Cervical carcinoma is a virus-associated cancer, induced by a persistent infection of the cervix epithelium with high-risk human papillomavirus (HPV).1 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.2 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.3-5 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).6 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.8 Therefore, many immunotherapeutic strategies are aimed at blocking these checkpoints in the tumor and/or draining lymph node microenvironment thereby inducing anti-tumor immunity.8 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.9-15 Also, small molecule inhibitors for indoleamine 2,3-dioxygenase (IDOi) have been tested in cancer patients, but no major clinical response has been achieved for IDOi as single therapy yet suggesting that they should be combined with other therapeutics.16-22 In cervical cancer, some clinical trials have been or are currently testing checkpoint inhibitors for PD-1 or CTLA-4.22,23 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 cancer24-28, 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 tumors29,30, 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. 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. 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 cells, 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. 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. 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 vitro 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 cells, with particularly 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 absent expression of classical HLA, and can be linked to invasiveness and metastatic potential. 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 activity and hereby potential tumor progression as shown in various tumor types. 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 survival, and also soluble HLA-G in serum did not correlate with clinicopathological characteristics in cervical cancer. This might be explained by the interaction of non-classical HLA with both inhibitory and activating receptors on T cells and NK cells, 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 cancer. We show that a combined HLA pattern with classical HLA class I downregulation and expression of non-classical 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. 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. 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. 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.

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 tumors and 10 cervical cancer cell lines used in our study expressed variable levels of EGFR. However, in line with clinical observations, 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. 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. 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). 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.\textsuperscript{5,70} 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.\textsuperscript{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)-\(\alpha\) and IFN-\(\gamma\)) 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 \textit{et al.}\textsuperscript{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 CD14\(^+\) 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-\(\gamma\) 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 immunosuppressive factors\textsuperscript{69} and (isolated) tumor cells\textsuperscript{77} 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)\textsuperscript{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.\textsuperscript{79} Of note, patients lacking CCL2 manifested with a significantly better survival than patients with
low or high CCL2 mRNA expression levels. 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. Indeed, M2-like CD163+ cells are present in primary cervical cancer samples, 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. 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. 201587).
Figure 1 (C) continued: Overview of complex suppressive interactions in the cervical tumor microenvironment. 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. 201587).

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 vaccines88,89 and the incidence of cervical AC, which is considered as a more aggressive disease type than SCC, is still rising90,91. 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.92 For patients with advanced or recurrent disease, there are currently no effective therapies and these women face a very poor prognosis93. 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 (aPD-L1), injected intratumorally in patients with early-stage cervical cancer (NTR6119). We believe that durvalumab
will modulate the microenvironment in the primary tumor and via the lymphatic draining flow will reach the TDLNs and will interrupt the immunosuppressive cycle and induce and/or boost anti-tumor immunity. Future research should focus on other checkpoints as well, like CTLA-4, TIM-3, LAG-3, VISTA, TIGIT, and BTLA as these molecules can be expressed in the cervical TME or in TDLN, or can be upregulated upon immunotherapy.\textsuperscript{61,127}

In conclusion, the studies in this thesis have contributed to a deeper insight in the immune characteristics of the primary tumor and TDLNs in patients with cervical cancer, identifying potential therapeutic targets to halt early metastatic spread. Only a part of the highly complex microenvironment is unravelled, and more extensive research is still required. Novel high-dimensional analytical methodologies such as mass cytometry, proteomics, and single-cell RNA sequencing may prove instrumental in this regard. Finding a single biomarker (such as HLA, PD-L1, or IDO) in cervical cancer is implausible to adequately reflect the complex interplay between tumor cells and the immune system. Understanding the strategies that tumors use to evade an effective immune response may aid in the exploration of less invasive, patient-tailored, combinatorial immunotherapeutic strategies aiming for complete and durable clinical responses in patients with cervical cancer. Most likely, combinatorial local therapies involving checkpoint inhibitors (e.g. α-PD-(L)1/α-CTLA-4/IDOi), immune stimulators (e.g. Toll-like agonists or radiotherapy), and adoptive-cell transfer (including T- and NK cells) will improve curative outcome. Future translational and clinical research will no doubt reveal this.
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