Immuno-oncology of gynecological malignancies
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Summary and Discussion
SUMMARY

The immune system plays a crucial role in the recognition and elimination of (pre)malignant cells. Immune T cells in particular are highly skilled at surveying the body and distinguishing (pre)malignant from normal cells for elimination. When these immune T cells infiltrate the tumor microenvironment they are referred to as tumor-infiltrating lymphocytes (TIL). In line with their cancer-killing role, TIL have a pronounced effect on the survival of patients across malignancies. Moreover, it has also become abundantly clear that TIL can be targeted by cancer immunotherapy to induce lasting clinical benefit. In this thesis, we explored several aspects of TIL biology, including localization and differentiation, with the ultimate aim of developing novel therapeutic interventions to eradicate cancer.

Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TIL) are an important biomarker for improved prognosis and response to immune checkpoint blockade (ICB) therapy in several malignancies. However, not all TIL subsets contribute equally to the eradication of cancer cells. CD8-positive cytotoxic T lymphocytes (CTL) are generally known as the tumor-killing subset, and the presence of these CD8-positive CTL contributes to an improved prognosis of cancer patients. On the contrary, regulatory T cells (Treg), characterized by CD4 and FoxP3 expression, have been described as an immune suppressive subset. As such, the presence of this suppressive subset does not confer prognostic benefit. In addition to the phenotype of TIL, the localization within the tumor microenvironment also appears to influence their prognostic benefit.

In chapter 2, we first examined the prognostic effect of CD8-positive CTL localization in high-grade serous ovarian cancer (HGSC). We observed a strong prognostic benefit for patients from TIL that localize within the epithelial cancer islets of HGSC, in contrast to TIL localized in the connective tissue of the tumor. Distinguishing epithelial regions from stromal regions is based on histological assessment and could be a somewhat arbitrary process. A single marker to discriminate intraepithelial from stromal TIL populations is therefore of interest. Webb et al. showed that the epithelial and stromal CTL populations in HGSC can be discriminated by expression of CD103. In chapter 2, we first confirmed that CD103 is indeed a marker for the intraepithelial T cell population in HGSC. In line with this, we showed that the total number of CD103-positive TIL, irrespective of localization, was associated with improved prognosis in HGSC. In addition, we elucidated the mechanism underlying induction of CD103 on CD8-positive CTL and demonstrate that CD103-positive TIL comprise the cancer cell-reactive subset.

To see whether CD103 could be used as a pan-cancer biomarker for intraepithelial TIL, we investigated the localization and prognostic role of CD103-positive T cells in endometrial cancer and cervical cancer (chapter 3 and 4). We confirmed that CD103 performed consistently across
gynecological malignancies as a prognostic biomarker. In addition to the prognostic value of TIL, the presence of TIL has also been described as a predictive and/or response biomarker for therapeutic efficacy of immunotherapy. We therefore investigated in chapter 4 whether CD103 also demonstrates promise as a response biomarker in an animal model of cervical cancer treated with therapeutic vaccination and/or immuno-radiotherapy.

Along with the number and localization of TIL, their differentiation status is also an important prognostic factor. In brief, naïve T cells circulate through secondary lymphoid organs, which maximizes their opportunity for encountering an antigen presenting cell (APC) and subsequent activation. These naïve T cells are characterized by expression of CD45RA, CCR7, CD28 and CD27. Upon activation, these naïve T cells progressively lose many of these markers and, upon chronic antigen stimulation (e.g. in the context of cancer) acquire a so-called exhausted phenotype wherein they are in the end no longer able to exert tumor control. In line with this, in chapters 5 and 6 we identified that less differentiated "younger" CD27+ TIL in the cancer cell epithelium were of greater prognostic benefit to patients than "older" more differentiated TIL in HGSC.

