using a flow cytometer LSR Fortessa (BD Biosciences). Phenotypic analyses were obtained from at least two independent experiments performed on each cell line. Data were analyzed using Kaluza software (Beckman coulter) and calculated as specific (geometric) mean fluorescence intensity (MFI) (MFI; geometric mean fluorescence of marker - geometric mean fluorescence of isotype).

Table 1: Cell line characteristics.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Histology</th>
<th>HPV type</th>
<th>RAS status</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>AC</td>
<td>18</td>
<td>Wild type</td>
</tr>
<tr>
<td>SiHa</td>
<td>SCC</td>
<td>16</td>
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</tr>
<tr>
<td>CaSkI</td>
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<tr>
<td>C33A</td>
<td>SCC</td>
<td>Negative</td>
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</tr>
<tr>
<td>C5CC7</td>
<td>SCC</td>
<td>16</td>
<td>Wild type</td>
</tr>
<tr>
<td>CC8</td>
<td>ASC</td>
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<td>Wild type</td>
</tr>
<tr>
<td>CC10A</td>
<td>AC</td>
<td>45</td>
<td>Wild type</td>
</tr>
<tr>
<td>CC10B</td>
<td>AC</td>
<td>45</td>
<td>Wild type</td>
</tr>
<tr>
<td>CC11A</td>
<td>AC</td>
<td>67</td>
<td>Wild type</td>
</tr>
<tr>
<td>CC11B</td>
<td>SCC</td>
<td>67</td>
<td>Wild type</td>
</tr>
</tbody>
</table>

AC adenocarcinoma, SCC squamous cell carcinoma, ASC adenosquamous carcinoma.

\* Characteristics adapted from www.lgcstandards-atcc.org and \textsuperscript{36,37}

\*\* RAS status obtained from www.lgcstandards-atcc.org and RAS typing performed by the RAIDs FP7 Consortium and in own institute.

RAS typing

RAS status was obtained from rational molecular assessments and innovative drugs selection (RAIDs) project data (http://www.raids-fp7.eu/project-overview.html) and www.lgcstandards-atcc.org for cell lines HeLa, SiHa, CaSkI, C33A, CSCC7, CC10A and CC10B. In addition, full RAS typing \(\approx\) e., BRAF exon 15, KRAS exon 2-3-4 and NRAS exon 2-3-4) was performed for cell lines CC8, CC11A and CC11B at the molecular pathology lab of the Department of Pathology of the VU University Medical Center (Amsterdam, The Netherlands) using high-resolution melting assay followed by Sanger sequencing of using high-resolution melting PCR products with an aberrant melt curve, essentially as described previously.\textsuperscript{36,37}

PBMC isolation & NK cell isolation

Whole blood samples from four healthy volunteers were collected. PBMC were isolated using Lymphoprep\textsuperscript{TM} (STEMCELL Technologies, The Netherlands) density gradient centrifugation. CD56+ NK cells were isolated from PBMC using a MACS\textsuperscript{®} Human NK cell isolation kit (Miltenyi
Biotech, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The cell number and purity of the isolated PBNK was analyzed by flow cytometry. Isolated NK cells were activated overnight with 1000 U/mL IL-2 (Proleukin®, Chiron, München, Germany) and 10 ng/mL IL-15 (CellGenix) before use in cytotoxicity assays. NK cell purity and viability were checked by flow cytometry using the following antibodies: 7-Aminoactinomycin D (7AAD; SigmaAldrich), CD3 (labeled with VioBlue), CD56 (labeled with APC-Vio770), and CD16 (labeled with APC) (all from Miltenyi Biotech). Purity of NK cells obtained from NK donors was 87 ± 6%.

For cytotoxicity assays, only PBNK with CD16 expression rates exceeding 80% were used.

**UCB-NK isolation and cultures**

Allogeneic NK cells were generated from cryopreserved umbilical cord blood hematopoietic stem cells as previously described. CD34+ UCB cells (3 × 10⁴ per mL) were plated into 12-well tissue culture plates (Corning Incorporated, Corning, NY) in Glycostem Basal Growth Medium (GBGM) (Clear Cell Technologies, Beernem, Belgium) supplemented with 10% human serum (Sanquin Bloodbank, The Netherlands), 25 ng/mL of SCF, Flt-3L, TPO, and IL-7 (CellGenix, Germany). In the expansion phase II, from day 9 to 14, TPO was replaced with 20 ng/mL IL-15 (CellGenix). During the first 14 days of culture, low molecular weight heparin (LMWH) (Clivarin®, Abbott, Wiesbaden, Germany) in a final concentration of 20 μg/mL and a low-dose cytokine cocktail consisting of 10 pg/mL GM-CSF (Neupogen), 250 pg/mL G-CSF and 50 pg/mL IL-6 (CellGenix) were added to the expansion cultures. Cells were refreshed with new medium twice a week and maintained at 37 °C, 5% CO₂. On day 14, the NK cell differentiation process was initiated by addition of NK cell differentiation medium consisting of the same basal medium with 2% human serum but with high-dose cytokine cocktail consisting of 20 ng/mL of IL-7, SCF, IL-15 (CellGenix) and 1000 U/mL IL-2 (Proleukin®, Chiron, München, Germany). Cultures were refreshed every 2-3 days and maintained till day 35. For cytotoxicity assays, UCB-NK was used with CD56+ cells >85% purity.

**In vitro NK cytotoxicity assays**

Cervical cancer cell lines (target cells) were labeled with 5 μM pacific blue succimidyl ester (PBSE, Molecular Probes Europe, Leiden, The Netherlands) in a concentration of 1 × 10⁶ cells/mL for 15 min at 37 °C. After incubation, cells were washed and resuspended in DMEM culture medium and then re-suspended in GBGM medium to a final concentration of 1 × 10⁶/mL. PBNK and UCB-NK were washed with PBS and subsequently re-suspended in DMEM medium culture medium with a final concentration of 1 × 10⁶/mL. Target cells were co-cultured in triplicate with effector cells (PBNK or UCB-NK), with or without 5 μg/mL CET at an E:T ratio of 11:1 in a total volume of 100 μL in FACs tubes (5 × 10⁶ targets in 50 μL of DMEM culture medium incubated with 5 × 10⁴ effectors in 50 μL of GBGM medium). PBNK, UCB-NK and target cells alone were cultured in triplicate as controls. To measure degranulation by PBNK and UCB-NK, anti-CD107a PE (Miltenyi Biotech, Germany) was added at the beginning of the assay. After incubation for 4 h at 37 °C, cells were harvested and stained with 7AAD, CD56 (labeled with APC-Vio770) and CD16 (labeled with APC) (all from Miltenyi Biotech, Germany). For NK flow cytometry and blocking experiments, NKG2D PE (clone ON72, Beckman Coulter) and DNAM-1 FITC (clone DX11, BD Pharmingen®) were used at 10 μg/mL. Further, killer-cell immunoglobulin-like receptor 2D (PanKIR2D) FITC (clone NKVF5) and CD94/NKG2A PE-Vio770 (Clone REA110) (both from Miltenyi Biotech) were used to screen inhibitory receptor expression on PBNK and UCB-NK. BD LSR Fortessa™ was used for readout of the cytotoxicity assays. Data were analyzed using Kaluza software (Beckman Coulter). Percentages of specific NK degranulation were calculated as ΔCD107a+ NK cells [i.e., (target cells + NK cells) – (NK cells only)] and percentages of cytokotoxicity as Δ7AAD+ target cells [i.e., (target cells + NK cells) – (target cells only)]. See Supplementary Figure 1 for a representative gating example.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software. Statistical significance of differences between conditions were determined using a parametric paired t test, unpaired t test or a one-way ANOVA with Bonferroni’s multiple comparison test and a two-way ANOVA with multiple comparisons between column means. Correlation analyses between percentage NK degranulation, cytotoxicity and MFI were performed using Pearson’s analysis. A P value of <0.05 was considered statistically significant.

**RESULTS**

Comparative analysis of NK cell cytotoxic activity against cervical cancer cell lines

Initially, we compared the antitumor potency of healthy activated PBNK in the presence or absence of CET. Ten cervical cancer cell lines (EGFR-expressing except for C33A, and all RAS-; Table 1) were subjected to PBNK only, CET only, or to a combination of PBNK with CET in order to examine ADCC effects. In line with previous studies, CET as monotherapy did not induce cell death in any of the cell lines tested (data not shown). However, cervical cancer cell lines were sensitive in varying degrees to PBNK-induced cell lysis (Fig. 1a), independent of their EGFR expression levels (Fig. 1b), with consistently and significantly higher cytotoxicity rates when coated with CET (Fig. 1c). C33A (EGFR-negative cell line) was the only cell line that did not display a higher cytotoxicity rate when PBNK were combined with CET (Fig. 1a-c).

Next, activated PBNK were compared with UCB-NK for their ability to induce target cell death. UCB-NK were significantly more cytotoxic than PBNK, consistently inducing higher rates of tumor cell death in all tested cell lines (P < 0.001) (Fig. 2a, b). Note that the PBNK cytotoxicity data presented in Fig. 2a are the same as those in Fig. 1a. The cytotoxicity levels were similar for UCB-NK and PBNK + CET (Fig. 1a, 2a) This was further borne out by observed degranulation levels of NK cells in response to exposure to the cervical cancer cell lines, as measured by CD107a surface expression. These were comparably and significantly elevated in the PBNK + CET and UCB-NK conditions over PBNK alone (Fig. 2c; Supplementary Figure 2). UCB-NK were not tested in combination with CET due to their low surface expression of...
CD16a, which is essential for ADCC in combination with therapeutic mAbs (data not shown). Interestingly, PBNK degranulation levels were low in combination with CET upon exposure to cervical cancer cell lines expressing low levels of EGFR (C33A, HeLa and SiHa; denoted in Fig. 2c by triangles). In contrast, degranulation levels in UCB-NK were generally high. PBNK, PBNK + CET and UCB-NK cytotoxicity levels per histological subtype and HPV type of cervical cancer cell lines are shown in Supplementary Figure 3. It shows that irrespective of HPV or histological tumor type, highest cytotoxicity was consistently achieved by UCB-NK.

Expression of NK-activating receptors and their ligands and their contribution to mediating cytotoxicity

To investigate the involvement of activating receptors in mediating the cytotoxic activity of PBNK and UCB-NK, the expression of the two major NK-activating receptors DNAM-1 and NKG2D on the NK cells described to be involved in the recognition of cervical cancer cells, and their respective ligands, i.e., PVR and MICA/B, ULBPs, on the tested cervical cancer cell lines, were assessed. Similarly, high levels of DNAM-1 and NKG2D were observed on both PBNK and UCB-NK (Fig. 3a). The cell lines showed differential expression of the NK-activating ligands, but all were positive for PVR, the DNAM-1 ligand, and at least one of the NKG2D ligands (Fig. 3b). From the panel of cell lines, SiHa (with highest expression levels of PVR and ULBP-2/5/6) and C33A (with lowest expression levels of PVR and ULBP-2/5/6) were selected as target cells in functional blocking studies. The relatively low ligand expression levels on C33A required combined blocking of DNAM-1 and NKG2D to achieve a significant reduction in either PBNK- or UCB-NK-mediated cytotoxicity (Fig. 3b). In contrast, blocking either DNAM-1 or NKG2D already led to significant reductions of cytotoxicity in SiHa cells (Fig. 3c). These data show dependence (at least in part) of both PBNK and UCB-NK on DNAM-1 and NKG2D for their cytotoxic potency.

Differential expression of NK inhibitory receptors and their ligands in relation to level of cytolysis

To investigate the effect of NK inhibitory receptors on the observed cytotoxic efficacy, the expression levels of KIR2D and NKG2A on the NK cells, and of their respective ligands, i.e., HLA-ABC/G and HLA-E\(^\text{a,b}\), on the cervical cancer cell lines, were assessed (Fig. 4a, b). Irrespective of overnight activation with IL-2/IL-15, PBNK expressed high levels of both KIR2D and NKG2A, whereas UCB-NK only expressed equivalent levels of NKG2A, but no KIR2D. All classical and non-classical HLA molecules were expressed on all ten cervical cancer cell lines, but in widely varying degrees (Fig. 4b). Correlation analyses showed a relationship only between HLA-ABC expression levels and levels of cytotoxicity achieved by PBNK, with lower HLA-ABC levels allowing for higher levels of cytotoxicity (\(P = 0.036\), Fig. 4c). In contrast, PBNK + CET (Fig. 4d) and UCB-NK cytotoxicity were totally independent of HLA-ABC expression levels (Fig. 4e). No other correlations were found between cytotoxicity levels and HLA-E or HLA-G expression levels on cervical cancer cell lines (data not shown).
Figure 2: PBNK and UCB-NK cytotoxicity against cervical cancer cells

(a) Cytotoxicity levels (Δ7AAD) of PBNK (open bars) and UCB-NK (hatched bars) against ten cervical cancer cell lines. Bars are means of triplicate values from four experiments with four different PBNK donors for C33A, HeLa, SiHa, CC11B, CC11A, CC10B, CC10A, CaSki and two experiments with two different donors for CSCC7 and CC8 and five experiments for UCB-NK using five different UCB-NK donors for all cell lines; Bars represent mean ± SEM. PBNK data used to compare with UCB-NK in a are the same dataset as Fig. 1a. (b) Significantly higher cytotoxicity levels (Δ7AAD) were observed in all cell lines after co-culture with UCB-NK compared to PBNK. (c) Significantly higher levels of NK degranulation (ΔCD107a) in PBNK + cetuximab (CET) and UCB-NK conditions compared to PBNK only condition. Triangles denote cell lines with low EGFR levels, i.e., C33A, HeLa, and SiHa. *P < 0.05, **P < 0.01 and ***P < 0.001 calculated in A and B with unpaired t-test, in C with one-way ANOVA, Bonferroni’s multiple comparison test.