**Effects of standard treatment on local and systemic immunity**

As immunotherapy by itself is insufficient for most patients, it is often proposed as combination therapy in addition to conventional treatments such as chemotherapy. As chemotherapy is known for its effects on dividing cells, chemotherapy might also affect tumor-reactive immune cell subsets. To this end, we describe in chapters 2, 5-7 whether, and at which time point, immunotherapy might best be combined with conventional chemotherapy. The effects of Carboplatin/Paclitaxel containing chemotherapy on peripheral blood immune cell subsets was investigated in a cohort of ovarian cancer patients. No systemic changes in the proportion of T cell subsets were observed during the course of Carboplatin/Paclitaxel chemotherapy. In line with this, we observed equal levels of tumor-infiltrating T cells in tumors of patients obtained during primary debulking surgery (PDS) versus tumors of patients that were pretreated with neoadjuvant Carboplatin/Paclitaxel chemotherapy (NACT). Interestingly, while we did not observe systemic or local immune modulating effects of chemotherapy on the T cell subsets, we identified a pronounced depletion of circulating myeloid cells during treatment with Carboplatin/Paclitaxel in ovarian cancer patients. Surprisingly, unpublished work by our group has so far revealed no such differences in infiltrating myeloid cells in the tumors of PDS treated patients versus NACT treated patients. Nevertheless, myeloid cells have been proposed to suppress the immune response even outside of the tumor microenvironment, thus highlighting a possible route for immunotherapy in ovarian cancer patients during the systemic depletion of these myeloid cells by standard-of-care chemotherapy.
Summary and discussion

Immunotherapy in cervical neoplasia and cervical cancer

As persistent infection with human papillomavirus (HPV) has been established as the causative agent for cervical cancer, all HPV infected cells are thought to express the viral oncoproteins E6 and E7. The oncoproteins E6 and E7 are therefore bona fide targets for immunotherapy in the form of therapeutic vaccination. E6/E7-targeted therapeutic vaccination aims to activate the immune system of the host against E6 and E7 expressing (pre)malignant cells. Indeed, in an animal model for cervical cancer described in chapter 4, therapeutic vaccination using a Semliki forest virus (SFV)-based vaccine targeting the oncogenes E6 and E7 induced HPV-specific immune responses and infiltration of tumor-reactive CD103-positive TIL into the tumor. In an effort to translate these findings into the clinic, a clinical batch of SFV-based therapeutic vaccine, named Vvax001, was evaluated for immunogenicity and tolerability in a phase first-in-human clinical study described in chapter 8. Taken together, the data described in this thesis contributes to our understanding of the tumor immune biology of gynecological malignancies and may help guide therapeutic intervention in the near future.

DISCUSSION

This thesis contains both translational studies and clinical studies. First the translational results from the bench will be discussed. Second, the immediate clinical implications of our work will be discussed and taken to the bedside…

From bench….

Tumor-infiltrating lymphocytes

In this thesis, we establish the prognostic value of localization and differentiation of TIL in high-grade serous ovarian cancer, endometrial adenocarcinoma, and cervical cancer. The presence of TIL has been widely described to be associated with improved prognosis across several malignancies, including breast cancer, colorectal cancer, ovarian cancer, non-small cell lung cancer, melanoma, renal cell cancers, head and neck cancer. However, there are many different TIL subsets and not all subsets contribute equally to an improved prognosis. CD103, the αE integrin subunit, has been described as a marker for prognostically favorable intraepithelial CD8-positive CTL in ovarian cancer, lung cancer, bladder cancer and, more recently, breast cancer. Within this thesis, we confirmed the prognostic benefit of CD103-positive TIL in ovarian cancer and extended this observation to endometrial cancer and cervical cancer.

We furthermore determined the ontogeny of CD103-positive TIL in HGSC, and were the first to demonstrate that CD103-positive TIL represent adaptive immune cells that are formed as part
of an ongoing anti-cancer immune response. Interestingly, we found that CD103 expression is induced by dual T cell-receptor- and TGF-β-receptor-signaling. TGF-β is generally known as an immunosuppressive molecule. However, our results indicate that TGF-β also plays an important role in T cell retention via binding of CD103 to E-cadherin on the tumor epithelium. This interaction of CD103-positive CTL in the tumor is important for the lysis of cancer cells as engagement of CD103 on CTL with E-cadherin triggers lytic granule polarization and cancer cell death. Until now, TGF-β secretion by tumor cells was only thought to paralyze infiltrating CTL. Hence, TGF-β has been identified as a therapeutic target because of its tumor-promoting roles. However, when applying TGF-β antagonistic agents, CD103 expression on CTL might be abrogated, as we show in chapter 2. As a consequence, CTLs might lose the ability to exert their cytolytic activity, for which near contact with E-cadherin on the tumor cells is needed.

Supporting evidence for this hypothesis comes from mouse studies on the immune system in the gut. Expression of CD103 has been described on a resident-memory population of intra-epithelial lymphocytes (IEL) in the gut of all vertebrate possessing a thymus. IEL play an important role regulating mucosal immune surveillance. Mice with either a null mutation in the gene encoding TGF-βr1 or T cell–specific deletion of TGF-βr1 lacked IEL, whereas mice with transgenic overexpression of TGF-βr1 had a larger population of IEL. In humans, TGF-βr1/2 mutations have also been described in the context of Loeys-Dietz syndrome (LDS). Like in the overexpressing mice, a mutation in either human TGF-βr1 or TGF-βr2 in LDS leads to an increase in the availability of TGF-β which appears to underlie the formation of abnormally weak connective tissue. Interestingly, the increased availability of TGF-β in LDS is associated with a higher prevalence of inflammatory bowel disease (IBD) when compared to the general population. Since IBD has been linked to the expansion of IEL in Crohn’s disease, it would therefore be of great interest to investigate the presence, number and functionality of CD103-expressing cells in these patients.

**Immunoscore**

As discussed above, our results from chapters 2 and 3, demonstrate a superior prognostic value of CD103-positive T cells over CD8-positive T cells, suggesting that CD103 may be used as an immune marker to predict prognosis and/or response to standard therapy. In line with this notion, worldwide taskforces are currently making an effort to stratify patients based on their immune infiltrates for the prediction of prognosis and response to therapy. Galon et al. developed the Immunoscore as a method for standardized quantification of immune infiltrates within the tumor contexture. This standardized scoring system is based on densities of two lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO) infiltrating the core and invasive margin of the tumor. The Immunoscore can provide a score ranging from Immunoscore 0, when low densities of both cell types are found in both regions, to Immunoscore 4, when high densities are found in both regions. To illustrate this, the Immunoscore approach was applied to
two large independent cohorts of colorectal cancer patients. Only 4.8% of patients with a high Immunoscore, relapsed after 5 years and 86.2% were alive. In comparison, 72% of patients with a low score experience tumor recurrence and only 27.5% were alive at five years. These low Immunoscore patients potentially could have benefited from adjuvant (immuno)therapy that increases T cell infiltration to (re)populate the tumor environment. In line with this, Galon et al. advocate to incorporate the Immunoscore into the TNM-classification (tumor burden, lymph nodes, metastasis) for tumor staging as a tool for the prediction of prognosis and response to therapy.

Based on our data, it could therefore be argued that the Immunoscore might even be improved by replacing CD3/CD8 or CD8/CD45RO with CD103 as marker for intratumoral T cells. This (modified) Immunoscore as described by Galon et al. in colorectal cancer, could also be of use for the prediction of prognosis and response to therapy in gynecological malignancies. Within the Netherlands, large randomized clinical trials have been performed in endometrial cancer patients, the Post-Operative Radiotherapy Endometrial Cancer 1-3 (PORTEC1-3) studies. The PORTEC studies investigate the effect of different postoperative radio(chemo)therapy strategies in endometrial cancer patients. These randomized PORTEC cohorts would therefore be ideal for validation of CD103 as prognostic biomarker for endometrial cancer patients in general, and in this case, response to adjuvant radio(chemo)therapy in particular.