Figure 3: NK-activating receptors in PBNK and UCB-NK and their ligand expression in cervical cancer cell lines influencing NK cytotoxicity

(a) Percentage of positive cells within the NK cell population for NK-activating receptors DNAM-1 and NKG2D for PBNK only, PBNK stimulated with cytokines (IL-2 + IL-15) and UCB-NK only were determined by flow cytometry. The data presented is from three representative donors for PBNK and UCB-NK. PBNK only are denoted by open circles, PBNK (IL-2 + IL-15) are denoted by closed circles and UCB-NK only by closed squares. (b) Representative example of histograms showing geometric mean fluorescence intensity (MFI) for NK-activating ligands PVR (ligand of DNAM-1 receptor), MICA/B, and ULBP1, -3 and -2/5/6 (ligands of NKG2D receptor). (c) PBNK and UCB-NK were coated with NKG2D and/or DNAM-1 blocking antibodies and incubated with C33A and SiHa cells. Cytotoxicity levels (Δ7AAD) were measured from 7AAD+ C33A and SiHa cells at the end of a 4 h assay. Data presented are means of triplicate values from three different PBNK and three different UCB-NK donors; Bars represent mean ± SEM. *P < 0.05 and **P < 0.01 calculated with paired, two-way ANOVA multiple comparisons of column means.
Discussion

Cervical cancer is the fourth most common malignancy in women worldwide. Survival is severely reduced in case of lymph node metastasis, with no curative treatment options available. In cervical cancer, ACT involving T cell or NK cell-based therapies have not yet been widely explored, but they have been successfully applied in the treatment of various other cancer types. In one clinical trial adoptive transfer of tumor-infiltrating T cells in metastatic cervical cancer resulted in tumor responses in 3/9 patients with complete remission in 2/9 patients. These findings suggest that ACT could be a viable treatment option for some patients with cervical cancer. However, most cervical tumors have HLA class I alterations and will therefore not respond completely to T cell-based therapies. NK cell-based therapies present a viable alternative in that case, but in advanced cervical cancer, these effector cells are often impaired in their functionality. In this study, we therefore explored the possible therapeutic efficacy of allogeneic NK cells. Clinically applicable NK cells may be derived from two sources, i.e., NK cells derived from peripheral blood and NK cells cultured and expanded from umbilical cord blood stem cells. We tested their cytotoxic efficacy (with and without CET for PBNK) on ten cervical cancer cell lines representing different histological subtypes, HPV types and expressing differential levels of NK-activating and inhibitory ligands.

Initially, we investigated the effect of PBNK alone and a combination of PBNK with CET on the cervical cancer cell lines. From literature it is known that cervical tumors often present with variable levels of EGFR. In colorectal cancers, mutant KRAS is associated with resistance to CET. Although most of the cervical cancer cell lines were EGFR-positive and all were RASwt, their EGFR expression levels were relatively low, and, in keeping with clinical observations for cervical cancer, they did not respond to CET as a single agent. Our observation of increased PBNK cytotoxicity upon combination with CET is in line with a report by Meira et al. who showed that one of the antitumor effector mechanisms upon combined CET and chemoradiation actually was ADCC.

Next, we compared the functionality of PBNK with that of ex vivo generated UCB-NK derived from cord blood stem cells and showed that UCB-NK were significantly more cytotoxic than PBNK (Fig. 2). NK cytotoxicity and NK degranulation levels were equivalent for UCB-NK and PBNK + CET. Further study of the NK killing mechanism revealed that the cytotoxic activity of both PBNK and UCB-NK was at least in part dependent on DNAM-1 and NKG2D receptors, as also previously reported for an NK cell line (NKL) and cytotoxicity it induced in the CaSki and SiHa cell lines. This was in keeping with high expression levels of both NKG2D and DNAM-1 observed on both PBNK and UCB-NK. As complete abrogation of tumor cell killing was not achieved by combined blocking of DNAM-1 and NKG2D on activated PBNK.
and UCB-NK, other NK killing mechanisms such as NKp44/Nkp44L, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), and FAS (Fas Ligand interactions) also might contribute to the observed target cell lysis.46,47 Indeed, NKp44 has been previously reported as highly expressed on expanded UCB-NK, in sharp contrast to PBNK cells, which in the steady state do not express NKp44.33 The known ligands for NKp44 have mostly been associated with microbial responses, whereas the identity of cancer-associated ligands until recently has remained mostly obscure. A ligand for NKp44 has now been identified on tumor cells, designated Nkp44L, which opens the way for further exploration of the relative importance of this activating receptor axis in NK-mediated tumor cytolysis.50

Interestingly, in the present study, we have shown the predominant effect of HLA class I expression on the functionality of PBNK. In contrast to PBNK, UCB-NK have the ability to overcome resistance to cytolysis due to HLA-ABC expression as demonstrated by the correlative studies with all ten cell lines which revealed efficient UCB-NK-mediated cytolysis of both HLA-ABC high- and low-expressing cell lines (Fig. 4c). A lack of expression of inhibitory KIRs on UCB-NK may provide a mechanistic explanation for their ability for HLA class I independent cytolysis. Indeed, whereas PBNK and UCB-NK expressed similar levels of NKG2A, inhibitory KIRs, as measured by a panKIR2D antibody, were completely lacking from the UCB-NK cell surface. In keeping with this observation, we previously published the profiling of UCB-NK using an expanded panel of antibodies to inhibitory KIR, which revealed low expression levels of KIR2DL1/DS1, KIR2DL2/DL3/DS2, and KIR3DL1/DS1 as compared to PBNK.33 Cervical tumors have been shown to also have aberrant non-classical HLA class I expression which might help them to escape from NK cell killing.14 Remarkably, in our hands, NK cytotoxicity was not impaired by higher levels of HLA-E or HLA-G expression. The apparent ability of UCB-NK to overcome the possible resistance related to expression of both inhibitory classical and non-classical HLA molecules may offer an excellent treatment modality for cervical cancer. NK cells are often dysfunctional and low in number in cervical cancer patients.19,20,24 In order to achieve a more potent and effective cytotoxic effect of NK cells in patients with cervical cancer, it is therefore critical to have adequate numbers of functional effector NK cells. In regard to generating large numbers of NK cells for therapeutic purposes, NK cells expanded from PBMC and other sources have limited expansion capacity as compared to cord blood-derived NK cells.30 Adoptive transfer of large numbers of cytotoxic UCB-NK could be a viable treatment option, because UCB-NK have a highly activated phenotype with more than 75% stable expression rates of NKG2D, DNAM-1, NKp30, NKp44 and Nkp46 in all mature UCB-NK, and lack inhibitory KIRs, resulting in HLA independent cytolytic efficacy; additional advantages of UCB-NK over PBNK are fewer impurities (such as T and B cells) detected upon full NK maturation, thereby reducing chances of GVHD upon adoptive transfer.31 In this study, UCB-NK were not tested in combination with CET due to their low surface expression of CD16a in vitro; however, UCB-NK further mature upon adoptive transfer in vivo which is accompanied by an increase in CD16a expression32, and this feature could be exploited to enhance tumor killing even more via ADCC using CET and other IgG1 therapeutic antibodies.

To facilitate clinical application, a GMP-based NK cell expansion and differentiation protocol has already been established, approved by regulatory authorities and applied in a Phase-I clinical trial for elderly acute myeloid leukemia patients and numbers of over 3 x 10^5/kg body weight cytotoxic UCB-NK (oNKord®) can easily be achieved for therapeutic purposes (CCMO no NL31699 & Dutch trial register no 2818). Therefore, it is now entirely feasible to develop clinical protocols to explore, for the first time, adoptive transfer of UCB-NK in patients with solid tumors like cervical cancer.

In conclusion, our data provide a clear rationale for the use of UCB-NK to treat cervical tumors and also the possibility of using PBNK in combination with CET for EGFR-expressing tumors, with both significantly higher cytotoxicity and degranulation levels than in PBNK only conditions. Notably, treatment with UCB-NK might serve as a generally applicable treatment for cervical cancer enabled by HLA-, histology- and HPV-independent killing mechanisms.

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Conflicts of Interest
J.P. Veluchamy and J. Spanholtz: employees of Glycostem Therapeutics; D. Heideman serves on scientific advisory boards of Amgen and Pfizer. The other authors declare no conflict of interest.
activated NK cell receptor ligands are differentially expressed during progression to cervical cancer.


**SUPPLEMENTARY FIGURES**

Supplementary Figure 1: Representative example of flowcytometric analysis.

A representative example of flowcytometric analysis of Natural Killer (NK) cytotoxicity assay with cell line CC10A. (a) Identification of effectors and targets using SSC and FSC gating. (b) Target cells stained with PBSE were gated out from effector cells and cell debris. (c) PBSE-positive target cells were then gated against 7AAD to determine the percentage of cell death induced by NK cells. (d) Cells staining negative for PBSE and low on SSC were gated against CD56 marker to identify NK cells, further on NK cells, the levels of (e) NK degranulation (CD107a) and (f) NK CD16+ cells percentages were calculated and compared with respective target only controls.
Supplementary Figure 2: NK degranulation levels per cell line for PBNK, PBNK + CET, and UCB-NK.
Significantly higher levels of NK degranulation (ΔCD107a) in PBNK + cetuximab (CET) and UCB-NK conditions compared to PBNK only condition for SiHa, CC10A and CaSki. Bars are means of triplicate values from four experiments with four different donors for C33A, HeLa, SiHa, CC11B, CC11A, CC10B, CC10A, CaSki and two experiments with two different donors for C5CC7 and CCB using PBNK and five experiments for UCB-NK using five different donors for all cell lines. *P <0.05 and **P <0.01 calculated with one-way ANOVA Bonferroni’s multiple comparison test.

Supplementary Figure 3: Cytotoxicity levels per histological subtype and HPV type.
PBNK (open bars), PBNK + cetuximab (CET) (closed bars), and UCB-NK (hatched bars) cytotoxicity levels per (a) histological subtype and (b) HPV type of cervical cancer cell lines. Bars represent mean ± SEM. Four experiments with four different PBNK donors for C33A, HeLa, SiHa, CC11B, CC11A, CC10B, CC10A, CaSki, two experiments with two different PBNK donors for C5CC7 and CCB, and five experiments with five different UCB-NL donors were used for this experiment. AC: adenocarcinoma; SCC: squamous cell carcinoma; ASC: adenosquamous cell carcinoma.
CHAPTER 6

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


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Tanja D. de Gruijl

*Equal contribution to the manuscript.
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 IL-6, IL-10, 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 indicated that the CD14+PD-L1+ APC/Treg axis is a particularly attractive and relevant therapeutic target to specifically tackle microenvironmental immune suppression and thus enhance the efficacy of immunotherapy in patients with metastasized cervical cancer.

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).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 in cervical cancer 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 immunosuppressive 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 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 naive T and B cells.10,11 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 cervical TDLNs is therefore critical for 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.5-8 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.

MATERIAL & METHODS

Subjects and ethical approval
Women presenting with 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 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 pathological characteristics of the study cohort.

### Table 1: Clinical and pathological characteristics of the study cohort

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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.

### 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. 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 scrapings, 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 were 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. Further steps were performed with IMDM medium containing 25 mmol/L HEPES, L-Glutamine (BE12-722F; Lonza), 10% FCS, 50 IU/mL penicillin (Astellas), and Streptomycin (X-Gen Pharmaceuticals). After incubation, 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 (all from Pharmingen), CD1a, CD3, CD8, CD45RO, CD83 (all from Beckman Coulter), CD56 (IQ-products), and Foxp3 (eBioscience; all labeled with phycoerythrin (PE)); B7-H4 (AbD serotec), BDCA-2 (Milteny 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 4x concentrate in fix-perm diluent (eBioscience) for 30 minutes at 4°C. After fixation, the cells were treated with x1 permeabilization buffer (eBioscience) and 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 x1 permeabilization buffer, resuspended in flow cytometry buffer, and transferred to Micromics (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 diluted in flow cytometry buffer for 30 minutes at 4°C. After incubation, the cells were washed with flow cytometry buffer and used for analysis. IgG1, IgG2a, and IgM isotype antibodies were used as negative controls.

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 LN+</th>
<th>Phenotype LN-</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td><strong>T cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>CD3+CD4+</td>
<td>61.07 ± 4.40</td>
<td>79.62 ± 2.14</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>CD3+CD8+</td>
<td>32.45 ± 4.98</td>
<td>18.31 ± 2.16</td>
</tr>
<tr>
<td>Double-negative T cells</td>
<td>CD3+CD4-CD8-</td>
<td>4.24 ± 0.59</td>
<td>15.15 ± 0.86</td>
</tr>
<tr>
<td>Double-positive T cells</td>
<td>CD3+CD4+CD8-</td>
<td>2.25 ± 0.95</td>
<td>0.55 ± 0.08</td>
</tr>
<tr>
<td>Activated T cells</td>
<td>CD3+CD4+HLA-DR+</td>
<td>31.24 ± 9.43</td>
<td>15.25 ± 1.88</td>
</tr>
<tr>
<td>CD3+CD8+HLA-DR+</td>
<td>49.47 ± 15.52</td>
<td>20.73 ± 3.80</td>
<td>0.011</td>
</tr>
<tr>
<td>Inhibited T cells</td>
<td>CD3+CD4+ICOS+</td>
<td>28.00 ± 8.78</td>
<td>13.87 ± 2.54</td>
</tr>
<tr>
<td>CD3+CD8+ICOS+</td>
<td>9.57 ± 4.05</td>
<td>4.58 ± 0.57</td>
<td>0.060</td>
</tr>
<tr>
<td>Naive T cells</td>
<td>CD3+CD4+CTLA-4</td>
<td>27.01 ± 4.13</td>
<td>15.07 ± 1.19</td>
</tr>
<tr>
<td>CD3+CD8+CTLA-4</td>
<td>9.16 ± 3.31</td>
<td>4.77 ± 0.66</td>
<td>0.058</td>
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<tr>
<td>Nave T cells</td>
<td>CD3+CD4+CD27+CD45RA+</td>
<td>39.32 ± 12.62</td>
<td>49.50 ± 4.01</td>
</tr>
<tr>
<td>CD3+CD8+CD27+CD45RA+</td>
<td>42.22 ± 22.24</td>
<td>63.74 ± 6.04</td>
<td>0.202</td>
</tr>
<tr>
<td>Memory-like T cells</td>
<td>CD3+CD4+CD54+</td>
<td>62.14 ± 12.56</td>
<td>36.44 ± 4.29</td>
</tr>
<tr>
<td>CD3+CD8+CD54+</td>
<td>58.46 ± 17.00</td>
<td>24.21 ± 4.39</td>
<td>0.001</td>
</tr>
<tr>
<td>Effector-like T cells</td>
<td>CD3+CD4+CD54RA-CD27+</td>
<td>54.25 ± 23.19</td>
<td>29.39 ± 5.07</td>
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<tr>
<td>CD3+CD8+CD54RA-CD27+</td>
<td>9.98 ± 0.57</td>
<td>1.33 ± 0.29</td>
<td>0.801</td>
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<tr>
<td>CD4+ Tregs</td>
<td>CD3+CD4+FoxP3+</td>
<td>10.08 ± 2.27</td>
<td>2.91 ± 0.36</td>
</tr>
<tr>
<td>CD8+ Tregs</td>
<td>CD3+CD8+FoxP3+</td>
<td>1.36 ± 0.63</td>
<td>0.42 ± 0.09</td>
</tr>
<tr>
<td><strong>DC subsets</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>DDCs</td>
<td>CD1c-CD14+</td>
<td>0.15 ± 0.05</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>LCs</td>
<td>CD1c-CD14+</td>
<td>0.01 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td><strong>MOscs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytic MDSCs</td>
<td>Lin-CD33+CD14+CD16-CD15+</td>
<td>0.09 ± 0.03</td>
<td>0.06 ± 0.01</td>
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<tr>
<td>Granulocytic MDSCs</td>
<td>CD1b-CD33+CD15-</td>
<td>0.50 ± 0.41</td>
<td>0.17 ± 0.09</td>
</tr>
</tbody>
</table>

NOTE: Data are expressed as mean ± SEM.
Abbreviations: DDC, dermal-like dendritic cell; LC, Langerhans cell; LNDC, lymph node-resident dendritic cell.

A minimum of 10 x 10⁶ cells were required to perform all flow cytometric analyses. If fewer cells were obtained from the scrapings, we carried out a partial analysis based on available cell numbers. Analyses were performed by four-color flow cytometry on a BD FACSCalibur (BD).

Note that because of technical limitations, CD4+HLA-DR+ and CD4+ICOS+ T-cell subsets in this study were identified by gating CD3+CD8+HLA-DR+ and CD3+CD8+ICOS+ populations, whereas CD8+CTLA-4+ and CD8+PD-1+ T cell subsets were similarly quantified by gating CD3+CD4+CTLA-4+ and CD3+CD4+PD-1+ populations. Expression of these activation markers on double-positive CD4+CD8+ and double-negative CD4-CD8- T cells was thus not taken into account. Ranges of double-positive CD4+CD8+ and double-negative CD4 CD8 T cells for LN- were 0.09 to 1.12 and 0.67 to 2.77, and for LN+ were 0.76 to 8.80 and 1.29 to 6.77, respectively.

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 CD274 (PD-1, eBioscience). Afterwards, slides were washed three times in PBS for 5 minutes and incubated with 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 x100 and x400, 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 x 10⁶ 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. 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 FACSCalibur 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/IL-10 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 pathological 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 parameters were examined by linear regression (r-test) using GraphPad Prism software. Differences and correlations were considered 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 LN- versus LN+ of 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 = 0.034), ICOS (n.s.), CTLA-4 (P = 0.014), and PD-1 (P = 0.005) 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).

Figure 1: 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+ T cells (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 naïve 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.

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 = 0.01, CD45RO+) 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-.
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 15), including the migratory DC subsets CD11c+CD1a− dermal-like DCs (DDC) and CD11c+CD1a+ Langerhans cells (LC), and CD1a+CD11c−CD14+ LN-resident DCs (LNDC) as well as CD1a+CD11c+CD14− APCs. Our data showed higher frequencies in LN+ for CD11c+CD1a− DDCs ($P = 0.019$), CD11c+CD1a+ LCs ($P < 0.001$), CD14+ APCs ($P < 0.001$), and CD14− LNDC subsets ($P = 0.032$). In addition, we investigated the plasmacytoid 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 immunocytological 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-DR−/low (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 TNFα were 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).

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−. Triple immunofluorescence staining of CD14 (green), CD163 (red), and PD-L1 (blue).

[117x39 to 546x382]
Association between immunosuppressive immune cells and lymphovascular space invasion of tumor cells

Lymphovascular space invasion (LVSI) is a prognostic factor in early-stage cervical cancer and precedes metastasis to cervical TDLNs. In our study cohort, higher rates of LVSI were observed in patients with LN+ as compared to patients with LN- ($P = 0.015$; Table 1). Seven of 20 patients with LN manifested with LVSI. 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+CD45RA+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, 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 immunosuppressive checkpoint molecules PD-1 and CTLA-4, and, as expected, higher proportions of suppressive CD4+ and CD8+ Tregs were present in LN+. Also, increased levels of Tregs have been reported to be present at the site of the primary tumor and in peripheral blood of patients with cervical intraepithelial neoplasia (CIN) or cervical cancer. Of note, we previously reported high Treg frequencies in the peripheral blood were associated with high-grade CINIII lesions and persistent HPV16 infection. Patients with higher CD8+ T cell/Treg ratios in primary tumor tissue have a prolonged survival time compared with patients with lower CD8+ T cell/Treg ratios. 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 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. Moreover, we found two other potentially suppressive subsets, CD4+CD8- T cells and CD4-CD8+ T cells, to be significantly overrepresented in LN+. Similarly, elevated proportions of CD4+CD8- T cells were detected in breast cancer. Hodgkin lymphoma, and melanoma. The functional role of CD4+CD8- T cells, however, remains controversial, as they may represent a regulatory subset, involved in immune regulation and tolerance, but they have also been ascribed antitumor activity.

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. Interestingly, compared with LC proportions in skin-draining lymph nodes, 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. 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 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, gastric cancer, melanoma, and endometrial cancer. 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+ APC subset in LN+. This PD-L1+CD14+ myeloid subset harbors low expression levels of costimulatory molecules and may thus exert immunosuppressive effects through its ability to bind the PD-1 checkpoint on T cells, which negatively regulates T-cell activation. Of note, this immunosuppressive subset also lacks the characteristic DC maturation marker CD83 but often expresses the M2-macrophage-associated marker CD63 (Fig. 2) and is thus reminiscent of M2-macrophages arising in DC differentiation cultures in vitro under the influence of cervical cancer-derived IL6 and PGE2, which also expressed CD163, CD14 and PD-L1. This would also fit with our observation of high levels of IL6 released by single-cell suspensions of LN+. We and others have reported a phenotypically similar subset in a range of solid tumor types. PD-L1 is expressed by the primary cervical tumor cells and its receptor PD-1 is often expressed by T cells infiltrating the primary tumor, similar to the results we obtained for LN+. Indeed, our finding of a significant correlation between 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 immune-suppressive 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. Our study is the first to report the presence of MDSCs in human LNs with a specific enrichment in LN+. Through cellular cross-talk, these MDSCs may further amplify the immunosuppressive effects of the CD14+ APCs and Tregs. 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 LN+ by concerted elevated levels over LN 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. In contrast, higher IFNγ release levels specifically upon R848/TLR-7/8 stimulation in LN 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, calls for caution and indicates that the therapeutic use of TLR-L in the context.
REFERENCES


Supplementary Figure S1.
Triple immunofluorescence staining of CD14 (green), CD163 (red) and PD-L1 (blue) of representative tumor negative (LN-) and positive (LN+) lymph node specimen reveals the presence of elevated numbers of CD14+CD163+ cells expressing PD-L1 in LN+ and low to no-detectable expression levels of PD-L1 on sporadic CD14+CD163+ cells in LN-. (magnification, x400; scale bar, 10 μm).
Nodal Metastasis in Cervical Cancer Occurs in Clearly Delineated Fields of Immune Suppression in the Pelvic Lymph Catchment Area
ABSTRACT

In cervical cancer, high frequencies of regulatory T cells (Tregs) and immunosuppressive PD-L1+CD14+ antigen-presenting cells dominate the microenvironment of tumor-positive lymph nodes (LN+). It is unknown whether this is restricted to LN+ or precedes metastasis, emanating from the primary tumor and spreading through tumor-draining lymph nodes (TDLNs). To investigate immunosuppression in the lymphatic basin of cervical tumors, all dissected TDLNs of five cervical cancer patients (in total 9 LN+ and 74 tumor-negative lymph nodes (LN-)) were analyzed for FoxP3+ Tregs, CD8+ T cells, HLA-DR+ and PD-L1+ myeloid cells by immunohistochemistry.

Tregs and PD-L1+ cells were found to form an immunosuppressive cordon around metastatic tumor cells. Importantly, whereas high HLA-DR+ and PD-L1+ cell rates were strongly associated with LN+, elevated Treg levels and decreased CD8+ T cell/Treg ratios were found similar in LN+ and adjacent LN-, as compared to LN- at more distant anatomical localizations. These data suggest that delineated fields of Treg-associated immune suppression in anatomically co-localized TDLNs enable metastasis by creating metastatic niches. This may be of importance for decision-making regarding (surgical) intervention in cervical cancer. Future efforts should include the implementation of immunotherapeutic regimens to overcome this immune suppression, establish loco-regional control and halt systemic tumor spread.

INTRODUCTION

Cervical cancer is caused by a persistent infection with high-risk human papillomavirus (HPV) types, and is therefore an immunogenic disease which requires a highly immunosuppressive microenvironment in order to progress and metastasize. Various immune suppressive cells are recruited, expanded and activated at the site of the primary tumor. These cells are able to inhibit and suppress activation of the immune system, and promote an immune suppressive microenvironment which supports tumor growth, not only in the primary tumor but also in tumor-draining lymph nodes (TDLNs). Recently, we reported on the presence and abundance of suppressive PD-L1+CD14+ M2-macrophage-like cells, myeloid-derived suppressor cells (MDSCs), T cells expressing co-inhibitory molecules (PD-1 and CTLA-4), and regulatory T cells (Tregs) in tumor-positive lymph nodes (LN+) compared to tumor-negative lymph nodes (LN-) from cervical cancer patients. Tregs present in cervical TDLNs were previously found to be HPV-specific and functionally suppressive. In line with the excess of suppressive immune cell subsets, decreased levels of interferon-γ (IFNγ) and high levels of interleukin-6 (IL-6), IL-10, and vascular endothelial growth factor (VEGF) were detected in cervical metastatic lymph nodes. This immune suppression in metastatically involved lymph nodes will stand in the way of effective anti-tumor immunity and may have to be tackled before immunotherapy can be effective and halt metastatic spread. In particular PD-L1 expressed on tumor-associated M2-like macrophages may represent an attractive therapeutic target. While systemic immune checkpoint blockade in cervical cancer is currently being explored in several clinical trials, more localized targeting of the microenvironment of cervical tumors and their TDLN may be even more effective while minimizing side effects.

Information is lacking on the localization and distribution of immune cell subsets in pelvic lymph nodes with respect to lymphatic drainage patterns and tumor involvement. Only one study focused on the difference between proximal and distal lymph nodes in relation to the primary tumor and reported a significantly higher CD4+/CD8+ T cell ratio in the proximal lymph nodes. FoxP3+ Tregs and potentially immunosuppressive macrophage-like cells (expressing HLA-DR and PD-L1) are able to cross-talk and thereby mutually amplify their suppressive activity on antitumor (CD8+) T cells. One of the crucial check-point molecules involved in this process is PD-L1, since it can bind to PD-1 on T cells thereby inhibiting their function. Therefore, in the present study we analyzed the distribution and localization of FoxP3+ Tregs, CD8+ T cells, HLA-DR+ and PD-L1+ myeloid cells in all pelvic lymph nodes, including LN+ and LN-, removed during primary surgery of five patients with cervical cancer. We show the presence of an immune suppressive cordon of Tregs and PD-L1+ cells around metastatic tumors in the TDLN and provide evidence for anatomically delineated fields of immune suppression characterized by high numbers of Tregs, in the tumor lymph catchment area, co-localizing with LN+ and suggestive of metastatic niche formation.