**Differentiation of TIL**

Along with the number and localization of TIL, their differentiation status is also an important prognostic factor. Following activation, (cancer) antigen-specific naïve T cells undergo a progressive differentiation towards distinct effector and memory-precursor phenotypes. During e.g. acute infections, the effector T cells progress towards a terminally differentiated state associated with high cytolytic activity towards target cells followed by programmed cell death during the so-called “resolution phase” of the immune response. Concurrently, precursor memory T cells mature into long-lasting memory T cells capable of rapid response and protection upon re-exposure. In cancer, the chronic nature of the antigen-exposure is thought to significantly skew this normal differentiation leading to an “exhausted” state in the effector population and a defective formation of memory T cells. In chapters 5-7, we describe how T cells in the peripheral blood and in the tumors of ovarian cancer patients display highly heterogeneous differentiation patterns, associated with prognosis. Indeed, less-differentiated TIL in ovarian cancers, characterized by expression of CD27, were associated with a significantly improved prognosis, particularly when cytoreductive surgery was incomplete. Remarkably, we observed a similar heterogeneous expression of CD27 within the peripheral blood, although we were unable to directly link CD27 expression status in circulating and tumor-infiltrating
CD8+ T cells. Nevertheless, our data suggests prospective studies comparing differentiation of circulating T cells and TIL may help elucidate how chronic antigen-exposure in OC patients shapes the immune response and outcome for patients.

**Patient selection for immunotherapy**

Immunotherapy, and in particular ICB can induce long-lasting clinical responses and even curtailment of some advanced and metastatic cancers. ICB reinvigorates exhausted, tumor-specific T cells. Unfortunately, only a small subset of cancer patients responds to ICB. Other disadvantages of ICB are the high costs, and the occurrence of severe, but manageable, adverse events. Generally, patients with high levels of infiltrating T cells -immunological ‘hot’ tumors- are more likely to respond to ICB compared to patients with low levels of infiltrating T cells – immunological ‘cold’ tumors-. At the very least, patients with immunological hot tumors should therefore be selected for ICB. By contrast, checkpoint inhibition therapy is less likely to benefit patients with immunological cold tumors, as there are none or not enough T cells to reinvigorate. Immunological cold tumors should therefore first be (re)populated with tumor-specific T cells. This (re)population can be induced by stimulation of the immune system with a tumor-specific antigen ex vivo, by adoptive T cell transfer, or in vivo, by therapeutic vaccination.

As we show in chapter 2, one example of an immunologically more profoundly cold tumor type is ovarian cancer. Indeed, only 10% of HGSC tumors show high levels of tumor-infiltrating lymphocytes. This immunologically cold tumor type can roughly be explained by an intermediate mutational load. As a consequence of this intermediate mutational load, the abundance of neoantigens derived from point mutations is expected to be lower in this disease. Identifying patients with these neo-antigen rich and exceptional immunologically hot tumors for treatment with ICB is therefore of interest.

One possible identifier might be breast cancer 1 (BRCA1)-mutation status as it has been described that TIL are particularly prominent in BRCA1-mutant tumors. This can partially be explained by the finding that BRCA1/2-mutated ovarian cancers harbor a higher mutational load and a unique mutational signature with an elevated number of larger insertions or deletions (indels). This mutational signature can lead to the formation of more tumor-specific neoantigens that stimulate the recruitment of TIL. However, this does not completely explain why this increased levels of TIL is specifically observed in BRCA1-mutant tumors and not in BRCA2-mutant tumors. In addition, approximately 50% of HGSCs are defective in Homologous Recombination (HR) DNA repair pathways (genetic and epigenetic, including BRCA1/2 mutations). HR deficient HGSCs depend on alternative, low fidelity mechanisms for double-strand break (DSB) repair. This error-prone DSB repair mechanism leads to point mutations and indels. Theoretically, this should then also lead to similar formation of neoantigens. An explanation for the decreased immunogenicity of HGSC might be that factors other than point mutation load are therefore
likely to influence T cell infiltration. These other factors might involve other classes of tumor antigens, such as amplified or aberrant gene products arising from gene fusions, so-called copy number aberrations (CNA).42 High level of chromosomal aberration has been described to correlate with poor clinical outcome in ovarian cancer.43 Taken together, HGSC is characterized by chromosomal instability e.g. caused by HR-deficiency or CNA. However, for unknown reasons this chromosomal instability does not lead to subsequent formation of TIL-attracting tumor-specific neoantigens. Patients with immunological cold tumors could first be selected for therapeutic strategies that (re)populates the tumorenvironment with tumor-specific T cells by stimulation of the immune system with a tumor-specific antigen ex vivo, by adoptive T cell transfer, or in vivo, by therapeutic vaccination.

In contrast to ovarian cancer, endometrial cancer includes some tumors with the highest mutational loads across malignancies. In particular, ultramutated POLE exonuclease domain-mutant (POLe-mutant) and hypermutated microsatellite unstable (MSI) tumors are characterized by high levels of neoantigens. In follow up to our data on CD103 in endometrial cancer in chapter 3, Eggink et al. explored the immune profiles, including CD103 infiltration, of these subgroups within the TransPORTEC cohort, a high-risk endometrial cancer cohort.44 High levels of CD103-positive TIL were seen in the POLe-mutant/MSI subtypes, whereas low levels of CD103-positive TIL were observed in the PS3-mutant/no specific molecular profile (NSMP) subtypes. In line with this, significant therapeutic responses to ICB have recently been observed in patients presenting with mismatch repair-deficient (dMMR) and/or MSI tumors.45–49 Based on these results, the FDA has recently approved the immune checkpoint inhibitor Pembrolizumab (anti-PD1 monoclonal antibody) for the treatment of dMMR/MSI solid tumors that have progressed following prior treatment and who have no satisfactory alternative treatment options.50 This approval also includes MSI endometrial cancer patients. Importantly, this is the first tissue/site agnostic FDA treatment approval.

Finally, cervical cancer as an entity is arguably the most immunogenic of all gynecologic malignancies. Indeed, cervical cancer is characterized by the expression of the viral oncoproteins E6 and E7 of high-risk HPVs by both malignant and premalignant cells. As a result, E6 and E7 are recognized as non-self and therefore the perfect antigens to target with immunotherapy by therapeutic vaccination or autologous cellular transfer strategies.

In chapter 4 we describe such a therapeutic vaccination in an animal model in which mice were inoculated with E6,E7 expressing tumor cells (TC-1). Tumor-bearing mice were treated with a Semliki Forest Virus (SFV)-based therapeutic vaccine encoding the E6,E7 oncoproteins of HPV16 (rSFVeE6,7). Previously, we demonstrated that therapeutic vaccination with rSFVeE6,7 resulted in complete eradication of TC-1 tumors and survival of vaccinated mice.51–54 Secondly, therapeutic vaccination induced long-lasting systemic immune responses measured by E6,7-specific CTL.51–
These long-lasting immune responses even protected the mice 6 months after the initial immunization for the outgrowth of tumor upon a second tumor challenge, without additional vaccination. Leveraging this model, we show in chapter 4, that therapeutic vaccination with rSFV e6,7 induced infiltration of CD103-positive TIL into the tumor. Importantly, these CD103-positive TIL were largely E6,7-specific-CD8-positive-CTL. Within this experiment a suboptimal dosed vaccination scheme was used to be able to obtain tumor samples and analyze TIL. Furthermore, therapeutic vaccination was combined with radiation, which is used as standard-of-care in advanced-stage cervical cancer, showing a synergistic effect of this treatment combination resulting in an even higher increase in the infiltration of CD103-positive CTL into the tumor. Our results therefore indicate that CD103 should be explored further as a biomarker for response evaluation of immunotherapeutic (combination) strategies.