Cervical cancer is typically treated with radical hysterectomy and pelvic lymphadenectomy or chemoradiation. Our findings argue in favor of intratumoral immune potentiation.
pre-surgical or combined with chemoradiation) to stem immune suppressive lymph drainage from the tumor, avoid intranodal metastatic niche formation, and enable anti-tumor T cell activation.

**MATERIAL & METHODS**

**Patient population**

In order to study the immune cell subsets in pelvic lymph nodes relative to the distance from the primary tumor, all formalin-fixed paraffin-embedded lymph nodes were collected from different proximal and distal anatomical locations, including fossa obturator, ilaca externa, ilaca communis and parametrium, from five patients with cervical squamous cell carcinoma (SCC) presenting with FIGO stage IB (according to International Federation of Gynecology and Obstetrics), who underwent radical hysterectomy and pelvic lymphadenectomy according to Wertheim-Okabayashi or lymphadenectomy only as primary treatment between 2005-2008 at the Academic Medical Center (AMC, Amsterdam, The Netherlands). Patients were selected on FIGO stage, the presence of lymph node metastases and practicable, total number of dissected lymph nodes (Table 1). Patient samples were handled according to the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies.

<table>
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<tr>
<th>Table 1: Clinical characteristics of the study group</th>
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<td>Patient</td>
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LND = lymph node debulking
RH = radical hysterectomy and lymphadenectomy

Additionally, fresh lymph node samples were collected from one cervical SCC patient with FIGO stage IIb, primarily treated with lymph node debulking in the AMC (Amsterdam, The Netherlands). These cells were used for FACS analysis as described below. This study design was approved by the Medical Ethical Committees of the AMC (Amsterdam, The Netherlands). The patient gave written informed consent.

**Immunohistochemistry**

In total, 9 LN+ and 74 LN- (from 3 LN- insufficient material was present), were obtained from the archive of the Pathology department at the AMC (Amsterdam, The Netherlands), sectioned at 4 μm and mounted on Starfrost slides (Walderm Knittel, Germany).

For multicolor immunofluorescence staining of CD8, FoxP3 and HLA-DR, slides were deparaffinized for 3 x 5 min in Neo-Clear (VWR, Catalog# 1.09843.5000), and in series of decreasing concentrations of alcohol (1 x 5 min in 100%, 1 x 5 min in 96%, and in 1 x 5 min in 70%). Subsequently, the slides were placed in Milli-Q water for 5 min, and then antigen retrieval was achieved by 10 min boiling in Tris-EDTA buffer at pH 9.0. Afterwards, the slides were allowed to cool down for at least 45 min, and were washed for 3 x 5 min in PBS. Slides were incubated with a mixture of primary antibodies diluted in 1% BSA/PBS overnight at room temperature (RT); 1:100 mouse IgG1 anti-FoxP3 (Abcam, Catalog# ab20034), 1:75 mouse IgG2b anti-CD8 (Novocastra, Catalog# NCL-CD8-4b11), and 1500 rabbit anti-HLA-DR (Abcam, Catalog# ab137832). Next, slides were washed for 3 x 5min in PBS, and then incubated with a mixture of secondary antibodies diluted in 1% BSA/PBS for 1 hour at RT, 1:200 Alexa Fluor 488 goat anti-mouse IgG1 (Life Technologies, Catalog# A21121), 1:200 Alexa Fluor 647 goat anti-mouse IgG2b (Life Technologies, Catalog# A21242), and 1:200 Alexa Fluor 546 goat anti-rabbit (Life Technologies, Catalog# A11010). Next, the slides were washed for 3 x 5 min in PBS, and counterstained with 1:1000 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI), washed in PBS and mounted under coverslips with Mowiol.

For immunohistochemical staining of all 83 lymph node samples for PD-L1, an automated immunostainer (Ventana Medical Systems, Inc. Tucson, USA) was used for deparaffination, antigen retrieval, incubation of the primary antibody 1:200 rabbit anti-PD-L1 (Cell signaling, Catalog# 13684), detection, and visualization steps, according to the manufacturer’s instructions. Sections were counterstained with Hematoxylin, dehydrated, and mounted under coverslips.

**Imaging, scoring & analysis**

The stained slides were analyzed using a fully motorized digital imaging fluorescence microscope (Axiovert-200M, Zeiss, Germany) or a bright-field microscope (Olympus BX50, Olympus, USA).

From each LN-, three to five representative T cell areas (paracortical areas without B cell follicles) were selected and imaged; from each LN+ three different areas were selected and imaged three to five times; tumor area, peri-tumoral area and paracortical T cell area. All pictures were taken at a 200x magnification. Tumor fields were morphologically distinguished from normal tissue by the use of nuclear staining with DAPI. The area (in mm²) of the region of interest was determined using image J (National Institutes of Health (NIH), USA) and Slide-Book 5.5 Reader (Intelligent Imaging Innovations (3I), USA). Cell counting of positively stained...
FoxP3 and CD8 cells from digital images was performed manually using the cell counter function of Image J. Results were expressed as number of cells per mm². HLA-DR+ and PD-L1+ cells were semi-quantitatively scored because their density and irregular morphology made it difficult to reliably count these cells and therefore we distinguished three groups: [-/+] minimal-, (+) moderate-, and (++) high numbers of positive cells in both LN- and LN+ (see Supplementary Fig. 2). For some of the analyses, lymph node groups were categorized as follows: (1) LN+, (2) LN- (LN- located in the same anatomical localization [i.e., lymph node station] as LN+) and (3) all LN- in the remaining anatomical locations, distant from LN+. In the latter analysis, parametrical lymph nodes were excluded.

Fresh lymph node collection, processing and flowcytometric analysis
Two lymph node samples were collected from one macroscopically characterized two distinct regions: metastatic tumor and pre-existent lymphoid area. From both areas, cells were collected and processed to single-cell suspensions as previously described. To study T cell frequencies, four-color flow cytometry was performed on the single-cell suspensions using a set of antibodies: CD4-FITC, CD3-APC (BD), CD25-APC (BD), CD3-PerCp-Cy5.5 (BD), CD8-PE (all from BD Biosciences, USA), and FoxP3-PE (eBioscience, USA). To identify Tregs (CD3+CD4+CD25+FoxP3+), a membrane and intracellular staining was combined as previously described. Mouse-IgG1 and rat-IgG2a antibodies were used as isotype control. Analyses were performed on a BD FACSCalibur (BD) and data were analyzed using CellQuest Pro software (BD).

Statistical analysis
All statistical analyses were performed using GraphPad Prism 5. The one-way ANOVA Friedman test was used to compare Tregs, CD8+ T cells, and CD8+ T cell/Treg ratios between three groups (T cell area/peri-tumoral area/tumor area and LN+/LN-*/LN-). Comparisons between specific groups were performed with Dunn’s post hoc test. The two-sided Chi² Fisher’s Exact test was used to analyze differences between distribution of HLA-DR+ and PD-L1+ cells, between two groups, and between three groups (T cell area/peri-tumoral area/tumor area and LN+/LN-*/LN-). Kruskal-Wallis test was used for comparison between three independent groups. Results were significant when P < 0.05.

RESULTS

We studied the intranodal distribution and localization of FoxP3+ Tregs, CD8+ T cells, HLA-DR+ and PD-L1+ cells in five patients with cervical cancer by a triple FoxP3/CD8/HLA-DR immuno-fluorescence staining and immunohistochemical staining for PD-L1 HLA-DR+ and PD-L1+ cells were morphologically identified as myeloid-like cells, based on their dendritic cell (DC)- or macrophage-like shape. In the cervical lymph nodes we were able to identify several immune cell subsets, including single FoxP3+, single CD8+, single HLA-DR+ and PD-L1+ cells. In addition, we observed double positive FoxP3+HLA-DR+, CD8+HLA-DR+ and CD8+FoxP3+ cells. However, only a few double positive immune cells were present per image, therefore we did not include these in our analysis.

Distribution and localization of FoxP3+, CD8+ and PD-L1+ cells in LN+
We studied the distribution and localization of Tregs, CD8+ T cells, single HLA-DR+ and PD-L1+ myeloid cells in all LN+. Nuclear DAPI stain was used to distinguish tumor tissue from normal tissue and in case of doubt a sequential Hematoxylin & Eosin (H&E)-stained slide was consulted (Fig. 1A,B). A distinction between paracortical T cell area, peri-tumoral area and tumor area in LN+ was made (Fig. 1A-H), and we found a significant difference in the amount of Tregs per mm² between the three areas (P = 0.008). Accumulation of Tregs was observed in the peri-tumoral areas, whereas limited numbers of Tregs were found in the metastatic tumor fields (P < 0.01) (Fig. 1I). Furthermore, we found a significant difference in the amount of CD8+ T cells per mm² between the three areas (P = 0.009), with higher numbers in paracortical T cell areas and only a few infiltrating the metastatic tumor area (P < 0.05) (Figure 1J). Moreover, we observed a significant difference in the distribution of PD-L1+ myeloid cells among the three areas (P = 0.038), with more PD-L1+ cells in peri-tumoral areas than in tumor areas (P = 0.017) (Fig. 1K). Note, metastatic tumor cells of 5/9 LN+ were weakly positive for PD-L1, however we were still able to identify PD-L1+ tumor infiltrating myeloid cells by the dense membranous PD-L1 expression compared to the relative dim expression on tumor cells (Figure 1H). Together these data point to a cord in immune cells, heavily populated by Tregs and PD-L1+ myeloid cells around nodal metastases.

Anecdotally, we collected fresh samples of one LN+, including a sample of ‘white’ tissue, referred as ‘tumor-and peri-tumoral area’, and one sample of ‘dark’ tissue, referred as ‘T cell area’, macroscopically determined by an experienced pathologist. We studied CD4+ and CD8+ T cell ratios and Treg (identified by CD3+CD4+CD25+FoxP3+) frequencies in both samples by flow cytometry, and found in concordance with our immunohistochemistry data, a higher percentage of CD8+ T cells (46.2% vs. 25.1%) and a lower percentage of CD4+ T cells (48.1% vs. 72.3%) in the tumor area than in the T cell area. Additionally, we found more Tregs (12.5%) in the tumor area compared to the T cell area (2.8%) (Supplementary Fig. 1).

Patterns of immune suppression in the tumor lymph draining catchment area
In a previous flowcytometry-based study, we found a significant correlation between Treg and PD-L1+ macrophage-like cell rates in single-cell suspensions from TLN. Here, we confirmed these findings: a significant association was found in the studied lymph nodes between high Treg frequencies and high PD-L1+ myeloid cell numbers in non-tumor regions. These regions were defined as paracortical areas in case of LN- and combined paracortical and peri-tumoral areas in case of LN+ (P = 0.003) (Fig. 2).
Figure 1: Tregs, CD8+ T cells and PD-L1+ myeloid cells in the paracortical T cell area, peri-tumoral and tumor area in metastatic lymph nodes

(A) H&E staining of a representative tumor-positive lymph node (LN+) showing the peri-tumoral and tumor area. Triple immunofluorescence staining of FoxP3 (green), HLA-DR (red) and CD8 (blue) of a (B) LN+ (sequential section to the one shown in (A), magnification 100x, scale bar is 20 μm) showing the presence of Tregs, CD8+ T cells and HLA-DR+ cells in (C) the paracortical T cell area, (D) the peri-tumoral area, and (E) the tumor area in a LN+. Nuclei are counterstained with DAPI (grey) (magnification 200x, scale bar 20 μm). In a sequential section, also PD-L1+ cells (in brown) were present in (F) T cell area, (G) peri-tumoral area, and (H) tumor area. Nuclei are counterstained with Hematoxylin (blue) (magnification 200x, scale bar 20 μm). Significantly more (I) Tregs, (J) CD8+ T cells and (K) PD-L1+ cells were present in peri-tumoral areas compared to the metastatic tumor fields. Arrow heads indicate PD-L1+ infiltrating cells among the weakly positive tumor cells. Note: in (I) and (J), the numbers on the y-axis should be multiplied with factor 100 and overall P value was calculated with One-way ANOVA Friedman test, and Dunn’s post hoc test was used. *P = 0.01 to 0.05 and **P = 0.001 to 0.01.

Next, we investigated Treg, CD8+ T cell, HLA-DR+ and PD-L1+ myeloid cell numbers in paracortical areas, and in case of LN+, paracortical and peri-tumoral areas, in all lymph nodes that could be delineated according to succession in the lymphatic drainage of the primary tumor (from proximal to distal and therefore excluding parametrial lymph nodes) according to their anatomical position (iliaca externa left or right, fossa obturator left or right, and iliaca communis left or right, based on the pathology reports) (Fig. 3A, 4A). This allowed for the identification of tumor-draining lymphatic patterns per patient, based on fields of immune suppression. We found evidence of a unique immune suppression-delineating draining pattern per patient, with varying levels of Tregs (Fig. 3B), CD8+ T cell/Treg ratios (Fig. 3C), and HLA-DR+ and PD-L1+ myeloid cells (Fig. 4C) between LN. High Treg levels, low CD8+ T cell/Treg ratios, and high numbers of HLA-DR+ cells were consistently found in LN+ (Fig. 3 and 4). High numbers of PD-L1+ cells were found in 5 out of 8 LN+, while in the remaining three LN+, minimal to moderate numbers of PD-L1- cells were observed (Fig. 4C), but this could be due to the fact that two of these LN+ had micro-metastases and one LN+ consisted almost entirely of metastatic tumor cells. Interestingly, 39.7% of the LN- had high Treg levels and 45.2% of the LN- had low CD8+ T cell/Treg ratios, whereas only 15.1% of the LN- had high numbers of HLA-DR+ cells, and 8.2% of the LN- had high numbers of PD-L1+ cells. Of note, as shown in Figure 3 and 4, these apparently affected LN- with high Treg rates most often co-localized with LN+ at the same side and in the same anatomical lymph node stations relative to the primary tumor, and thus seemed to delineate an immune suppressive lymph flow from the primary tumor, apparently facilitating loco-regional tumor spread.