...to bedside.
The highly promising preclinical data on rSFV e6,7, described in part above, resulted in the initiation of a first-in-human clinical trial with rSFV e6,7 as Vvax001 (chapter 8). Perspectives on this Vvax001 therapeutic vaccination and other immunotherapeutic strategies in cervical cancer will be discussed in this “bedside section”.

Therapeutic vaccination using Vvax001
Therapeutic vaccination for the treatment of cervical cancer can be deployed in two phases of development of the disease. Firstly, in precancerous lesions enhancing the immune response to HPV could inhibit progression of disease towards invasive cancer. Secondly, in cervical cancer, therapeutic vaccination may result in cancer cell death, which on itself, or when combined with e.g. radiochemotherapy and/or other immunotherapeutic strategies such as immune checkpoint blockade (ICB), might be curative.

As a result of population-based screening in western countries, cervical cancer is mostly diagnosed in a precancerous stage, so called cervical intraepithelial neoplasia (CIN). Currently, CIN lesions are surgically treated by a large-loop-excision of the transformation zone (LLETZ). This is mostly an effective procedure, however, direct and indirect complications can occur such as bleeding, infection, infertility and insufficiency of the cervix during pregnancy. Furthermore, this surgical LLETZ procedure does not necessarily eradicate the HPV infection. Therefore, new therapeutic strategies are warranted.

The promising preclinical results of rSFV e6,7 therapeutic vaccination, published earlier and described in chapter 4, have led to the development of an investigational medicinal product, named Vvax001, for the treatment of (pre)malignant cervical lesions. Vvax001 therapeutic vaccine is currently under investigation in a phase I clinical trial in healthy women with a history of HPV-related (pre) malignant lesions of the cervix. Chapter 8 describes the design of the first-
in-human phase 1 clinical trial. The primary aim of this phase I clinical study is to determine the immunogenicity of Vvax001 in humans. In addition, safety and tolerability is being investigated. When proof of immunogenicity has been established, we envision a phase 2 clinical study, in which the clinical efficacy of Vvax001 will be established in patients with active disease of the cervix. To evaluate the clinical efficacy, we intend to immunize patients with HPV16-positive high-grade CIN lesions (CIN2/3) with Vvax001, instead of the standard LLETZ treatment. After the Vvax001 immunization, patients will enter a watchful waiting phase. During this watchful waiting phase patients will be monitored by regular colposcopy. At a to be defined interval after vaccination (e.g. 6 months), a biopsy or if necessary LLETZ excision, will be performed. If progression of the disease is suspected during the watchful waiting phase, LLETZ excision will be performed. Within this phase 2 study, pre- and post-vaccination biopsies will be collected to investigate immunological effects by monitoring CD103-positive CTL infiltration levels. Furthermore, systemic E6,7-specific immune response will be monitored before and after vaccination, and up to 24 weeks to establish long-lasting immunological responses. This phase 2 study can give insight in the relation between clinical efficacy (clearance/regression of CIN lesion) and systemic and local immune responses. A possible advantage for patients who would participate in this proposed phase 2 trial, is that these patients –mostly women in fertile life phase- might avoid LLETZ excision if biopsy proves regression of the CIN lesion. In addition, therapeutic vaccination might induce immune memory, protecting for recurrences of disease.

When Vvax001 is proven to be well tolerated, immunogenic and therapeutically effective in these phase I and II clinical trials, SFV can be further expanded as an immunization platform e.g. for the treatment of other HPV types or for the treatment of other HPV-related malignancies such as head and neck squamous cell carcinoma. Indeed, while Vvax001 therapeutic vaccine is the first viral vector vaccine using the Semliki forest virus (SFV) as viral vector, it can be readily modified to encode a variety of cancer antigens, including e.g. neo-antigens.