Subsequently, all lymph nodes from all patients were divided over three different groups:

Figure 2: Association between high Treg- and high PD-L1+ myeloid cell rates in cervical lymph nodes

Treg and PD-L1+ myeloid cell rates in non-tumor regions of all cervical lymph nodes were significantly correlated. Note: the numbers on the y-axis should be multiplied with factor 100. The overall P value was calculated with One-way ANOVA Kruskal-Wallis test. For testing of selected groups, Dunn’s post hoc test was used. ***P = 0.001 to 0.01 and ****P < 0.001.
LN+, LN-* (present in the same anatomical location, i.e. same side and lymph node station, as the LN+) and all remaining LN- (i.e., not co-localized with LN+). Numbers of Tregs, CD8+ T cell/Treg ratios, HLA-DR+ and PD-L1+ myeloid cells were compared between these groups. No differences were observed for Treg numbers and CD8+ T cell/Treg ratios between LN+ and LN-, whereas significant differences were observed between LN+ and LN-, confirming elevated Treg rates in LN- proximal to LN+. No significant differences were found for CD8+ T cell levels between the three different groups (Fig. 5A,B,C). In contrast, for HLA-DR+ and PD-L1+ myeloid cells, significant differences were found both between LN+ and LN- (\(P = 0.002\) for HLA-DR and for PD-L1) and between LN+ and LN- (for both \(P < 0.001\), but not between LN+ and LN- (Fig. 5D,E), revealing a more strict tumor-associated recruitment of potentially suppressive PD-L1+ myeloid cells.

Figure 3: Treg levels and CD8+ T cell/Treg ratios in the lymphatic basin of five patients with cervical cancer
(A) Reconstruction of the anatomical locations of pelvic lymph nodes from all patients, showing the following regions: iliaca externa (purple), fossa obturator (green), and iliaca communis (red) on both sides of the body (right and left). Graphs showing (B) Treg levels per mm² and (C) CD8+ T cell/Treg ratios per mm². Note: in (B), the numbers on the y-axis should be multiplied with a factor 100. Closed circles represent LN+, open circles represent LN-.

Figure 4: HLA-DR and PD-L1 levels in the lymphatic basins of five patients with cervical cancer
(A) Reconstruction of the anatomical locations of pelvic lymph nodes from five patients with cervical cancer, showing the following regions: iliaca externa (purple), fossa obturator (green), and iliaca communis (red) on both sides (right and left). Graphs showing (B) HLA-DR levels and (C) PD-L1 levels (in paracortical areas in tumor-negative lymph nodes (LN-) and in case of tumor-positive lymph nodes (LN+), paracortical and peritumoral areas) per lymphatic basin per patient. Levels for HLA-DR and PD-L1 are indicated by (+++) minimal, (+) moderate, and (+++) high numbers of positive cells. Closed circles represent LN+, open circles represent LN-.
Prior to metastasis, lymph nodes are conditioned by these Tregs to form a metastatic tumor spread. In contrast to Tregs, PD-L1+ myeloid cells were more strictly associated with the presence of metastatic tumor spread. In cervical cancer, only one study found higher CD8+ T cell/Treg ratios in proximal lymph nodes compared to distal lymph nodes in relation to the distance from the primary tumor, but for FoxP3+ Tregs no significant difference was found between the primary tumor localization and tumor flow.

Our results point to a strong immunosuppressive microenvironment in LN+ from patients with cervical cancer with high Treg levels, low CD8+ T cell/Treg ratio, and high levels of PD-L1+ and HLA-DR+ myeloid cells, which is consistent with previous data based from flowcytometric analyses and immunohistochemical stainings. In addition, we identified a peri-tumoral area with immunosuppressive FoxP3+ Tregs and PD-L1+ myeloid cells accumulation whereas only a minimal number of immune cells were observed infiltrating into the tumoral areas in lymph nodes from patients with cervical cancer. This cordon around the metastases might be caused by the presence of immunosuppressive factors such as PD-L1, IDO, IL-6 and Prostaglandin-E2 (PGE2), or extracellular matrix components (e.g. versican) shown to be expressed by primary cervical tumor cells, leading to immunosuppressive cell subset accrual and preventing antitumor cell subsets to enter the tumor area.

In order to unveil coordinated suppression through primary tumor-derived lymph flow, we have grouped pelvic lymph nodes according to anatomical location, which showed a unique lymphatic suppressive drainage pattern per patient. Next to the high numbers of Tregs, HLA-DR+ and PD-L1+ myeloid cells, and low CD8+ T cell/Treg ratios in LN+, interestingly, also in a number of LN-, we found high Treg numbers and low CD8+ T cell/Treg ratios. The measured PD-L1+ cells are most likely M2 (CD163+) macrophages, originating from monocytes in the presence of IL-6, PGE2 and IL-10.

Also, in lymph nodes located distal from the primary tumor, at the iliaca communis region, the microenvironment might be immunosuppressive as suggested by the here presented data. This might be explained by sentinel lymph node (SLN) identification studies in cervical cancer, in which first-line draining lymph nodes were found proximal, but also in distal locations including the iliaca externa region, the fossa obturator region, the iliaca communis region, and within the parametrium. This indicates that the (immunosuppressive) tumor flow is unpredictable and varies highly per patient. In cervical cancer, only one study found higher CD4+/CD8+ T cell ratios in proximal lymph nodes compared to distal lymph nodes in relation to the distance from the primary tumor, but for FoxP3+ Tregs no significant difference was found. This discrepancy might be explained by the fact that the latter study was performed irrespective of SLN localization and tumor flow.

Interestingly, we found similarly elevated Treg levels and CD8+ T cell/Treg ratios in LN+ and LN-*, and higher CD8+ T cell/Treg ratios were found in LN- compared to LN+.

### Figures

**Figure 5:** Frequencies of immunosuppressive cell subsets in tumor positive lymph nodes (LN+), proximal (to the LN+) uninvolved lymph nodes (LN-) and more distal uninvolved lymph nodes (LN-*) from patients with cervical cancer.

(A) High levels of Tregs in tumor-positive lymph nodes (LN+) and tumor-negative lymph nodes (LN-), located in the same region as LN+ (LN-*). (B) Constant levels of CD8+ T cells in LN+, LN-* and LN-. (C) Low CD8+ T cell/Treg ratios in LN+ and LN-*, and higher CD8+ T cell/Treg ratios were found in LN- compared to LN+. (D) The distribution of HLA-DR+ cells in LN+ was significantly different compared to LN- and LN-*. (E) The distribution of PD-L1+ cells in LN+ was significantly different compared to LN- and LN-*. Minimal (−/−), moderate (−/+), and high numbers (+/+ of positive cells. Note: in (A-C), the numbers on the y-axis should be multiplied with a factor 100. One-way ANOVA Friedman test P value in (A), (B), and (C), Fisher’s Exact test P value in (D) and (E) (**P < 0.001 to 0.01 and ***P < 0.001).

### DISCUSSION

Progression of cervical cancer manifests predominantly by local expansion and through lymphovascular space invasion, and is significantly associated with the risk of pelvic lymph node metastasis, which is an important indicator for poor prognosis. In this study, we investigated the localization and distribution of FoxP3-, CD8-, HLA-DR- and PD-L1+ myeloid cells in all pelvic lymph nodes from five SCC cervical cancer patients with lymph node metastases. This is the first study to investigate the microenvironment of pelvic lymph nodes in different locations within the pelvic lymphatic basin, in order to study immune suppression in LN+ in relation to other proximal lymph nodes and determine suppressive lymphatic draining patterns per patient.
astatic niche. Subsequently, metastatic tumor cells again recruit and convert PD-L1+ M2-like cells and facilitate the expansion of a next wave of Tregs preparing the way for further metastatic spread.

These observations support previous findings on an improved survival of patients with LN- after complete lymphadenectomy compared to patients with LN+ with an uncompleted lymphadenectomy, and might be important for surgical intervention and the exploration of therapies aimed at counteracting the immunosuppressive microenvironment in the primary cervical tumor and the tumor-draining lymph nodes by checkpoint inhibitors, e.g. anti-PD-L1 to inhibit M2-macrophages or anti-CTLA4 to deplete Tregs, thus inducing a robust antitumor T cell response and breaking the vicious cycle of immune suppression and metastatic spread.

Acknowledgements
We would like to thank Wim Vos for technical assistance, Jeroen Wierda for assisting with the PD-L1 scoring, Sanne Samuels and Debbie Ferns for patient inclusion. In addition, we would like to thank the Pathology department from AMC (Amsterdam, The Netherlands) for collecting the fresh lymph node samples.

REFERENCES

**SUPPLEMENTARY FIGURES**

Supplementary Figure 1: Flowcytometric analysis of CD8+ T cell and Treg frequencies in lymphoid vs. tumor tissue from a cervical tumor draining lymph node
A lower percentage of CD8+ T cells (46.2% vs. 25.1%, upper panel), a higher percentage of CD4+ T cells (48.1% vs. 72.3%, upper panel), and less Tregs (identified by CD3+CD4+CD25highFoxP3+ cells) (2.8% vs. 12.5%, lower panel) were found in the lymphoid area compared to the tumor area in one freshly collected lymph node as analyzed by flow cytometry.

Supplementary Figure 2: Representative images for semi-quantitative analysis of HLA-DR and PD-L1
(A) Minimal (+/-), (B) moderate, (+), and (C) high numbers of HLA-DR+ cells in lymph nodes from patients with cervical cancer. (D) Minimal (+/-), (E) moderate (+), and (F) high numbers of PD-L1+ cells in lymph nodes from patients with cervical cancer. NB: in (D) a very weak brown staining is visible in lymph node sinuses which was regarded as background. Scale bars are 20 μm.
CD14⁺ Macrophage-like Cells as the Linchpin of Cervical Cancer Perpetrated Immune Suppression and Early Metastatic Spread: a New Therapeutic Lead?


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ABSTRACT

A number of studies point to an aberrant differentiation and accumulation of CD14+PD-L1+M2-macrophage-like cells in the microenvironment of cervical cancer, which promote immuno-suppressive conditions and are associated with tumor invasion, angiogenesis and metastasis. Therapeutic targeting of these macrophages may tip the balance in favor of anti-tumor immunity. Cervical cancer is the fourth most common cancer among women worldwide and is caused by a persistent infection and subsequent integration of high-risk types of the human papillomavirus. Continuous expression of the viral oncoproteins E6 and E7 has been shown essential to maintain the transformed state of infected keratinocytes. As these non-self oncoproteins are immunogenic, cervical cancer requires a highly immune suppressed microenvironment to metastasize through lymphovascular space invasion (LVISI) to the pelvic tumor-draining lymph nodes (TDLN). Unraveling the mechanisms underlying this immune suppression may uncover novel therapeutic targets aimed at loco-regional control of cervical cancer.

Recently we uncovered a strong association in cervical TDLN between metastatic involvement and high rates of a CD14+CD163+ M2-macrophage-like subset with high programmed death-ligand 1 (PD-L1) expression and low levels of the co-stimulatory molecules CD80 and CD86. As these M2-like cells were virtually absent from uninvolved TDLN and phenotypically differed considerably from the CD14+ dendritic cell (DC) LN-resident subset, we hypothesized that they had originated from monocytes that were both attracted and conditioned by tumor-derived factors. Indeed, we and others have previously shown that monocyte-to-DC differentiation can be diverted to the development of M2-like cells in the presence of primary tumor supernatants, mostly due to high levels of prostaglandin-E2 (PGE2) and interleukin (IL)-6.2,3 In addition, maturation of DC in the presence of IL-10 can lead to their conversion into CD14+ M2-like cells with very similar phenotypic and functional features, i.e., up-regulated PD-L1 and CD163 expression, low levels of co-stimulatory molecules, reduced IL-12p70 and increased IL-10 release (upon CD40L and IFNγ stimulation), poor CD8+ effector T cell priming, and induction of CD25hiFoxP3+ regulatory T cells (Treg). Additionally, these M2-like cells expressed pro-angiogenic and pro-tumor invasive factors (Fig. 1).4

In primary cervical tumors, CD14+ cells are present in large quantities5, and can actually adopt a T cell-stimulatory M1-phenotype, which, in conjunction with CD8+ T cell infiltration, resulted in a prognostically favorable association with overall survival.6 Interestingly, we observed a reduced frequency of CD8+ effector T cells in metastasis-free TDLN of patients with LVISI. This is proof of early immune suppression in TDLN preceding actual metastatic spread and indicates the importance for tumor invasion of sabotaging effector T cells. PD-L1 is an inhibitory molecule also expressed on cervical tumor cells, which can bind to PD-1 on activated T cells, thus blocking their anti-tumor effector functions (Fig. 1). The strong association between metastasis and the presence of PD-L1+ M2-macrophages in TDLN suggests that metastasizing tumor cells have the ability to recruit and convert monocytes to M2-macrophages, likely through the release of chemo-attractants like CCL2 and of M2-inducing factors like PGE2, IL-6, IL-10, IL-4, and IL-13 (Fig.1).2,4,6,7 These M2-macrophages may aid tumor invasion not only through effector T cell inhibition, but also physically through the release of matrix metallo-proteinases.4 In cervical TDLN, we also found a clear and significant correlation between frequencies of Tregs and rates of CD14+ M2-like cells as well as their expression levels of PD-L1.Tregs are numerous at both primary and metastatic tumor sites1,5,9, and in TDLN were previously shown to be highly T cell suppressive.9 Their expansion and activation may be induced by interactions with M2-macrophages, and vice versa, Tregs can promote differentiation of monocytes to immunosuppressive M2-macrophages. Thus, a picture is emerging in metastatic TDLN of a concerted increase in M2-macrophages in TDLN and Tregs, associated with high IL-10 and IL-6 release levels (Fig. 1).1 We propose that metastasizing tumor cells condition the microenvironment through PGE2 and IL-6 release to engender PD-L1+ M2 skewing, leading to collateral Treg expansion and activation. Cross-talk with co-mobilized myeloid-derived suppressor cells (MDSC) may further amplify this vicious cycle of immune suppression, thus enabling immune escape (Fig. 1).
CD14+ M2-LIKE CELLS AS THE LINCHPIN OF CERVICAL CANCER: A NEW THERAPEUTIC LEAD?

Figure 1: Model showing the central role of CD14+ M2-macrophage-like cells in the immune suppression in cervical cancer. CD14+ monocytes are recruited from the peripheral blood by tumor-derived CCL2, produced by metastasizing tumor cells. In the presence of interleukin (IL)-6 and prostaglandin-E2 (PGE2), these monocytes become converted in the microenvironment of the tumor-draining lymph nodes and the primary tumor into CD14+ M2-macrophages expressing programmed death-ligand 1 (PD-L1). These suppressive CD14+ M2-macrophages promote progression and early metastasis of the primary tumor by producing pro-angiogenic and tumor-invasive factors. In addition, they can bind to PD-1 on anti-tumor effector T cells, thereby inhibit an immune response against the primary and metastatic tumor cells. This immunosuppressive cycle is further amplified by the interplay and crosstalk of regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC).

How can we therapeutically target M2-macrophages to lift the immunosuppressive barriers in the microenvironment of cervical tumors and TDLN? A first option would be to interrupt the immunosuppressive cycle through PD-1/PD-L1 checkpoint inhibition. This intervention is already used to great clinical effect in other types of cancer, and is likely to be effective also in cervical cancer. This notion is supported by our observation of increased PD-1 levels on T cells in tumor-involved TDLN.7 Aberrant myeloid differentiation has been linked to JAK2/STAT3 signaling. A promising in vitro study has shown combined inhibition of the STAT3 and p38-MAPK signaling pathways to facilitate CD1a+ DC differentiation in primary tumor suspensions while reducing frequencies of CD14+ cells and inducing a Th1 response.6 Another approach is to block upstream-acting IL-6 or cyclooxygenase (COX) -1/-2 (i.e., the catalytic enzymes controlling PGE2 production)2,7, possibly in combination with conventional chemotherapy. Indeed, combined in vitro treatment of tumor cell lines with cytostatic drugs and COX-1/-2 and/or IL-6R inhibitors prevented subsequent M2-skewing.7 A recent publication suggested CSFIR blockade as a means to decrease the number of cervical tumor-associated M2-macrophages through interrupted recruitment, differentiation and turn-over rates.6 Alternatively, M2-macrophages may be re-programmed to M1-macrophages through effector molecules associated with Th1 cells such as CD40L and IFNγ, or by the use of TLR ligands.2 However, our own results suggest that this may not suffice.1,4,6 Ideally, a combinatorial immunotherapy should be pursued by blocking immune suppression (e.g., through blockade of PD-L1 or STAT3/p38 signaling), thereby minimizing the negative impact of CD14+ M2-macrophage-like cells, while promoting an anti-tumor response in the TDLN, e.g., by immune potentiation or vaccination. Such a “push and pull” approach could be applied locally to minimize unwanted side effects and might result in effective loco-regional control of cervical cancer. This promising therapeutic approach certainly deserves further (pre-)clinical exploration.
REFERENCES


CHAPTER 9

Summarizing discussion and future directions
Cervical carcinoma is a virus-associated cancer, induced by a persistent infection of the cervix epithelium with high-risk human papillomavirus (HPV). This infection may lead to the development of low-grade and high-grade dysplastic lesions and eventually it could lead to the formation of invasive carcinoma. Cervical cancer is mainly a locally invading disease spreading to the adjacent organs (vagina and bladder) and pelvic lymph nodes first and the lymph node status is the most important prognostic factor. Similarly to the majority of cancers, HPV-infected cervical tumor cells employ various immune evasion strategies leading to progression and metastasis.

One of these escape mechanisms is the expression of checkpoint molecules that hamper tumor-specific T cell activation in the tumor microenvironment (TME). These co-inhibitory checkpoints are crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses at peripheral sites. However, tumors are able to abuse these checkpoint pathways as an important escape mechanism to evade an anti-tumor T cell response. Therefore, many immunotherapeutic strategies are aimed at blocking these checkpoints in the tumor and/or draining lymph node microenvironment thereby inducing anti-tumor immunity. To this end, monoclonal antibodies targeting the interactions of programmed death (PD)-1:PD-Ligand-1/-2 and Cytotoxic T Lymphocyte-associated Antigen 4 (CTLA-4):CD80/86 are administered to cancer patients. Durable complete clinical responses have been achieved by the implementation of monoclonal antibodies as single or combinatorial therapies in various tumor types, e.g., in melanoma, bladder cancer, lung cancer, and renal-cell cancer. Also, small molecule inhibitors for indoleamine 2,3-dioxygenase (IDO) have been tested in cancer patients, but no major clinical response has been achieved for IDO-i as single therapy yet suggesting that they should be combined with other therapeutics.

In cervical cancer, some clinical trials have been or are currently testing checkpoint inhibitors for PD-1 or CTLA-4. However, no impressive clinical responses are achieved yet, and therefore, the focus of research should be on the complete characterization of the cervical tumor immune microenvironment in order to find predictive and prognostic biomarkers to achieve higher immunotherapy response rates and avoid unnecessary over-treatment or to find novel therapeutic targets.

In this thesis, the expression of checkpoint molecules PD-L1 (chapter 2, n=332) and IDO (chapter 3, n=71) in the TME was evaluated in formalin-fixed, paraffin-embedded (FFPE) tissue from patients with cervical cancer. Consistent with other reports on cervical cancer, PD-L1 and IDO positivity was observed abundantly in primary and metastatic tumor cells and myeloid immune cells, making both checkpoints an interesting target for immunotherapy against cervical cancer. However, conflicting data exist on the prognostic value of PD-L1 and IDO in solid tumors, and in the reports presented in this thesis we did not find a straightforward association between PD-L1 and IDO expression per se and survival. Strikingly, protein expression patterns for these markers seem vital for patient outcome as we observed an unambiguous survival benefit (with 100% 5-year survival) for cervical squamous cell carcino-
ma (SCC) patients with marginal expression (at the tumor-stromal interface) of PD-L1 or IDO as compared to patients with diffusely PD-L1- and/or patchy IDO expressing tumors. These data are in concordance with another IDO study in cervical cancer.24 This marginal expression effect for both PD-L1 and IDO was proposed to be induced by interferon (IFN)γ secretion by co-localized tumor-specific T cells in the TME.33,34 Indeed, we observed a significant correlation between IDO1 and IFNG transcripts and patients with high levels of IFNG, whether or not combined with high levels of IDO1 expression, had a survival advantage. In contrast, diffuse (PD-L1) and patchy (IDO) expression patterns may result from activation of oncogenic signaling pathways leading to intrinsically elevated PD-L1 and IDO expression levels in tumor cells35-37, which leads probably to a more aggressive tumor cell phenotype and consequently a worse patient outcome than patients with marginal expression patterns of PD-L1 and IDO. Unexpectedly, our findings for IDO expression point to a prognostically favorable association with tumor size, lymph node metastases and infiltration of proliferating cytotoxic CD8+ T cells. We next examined if the kynurenine/tryptophan (kyn/trp) ratio in serum, as an indication of IDO activity, could serve as a prognostic marker for patients with cervical cancer and as possible predictive marker for patient stratification for e.g. treatment with IDO inhibitors.26 We found an association between IDO expression in the tumor and the kyn/trp ratio in serum of patients with early stage cervical SCC. However, this association was only evident for patients with patchy IDO expression in the primary tumor, independent of any simultaneous marginal expression and showed no direct relation to survival. Based on these data, we conclude that for IDO (and the same seems to hold true for PD-L1) protein expression patterns in the tumor, rather than serum levels, provide the most definitive prognosticators, and possibly valid markers for stratification of patients entering clinical trials.

Another immune escape mechanism of tumor cells is aberrant expression of human leukocyte antigen (HLA) class I molecules, involved in tumor-derived antigen presentation for recognition and subsequent killing by CD8+ cytotoxic T cells.37 In chapter 4, we compared the expression of classical and non-classical HLA molecules between the two common histological subtypes SCC and adenocarcinoma (AC) in cervical primary tumors and paired metastatic lymph node samples in a large patient cohort (n=136). The vast majority (80-90%) of SCC and AC tumors, and especially large tumors, manifested with downregulation of classical HLA at the site of the primary tumor and an even lower expression in the metastatic tumor cells. This will likely result in a hampered recognition by T cells, which has been shown in vitro38 and is supported by immunohistochemistry (IHC) studies on cervical cancer tissues showing a significant association between HLA class I downregulation and low numbers of tumor-infiltrating CD8+ T cells37, with particularly lower numbers of CD8+ T cells in primary tumors with weak HLA-A expression.26 Moreover, this outcome fits with the concept that tumor cells are positively selected based on low or absent expression of classical HLA, and can be linked to invasiveness and metastatic potential.41,42 Additionally, 30% and 25% of the cervical tumors (both SCC and AC) expressed non-classical HLA-E and HLA-G, respectively. In theory, this could lead to decreased natural killer (NK) cell and/or T cell effector activity43-45, and hereby potential tumor progression as shown in various tumor types.46-48 However, we were not able to link HLA-E and -G expression to poor survival outcome. Rather, HLA-E protein expression has been associated with improved survival49, and also soluble HLA-G in serum did not correlate with clinicopathological characteristics in cervical cancer50. This might be explained by the interaction of non-classical HLA with both inhibitory and activating receptors on T cells and NK cells51-53, or due to the fact that the HLA genotype has not been tested since there are multiple genetic alterations in HLA alleles known for cervical cancer54. We show that a combined HLA pattern with classical HLA class I downregulation and expression of non-classical-class HLA-class I (specifically HLA-G) is responsible for a poorer survival in patients with cervical cancer, which has been shown in other tumor types as well.47,55 Likely these HLA class I alterations, leading to resistance to T cell cytotoxicity, will result in low response rates to T-cell based or targeted therapies, including adoptive T-cell transfer and checkpoint blockade.56 However, no association was found between response to checkpoint inhibition and HLA class I expression in melanoma and Hodgkin lymphoma, rather HLA class II expression by tumor cells was correlated to clinical response.57,58 Though, there are some indications that acquired resistance to α-PD-1 therapy is associated with downregulation of HLA class I and mutations in β2-microglobulin required for HLA complex formation and antigen presentation suggesting a critical role for HLA expression and T cell-based therapies.59-61

In chapter 5 we aimed to shed more light on NK cell-based therapy as a possible alternative in overcoming aberrant classical and non-classical HLA class I expression by cervical tumor cells. We explored the anti-tumor efficacy of umbilical cord blood-derived NK (UCB-NK) cells and healthy peripheral blood NK (PBNK) cells against 10 different cervical cancer cell lines stratified for NK activating and inhibitory ligands. We observed increased PBNK degranulation and cytotoxicity against cervical cancer cell lines upon combination with cetuximab (a monoclonal antibody against epidermal growth factor receptor (EGFR)). Primary cervical tumors52,53 and 10 cervical cancer cell lines used in our study expressed variable levels of EGFR. However, in line with clinical observations64,65, cetuximab as single agent did not have an effect on tumor cell viability, independent of EGFR status, and the added cytotoxic effect of cetuximab to PBNK was mainly provoked by antibody-dependent cell-mediated cytotoxicity (ADCC), which is mediated through binding of the Fc part of cetuximab (in this particular case) to Fc receptors (like FcγRIIIA and FcγRIIC) expressed on NK cells.66 Interestingly, we showed that UCB-NK cells had a significantly higher cytotoxic capacity than PBNK cells and that these cord blood-derived cells had the ability of HLA-independent tumor cell killing. This effect might be explained by the lack of cell surface expression of inhibitory killer-cell immunoglobulin-like receptors on UCB-NK cells as compared to PBNK cells.64 These findings provide a clear rationale for the use of NK cell adoptive transfer in patients with cervical cancer, using either UCB-NK cells or PBNK cells plus cetuximab or a different more cervical cancer-specific antibody. Primary tumor growth and spread is controlled by the microenvironment in tumor-draining lymph nodes (TDLNs).67 Ideally, an effective anti-tumor response is generated in these lymph...
nodes making their microenvironment critical in the initial decision between activation and suppression of the immune system by the primary tumor.6,7 Interestingly, several studies point to a survival benefit for cervical cancer patients treated primarily with surgery undergoing complete lymphadenectomy compared to patients undergoing incomplete lymphadenectomy or solely the removal of the sentinel lymph node (SLN) indicating the presence of an unfavorable immune microenvironment in the pelvic lymph nodes.71-75 To understand this phenomenon and find new (immuno)therapeutic targets that would allow immune stimulatory conversion of their microenvironment, we studied the immune composition of cervical TDLNs. In chapter 6, various T cell populations, myeloid cell subsets (including antigen-presenting cells (APCs) and myeloid-derived suppressor cells (MDSCs)); and the cytokine release profile (interleukin (IL)-4, IL-6, IL-10, and tumor necrosis factor (TNF)-α and IFN-γ) in cervical tumor-negative lymph nodes (LN-, n=20) vs. tumor-positive lymph nodes (LN+, n=8) was assessed using four-color flow cytometry. Consistent with findings of Battaglia et al.,76 we observed a highly immune-suppressed microenvironment in LN+ compared to LN- in patients with cervical cancer. Despite signs of activation of the immune system, characterized by elevated levels of activated cytotoxic CD8+ T cells and increased numbers of CD1a+ migratory DCs, we found the microenvironment in cervical LN+ to be mainly dominated by immunosuppressive-cell subsets including Tregs, T cells expressing co-inhibitory checkpoint molecules PD-1 and CTLA-4, suppressive M2-like CD14+PD-L1+ APCs, and MDSCs. Also, after stimulation with Toll-like agonists, higher levels of the immune suppressive cytokine IL-10 coupled to lower levels of IFN-γ were found in cervical LN+ compared to LN-. Interestingly, the observed high and interrelated rates of CD14+ APCs expressing PD-L1 and Tregs in cervical LN+ might be indicative of their co-regulation and important role in facilitating tumor immune escape. In an immunohistochemical study in chapter 7 where we analyzed the distribution and localization of CD8+ T cells, FoxP3+ Tregs, HLA-DR+ and PD-L1+ myeloid cells in delineated cervical lymph nodes (LN+, n=9, LN-, n=74) of five patients with metastatic disease; we demonstrated that these Tregs and PD-L1+ myeloid cells form a suppressive cordon around metastatic tumor cells possibly preventing the infiltration and cytotoxic action of CD8+ T cells. Furthermore, we demonstrated an altered, already suppressed microenvironment with high levels of Tregs and low CD8+ T cell/Treg ratios in LN- adjacent to LN+ as compared to LN- at more distant anatomical locations. This is indicative of the influence of a draining flow carrying immuno-suppressive factors75 and (isolated) tumor cells77 from the primary tumor and/or metastases. In contrast to Tregs, high numbers of PD-L1+ myeloid cells were strictly found in LN+ only. Based on these findings, in chapter 8 we postulate a model for metastatic niche formation in cervical TDLN, involving monocytes recruited to tumor nests and converted to CD14+PD-L1+ M2-like cells and subsequent expansion of Tregs. We propose that primary tumors are able to recruit (possibly via CCL2)78 and polarize CD14+ monocytes into suppressive PD-L1+ M2-like macrophages. This hypothesis is supported by the relationship between CCL2 mRNA expression and the number of tumor-associated macrophages in cervical tumor tissue.79 Of note, patients lacking CCL2 manifested with a significantly better survival than patients with low or high CCL2 mRNA expression levels.79 Furthermore, in vitro studies have shown that prostaglandin-E2 (PGE2) and IL-6 produced and secreted by tumor cells are responsible for the skewing of CD14+ monocytes towards the development of M2-like cells which in turn are able to induce Treg expansion.60,65 Indeed, M2-like CD163+ cells are present in primary cervical cancer samples62,63, and can express PD-L1 (chapter 2), most likely due to the presence of IL-10 as shown during the in vitro maturation of DCs.64-66 Findings in chapter 7 suggest that prior to metastasis, lymph nodes are conditioned by Tregs to form a (pre-)metastatic niche. Subsequently metastatic tumor cells recruit and convert PD-L1+ M2-like cells and facilitate the expansion of new suppressive waves of Tregs preparing the way for further lymphatic spread. Figure 1 provides an overview of immune escape mechanisms at play in primary cervical tumors and their draining lymph nodes that facilitate local invasion and metastatic spread.
Different immune escape mechanisms have been identified in this thesis as being active in the cervical tumor microenvironment: (A) PD-L1 and IDO expression, (B) aberrant classical HLA class I expression, and (C) PD-L1-expressing macrophages (Figure based on Heeren et al. 2015).

**FUTURE DIRECTIONS**

Despite early detection screening programs and the recently introduced prophylactic HPV vaccines, cervical cancer will in the upcoming decades remain a common cancer in women. Not all high-risk HPV types are covered by the prophylactic vaccines and the incidence of cervical AC, which is considered as a more aggressive disease type than SCC, is still rising. Only a few therapeutic options for patients with cervical cancer are available, including invasive surgery and/or chemoradiation with both having a high impact on the quality of life. For patients with advanced or recurrent disease, there are currently no effective therapies and these women face a very poor prognosis. New precision medicine therapies...
and companion predictive markers should improve this since at the moment all patients with cervical cancer are treated the same way without stratification for histology subtype, mutational- and immune status.

Based on literature and on the findings described in this thesis, we propose different therapeutic strategies for SCC and AC cervical tumors. These two histological subtypes differ significantly in clinical outcome, HPV status, gene expression signature, and immune characteristics such as the presence of HLA-E, HLA-DR, FoxP3, and IL-17 cells. Of note, we found significantly more PD-L1 expression by tumor cells (54% vs. 14%) and higher rates of tumor-associated macrophages (53% vs. 12%) in SCC tumors compared to AC tumors (chapter 2). Furthermore, SCC patients had more often loss of HLA class I expression as compared to AC patients (chapter 4). Among patients with cervical SCC, the TME and its relation to clinical outcome can be very diverse, with major differences in immune cell infiltration and HLA- and checkpoint molecule expression by tumor cells. We conclude from our findings that SCC tumors appear to be under more immunological pressure than AC tumors, but the underlying causes for this remains unclear. As both tumor types are related to HPV infection, one could speculate that SCC might be intrinsically more immunogenic, e.g. through the presence of exclusive mutations in HLA-A and HLA-B and the activation of different pathways compared to adenocarcinoma. Checkpoint inhibitors would be a promising therapy for patients with ‘hot’ tumors, i.e., with a pre-existing anti-tumor T cell response and high TIL rates, whereas patients with ‘cold’, i.e., poorly infiltrated tumors, will benefit more from immune activators such as therapeutic HPV vaccines and focal radiation to induce proper DC maturation, priming of T cells, and intratumoral infiltration of tumor-specific T cells. To block local tumor growth and lymphatic spread in patients with cervical cancer, we propose to use local immunotherapy as a therapeutic strategy, since cervical cancer is a very immunogenic disease due to the presence of HPV antigens, neo-antigens and a diverse repertoire of tumor-specific T cells, and is characterized by loco-regional invasion and metastatic spread. Currently, some clinical trials have been or are testing angiogenesis inhibitors, checkpoint inhibitors and vaccine-based immunotherapy, all administered systemically, in patients with cervical cancer. However, we believe that since cervical cancer is mainly a locally invading disease comprising the pelvic lymph nodes rather than distant sites of the body, it makes it pre-eminently suitable for local intratumoral interventions instead of systemic therapy. Of note, the cervix is easily accessible for local intervention therapies. For intratumoral immunotherapy, lower doses can already be sufficient to induce systemic immunity as well, thereby avoiding high-dose systemic-induced severe toxicities such as auto-immunity. Highly promising data of two clinical trials testing local low-dose CpG-B in early-stage melanoma patients boosting loco-regional and systemic immunity and findings of this thesis showing PD-L1 (chapter 2, 6 and 7) as a promising immunotherapeutic target on tumor cells and M2-macrophages, have led to initiation of the phase I clinical trial ‘DURVIT’, testing the safety and feasibility of the administration of a single low-dose of durvalumab (αPD-L1), injected intratumorally in patients with early-stage cervical cancer (NTR6119). We believe that durvalumab...
REFERENCES

40. Jordanova, E.S., et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+regula-
Immunol Today phages.

Melanoma-specific MHC-II expression represents a tumour-autonomous phenotype and pre-


CHAPTER 9 SUMMARIZING DISCUSSION AND FUTURE DIRECTIONS


45. Steele, J.C., et al. T-cell responses to human papillomavirus type 16 among women with different grades of cervical...


Nederlandse samenvatting

Immuuntherapie wordt nog niet als standaard behandeling gegeven aan patiënten met baarmoederhalskanker. Op dit moment zijn er een aantal nog lopende experimentele klinische onderzoeken naar de effectiviteit van monoklonale antilichamen die immuun checkpoints kunnen blokkeren, zoals aPD-1 en aCTLA-4. Echter, er zijn nog geen duurzame klinische responsen bereikt en daarom ligt de focus van dit proefschrift op de karakterisering van het tumor immuun-nieuil om zo voorspellende en prognostische factoren, genaamd biomarkers, te vinden en om nieuwe therapeutische targets te identificeren om daarop toepassingen te ontwikkelen, om hogere frequenties aan klinische responsen te bereiken en onnodige overbehandeling te vermijden.

In hoofdstuk 2 van dit proefschrift hebben we de expressie van het checkpoint molecuul PD-L1 in formaline-gefixeerde en paraffine-ingebedde cervixcarcinoom monsters bestudeerd. PD-L1 kan binden aan PD-1 op T-cellen en op deze manier de killer T-cellen remmen in hun functie. We onderzochten in twee patiëntgroepen het verschil tussen plaveiselcelcarcinoom (PCC) vs. adenocarcinoom (AC; patiënten cohort I, \( n = 205 \)) en primaire tumoren vs. gemaakte lymfeklier metastasen (patiënten cohort II, \( n = 127 \)). Daarnaast hebben we de PD-L1 expressie
data gecorreleerd aan klinisch-pathologische kenmerken en overleving van de patiënten. Immunohistochimische kleuringen laten zien dat er veel PD-L1 tot expressie wordt gebracht door tumorcellen en door myeloïde immuuncellen in de tumor. PCC tumoren brengen significant meer PD-L1 tot expressie dan AC tumoren (54% vs. 14%, respectievelijk). Een opvallende bevinding binnen de groep met PCC tumoren zijn de verschillende expressiepatronen van PD-L1 in de primaire tumor. Patiënten met expressie van PD-L1 aan de rand (in de marge) van de tumorvelden hadden een betere 5-jaars (100%) overleving dan patiënten met diffuse PD-L1 expressie in de primaire tumor. De PD-L1 "marginale" expressie wijst mogelijk op een effectieve T-cell afweerreactie met IFNγ productie, wat zorgt voor de op-regulatie van PD-L1 expressie, terwijl het diffuse PD-L1 patroon meer duidt op activering van een interne oncogene signaleringsroute wat resulteert in hoge PD-L1 expressie. Op basis van deze resultaten concluderen we dat PD-L1 eiwitexpressiepatronen in de tumor van belang kan zijn voor prognose en in de toekomst ook toegepast zouden moeten worden als biomarker voor stratificatie van patiënten met cervixcarcinoom die deelnemen aan experimentele klinische onderzoeken naar αPD-(L)1 therapie.

In hoofdstuk 3 hebben we de expressie van een ander checkpoint molecuul onderzocht, indoleamine 2,3-dioxygenase (IDO). IDO kan via een enzymatische reactie tryptofaan omzetten in kynurenine. In vitro en in vivo studies hebben aangetoond dat met name geactiveerde T- en NK-cellen negatief worden beïnvloed door de resulterende tryptofaan depletie en dat immun suppressieve regulatoire T-cellen (Tregs) worden geïnduceerd door de aanwezigheid van IDO. Eerder onderzoek in patiënten met baarmoederhalskanker heeft aangetoond dat een lage kynurenine/tryptofaan (kyn/trp) ratio in serum is geassocieerd met een slechtere prognose. We onderzochten het verband tussen IDO eiwitexpressiepatronen in 71 primaire PCC tumoren en 14 gepaarde lymphkliermetastasen en de concentratie van IDO metabolieten (kynurenine en tryptofaan) in het serum van baarmoederhalskankerpatiënten. Verder correlerden we IDO expressiepatronen in het tumor milieu met klinisch-pathologische kenmerken en de aanwezigheid van prolifererende (delende) killer T-cellen en Tregs. We vergeleken ook de relaties tussen IDO1- en IFNG-geneexpressie en klinische parameters met behulp van RNAseq-data van 144 PCC cervixcarcinoom monsters on-line gepubliceerd door The Cancer Genome Atlas (TCGA). Immunohistochimische kleuringen lieten zien dat er naast PD-L1, ook veel IDO tot expressie wordt gebracht door tumorcellen en door myeloïde immuuncellen. Net als bij PD-L1, zagen we ook voor IDO verschillende eiwitexpressiepatronen in de primaire tumoren, welke vergelijkbare associaties met overleving lieten zien als voor de verschillende expressiepatronen van PD-L1 (d.w.z. betere overleving voor patiënten met een "marginale" IDO expressie). Van IDO is bekend dat het net als PD-L1 door T-cellen afkomstig IFNγ op-gereguleerd kan worden. Met behulp van de TCGA data set vonden we een significante correlatie tussen IDO1- en IFNG-geneexpressie, wat inderdaad wijst op een IFNγ-gemedierte op-regulatie van IDO middels een effectieve anti-tumor T-cell respons. Dit ondersteunt onze bevindingen dat IDO expressie in het tumormilieu een prognostisch gunstige associatie heeft met tumurgrootte, lymphkliermetastasen en infiltratie van geactiveerde en delende killer T-cellen. Een belangrijke bevinding binnen deze studie is het verband tussen IDO expressie in de tumor en de kyn/trp ratio in serum van patiënten met PCC tumoren in een vroeg stadium. Deze associatie was echter alleen duidelijk voor patiënten met een niet-marginaal IDO expressie in de primaire tumor. Gebaseerd op deze resultaten concluderen we dat de IDO eiwitexpressiepatronen in de primaire tumor meer informatie geven dan kyn/trp serum levels en mRNA levels ten behoeve van prognose en stratificatie van patiënten met cervixcarcinoom die deelnemen aan experimentele klinische onderzoeken naar IDO-remmers.

Een ander ontsnappingsmechanisme van tumorcellen is via de afwijkende expressie van HLA klasse I-moleculen, die betrokken zijn bij de presentatie van (tumor)antigenen aan killer T-cellen en zo een anti-tumor T-cell respons tegen de tumor mogelijk maken. In hoofdstuk 4 vergeleken we de expressie van klassieke (HLA-A, -B, -C) en niet-klassieke (HLA-E en -G) HLA-moleculen tussen baarmoederhalskankerpatiënten met PCC- en AC tumoren in primaire tumoren en gepaarde tumor-positieve lymphklier samples (n=136). In 80-90% van de gevallen, en met name in grote tumoren (>4 cm), was er sprake van HLA down-regulatie in cervix tumoren. Bovendien hadden patiënten met PCC tumoren vaker verlies van klassieke HLA expressie dan patiënten met AC tumoren. Ook was er een duidelijk verband tussen het verlies van klassieke HLA expressie in de primaire tumor en de gepaarde lymphkliermetastasen. In de lymphklieren van patiënten met PCC tumoren vonden we zelfs een significantaal lagere expressie van klassieke HLA moleculen. Dit zal waarschijnlijk resulteren in een belemmering van herkenning door T-cellen. Deze uitkomst past bij het idee dat met name tumorcellen met een lage of afwezige expressie van klassieke HLA aan killer T-cellen kunnen ontsnappen en vervolgens kunnen metastaseren naar de draininger lymphklieren. Daarnaast brachten 30% en 25% van de cervix tumoren, respectievelijk, HLA-E en HLA-G tot expressie. Omdat deze niet-klassieke HLA moleculen kunnen functioneren als afweer remmers, zou dit kunnen leiden tot verminderde T-cell activiteit en dus kunnen leiden tot progressie van de tumor. We vonden dat klassieke HLA downregulatie en expressie van niet-klassieke HLA-G verantwoordelijk is voor een slechtere overleving bij patiënten met baarmoederhalskanker. Deze veranderingen in HLA expressie kunnen zorgen voor lagere klinische responders na therapieën specifiek gericht op killer T-cellen, zoals selectieve T-cell therapie en checkpointblokkade. Daarom onderzochten we in hoofdstuk 5 een alternatieve therapie gericht op Natural Killer (NK) cellen die onafhankelijk van afwijkende HLA expressie tumorcellen toch kunnen aanvallen en elimineren. We bestudeerden de anti-tumorale werking van deze cellen, afgeleid van volwassen donor bloed (PBNK cellen) of van stamcellen uit navelstrengbloed (UCB-NK cellen), in combinatie met cetuximab, een monoklonaal antilichaam tegen epidermale groeifactor receptor (EGFR). Cetuximab als monotherapie had geen toegevoegd anti-tumor effect, zoals eerder onderzocht in patiënten met baarmoederhalskanker. We observeerden daarentegen wel een verhoogde killer activiteit van PBNK cellen tegen de tumorcellen na toediening van cetuximab, middels een proces dat bekend staat als antilichaamafhankelijke celgemediëerde cytotoxictiteit (ADCC), d.w.z. de NK cellen herkenden en bonden aan de tumorcellen middels een alternatieve therapiemaatregel gericht op Natural Killer (NK) cellen.
het cetuximab dat gebonden was aan het oppervlak van de tumorcellen. Interessant genoeg toonden we aan dat UCB-NK-cellen een significant hoger tumor dodend vermogen hadden dan PBNK-cellen en dat deze uit navelstrengbloed verkregen cellen het vermogen hadden om via een HLA-onafhankelijk manier de tumorcellen te doden. Dit effect kan worden verklaard door de afwezigheid van remmende receptoren op het celmembraan van UCB-NK cellen. Deze bevindingen bieden een rationale voor het gebruik van adoptieve NK-cell therapie bij patiënten met baarmoederhalskanker, in combinatie met cetuximab of een ander meer cervix tumor-specifiek antilichaam.

De groei van de primaire tumor en het proces van metastasering wordt gemoduleerd door de afweercellen aanwezig in de tumor-draherende lymphklieren. Idealiter wordt er in deze lymphklieren een effectieve anti-tumorrespons gegenereerd en daarom is het micromilieu van de klieren van groot belang bij de initiële beslissing tussen activering of onderdrukking van het immuunsysteem ten behoeve van de tumor afweer. Om dit fenomeen beter te begrijpen en om nieuwe (immuun)therapeuten te ontwikkelen die o.a. ook aangrijpen op het immunologische kanker geconditioneerde micromilieu in de lymphklieren, hebben we de aanwezige afweercellen in cervix tumor-draherende lymphklieren bestudeerd. In hoofdstuk 6 werden verschillende T-cel-populaties, myeoloïde cel subsets (waaronder antigen-presenteerende cellen (APCs) en myeoloïd-agafelede suppressor cellen (MDSCs)); en de cytokineprofiel (interleukine (IL)-4, IL-6, IL-10 en tumornecrosefactor (TNFα en IFNγ)) in tumor-negatieve lymphklieren (LN-; n=20) vs. tumor-positieve lymphklieren (LN+; n=8) onderzocht met behulp van flow cytometrie. We vonden een zeer suppressief milieu in LN+ van patiënten met cervixcarcinoom. Ondanks immuun activering, gekenmerkt door verhoogde aantallen geactiveerde killer T-cellen en meer uit de tumor migrerende dendritische cellen (DCs), werd het milieu voornamelijk gedomineerd door suppressieve afweercellen zoals Tregs, T-cellen positief voor de checkpoints PD-1 en CTLA-4, suppressieve M2-achtige CD14+PD-L1+ APCs en MDSCs. Ook vonden we na stimulatie met ‘toll-like receptor’-agonisten hogere levels van het suppressieve cytokine IL-10 en lagere levels van IFNγ in LN+ ten opzichte van LN- Daarnaast vonden we een interessante correlatie tussen de aanwezen CD14+PD-L1+ APCs en Tregs in LN+, wat mogelijk duidt op een co-regulatie en onderlinge samenwerking om de tumorcellen te laten ontsnappen van het immuunsysteem. In een immunohistochemische studie in hoofdstuk 7, bestudeerden we de distributie en lokalisatie van killer T-cellen, Tregs, HLA-DR+ en PD-L1+ myeoloïde cellen in cervix tumor-draherende lymphklieren (LN+; n=9, LN-; n=74) van vijf patiënten met cervixcarcinoom. In deze studie zagen we dat Tregs en PD-L1+ myeoloïde cellen een suppressief cordon vormden rondom gemetastaseerde tumorcellen, wat mogelijk zorgt voor hindering van de infiltratie en tumor dodende werking van killer T-cellen. Bovendien toonden we een veranderd, reeds suppressief immuun milieu met hoge aantallen Tregs en lage killer T-cel/Treg ratio’s aan in LN+ die zich in hetzelfde bassin bevonden als LN+ in vergelijking met LN- in een verder gelegen bassin zonder LN+. Deze nabij LN+ gelegen LN- zijn hoogst waarschijnlijk al beinvloed door een immuun suppressieve drainerende-flow die immunsuppressieve factoren en (geïsoleerde) tumorcellen meebrengt vanuit de primaire tumoren en lymphklieren. In tegenstelling tot Tregs werden hoge aantallen PD-L1+ myeoloïde cellen alléén gevonden in LN+. Op basis van deze bevindingen presenteren we in hoofdstuk 8 een model voor de necroïeving voor metastasen in cervix tumor-draherende klieren. Wij hypothesizeren, mede op basis van de bestaande literatuur, dat primaire tumoren in staat zijn om CD14+ monociten te rekruteren en te polariseren naar suppressieve PD-L1+ M2-achtige macrofagen die op hun beurt Tregs kunnen activeren en expanderen. Ondersteund door de bevindingen in hoofdstuk 7 suggereren wij dat lymphklieren voorafgaand aan de metastasering van primaire tumorcellen naar de lymphklieren, door Tregs worden geconditioneerd en een pre-metastatische niche creëren met een gebrek aan functionele killer T cellen. Hierdoor kunnen tumorcellen zich vestigen in de lymphklier, om vervolgens weer suppressieve PD-L1+ M2-achtige macrofagen te rekruteren die op hun beurt weer een nieuw leger Tregs mobiliseren om zo de weg vrij te maken voor verder verspreiding van de tumor.

Addendum

PhD portfolio and List of publications

About the author

Dankwoord
PHD PORTFOLIO

PhD student: Anne Marijne Heeren
PhD period: January 2014 – May 2018
PhD supervisors: Prof. dr. G.G. Kenter, Prof. dr. T.D. de Gruijl, dr. E.S. Jordanova

1. PhD training

Courses
- 2014 Basic Medical Statistics/SPSS 1.5 ECTS
- 2014 Advanced Immunology 3.0 ECTS
- 2015 Basic course oncology 1.5 ECTS
- 2015 In the footstep of AvL/Basic Microscopy 1.5 ECTS
- 2016 Research Integrity 2.0 ECTS
- 2017 Matlab programming for beginners 1.0 ECTS
- 2018 Using R for data analysis 1.5 ECTS

Workshops
- 2015 BD Horizon, UMC, Utrecht, NL
- 2016 PhD as a steppingstone to success, VUMC, Amsterdam, NL
- 2016 Thesis Layout, VUMC, Amsterdam, NL
- 2016 Science Exchange Day, VUMC, Amsterdam, NL

Presentations
- 2014 ‘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.’ Dutch Tumor Immunology Meeting (DTIM), NL (Oral presentation).
- 2014 ‘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.’ Society for Immunotherapy of Cancer (SITC), Washington D.C., USA (Poster presentation).
- 2015 ‘Unique suppressive tumor-draining flow in pelvic lymph nodes from patients with cervical cancer’. European Society for Gynaecological Oncology (ESGO), Nice, France (Poster presentation).
LIST OF PUBLICATIONS


*contributed equally to the manuscript
ABOUT THE AUTHOR

Anne Marijne Heeren was born on the 16th of December 1988 in Aalsmeer, The Netherlands. Marijne still lives in Aalsmeer, is married to Jacco Keessen and they have an one year old son called Senn.

In 2007, she graduated in VWO Economy & Society at the Alkwin Kollege in Uithoorn. Because she was very interested in beta sciences, consequently, she followed the ‘Bêtabrugtraject’ at the University of Amsterdam (UvA) to obtain additional courses for Biology, Chemistry, Mathematics and Physics in 2008. Thereafter, she studied Biomedical Sciences followed by the master Biomolecular Sciences, both at the VU University in Amsterdam.

She chose carefully her internships based on her interest for the research fields gynecology and reproductive medicine. In 2011, during her bachelor internship, she worked on the up- and downregulation of EBI3/HLA-G genes related to preeclampsia using a lentiviral vector system at the dept. of Gynecology & Obstetrics at the Academic Medical Center in Amsterdam (supervisors: dr. C Ris and dr. G Alink). During her first master internship in 2012, she studied ‘The viability of cryopreserved human ovarian tissue’ at the dept. Anatomy & Embryology at Leiden University Medical Center (LUMC) in Leiden (supervisors: dr. SM Chuva de Sousa Lopes and dr. LAJ van der Westerlaken). After this internship, she completed several research projects and manuscripts (2012-2013) on human and chicken reproductive systems and finished her master thesis on ‘Fertility preservation in men after they have received cancer therapy’ (supervisors: dr. LAJ van der Westerlaken and Prof.dr. FM Helmerhorst). Her second master internship project (2013) was about ‘The characterization of immune cells in (fresh) tumor-draining lymph nodes and HLA-A, -B, -C, -E and -G expression in primary and metastatic (paraffin-embedded) cervical cancer’. This was a collaboration between the departments Obstetrics & Gynecology and Medical Oncology at VU University Medical Center (VUMC) in Amsterdam. After this internship, she got the opportunity to stay and to acquire a PhD position (2014 – 2018) on the KWF-granted project ‘Translating immune characteristics of metastatic tumors into therapeutic targets for cervical cancer’ supervised by Prof.dr. GG Kenter, Prof.dr. TD de Grujl, and dr. ES Jordanova. On May 11th 2018, she was appointed as junior Postdoctoral Researcher to study the microenvironment of tumor-draining lymph nodes from patients with breast cancer at the department of Medical Oncology, Amsterdam UMC.
DANKWOORD

Met het afronden van mijn PhD sluit ik een hectische, maar ook een mooie en zeer leerzame periode in mijn leven af. Veel mensen hebben op allerlei verschillende manieren bijgedragen aan de totstandkoming van dit proefschrift. Heel veel dank daarvoor!

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Daarnaast wil ik ook graag de leden van de promotiecommissie: Prof. dr. Y. van Kooyk, Prof. dr. I.J.M. de Vries, dr. R. van de Ven, Prof. dr. C.A.H.H. Daemen, Prof. dr. R.M. Luiten en dr. P.J. de Vos van Steenwijk bedanken voor de tijd en moeite voor het lezen en beoordelen van mijn proefschrift en voor de discussie tijdens de aanstaande verdediging op 19 september 2018.


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ADDENDUM

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