Other immunotherapy in cervical neoplasia and cervical cancer

In addition to Vvax001, a number of agents aiming to stimulate the immune system against cervical lesions are currently under evaluation in the field of cervical lesions. A search on ClinicalTrials.gov revealed that interventional clinical studies have been registered in the past decade for the treatment of cervical intraepithelial neoplasia and/or cervical cancer involving immunotherapeutic agents as monotherapy or in combination with radio(chemo)therapy. Most agents target HPV-16 and/or 18, but also other HPV serotypes are targeted.

The majority of the registered clinical trials involve therapeutic vaccination strategies (27 studies). Most therapeutic vaccines target the E6 and E7 viral proteins of HPV-16 and/or HPV-18. In two studies, prophylactic HPV vaccines (Gardasil® and Gardasil®9) are being evaluated for their therapeutic efficacy in women with cervical intraepithelial neoplasia. Thus far, therapeutic
vaccines targeting HPV-16 or HPV-18 have shown clinical efficacy with clearance of HPV infection and clearance of precancerous lesions in phase 2 studies.\textsuperscript{55–58} In advanced cervical cancer, therapeutic vaccination resulted in induction of immune responses but no clinical effect was observed.\textsuperscript{59} In addition, also vaccination studies aiming for targets other than HPV were open for cervical cancer patients; e.g. studies investigating a peptide-vaccine targeting VEGFR2, ‘preferentially expressed antigen in melanoma’ (PRAME) and ‘prostate-specific membrane antigen’ (PSMA).\textsuperscript{60}

The second most used immunotherapeutic strategy that is used in the field of cervical cancer is adoptive cellular transfer (ACT). Currently, 8 studies are registered on clinicaltrials.gov using ACT strategies. In advanced metastatic cervical cancer, E6,7-reactive autologous TIL were selected and transferred back into the patients. Initial results showed impressive clinical responses with the clearance of tumor masses.\textsuperscript{61} Recently, the results of a clinical study administering genetically modified T cell receptor (TCR) t cells targeting melanoma-associated antigen-A3 (MAGE-A3) were published in which also 3 cervical cancer patients were included. A complete response, that is still ongoing at 29 months, was observed in one patient with metastatic cervical cancer. The other two cervical cancer patients showed no response to the therapy.\textsuperscript{62} Ongoing studies use a variety of ACT strategies for the treatment of cervical cancer such as natural killer (NK) cellular transfer and chimeric antigen receptor (CAR) T cells targeting Mesothelin.

Lastly, immune checkpoint blockade is also under investigation for the treatment of cervical cancer. Preliminary results from the KEYNOTE-028 study, evaluating the safety and efficacy of Pembrolizumab (anti-PD1) in patients with advanced solid tumors were presented in 2016. This study included a cohort of 24 patients with advanced cervical cancer. Out of these 24 patients, 3 patients showed a partial response leading to stable disease with a medium duration of 19.6 weeks.\textsuperscript{63} As these results are preliminary, the clinical benefit of Pembrolizumab will be further investigated. ICB monotherapy can be given to boost pre-existing immunity against E6,E7 antigens, in patients with a pre-existing immune response or maybe even CIN-patients for the prevention of transformation to invasive cancer. However, according to the preliminary results in advanced cervical cancer, ICB monotherapy does not induce complete responses. Therefore, ICB is now mostly being tested in combination with other immunotherapeutic strategies (ACT and therapeutic vaccination) that have shown to initiate an immune response or to augment the pre-existing immune response.

In current ongoing trials, 4 studies investigate multiple immunotherapeutic combination strategies. Examples of therapeutic vaccination combined with ICB are Atezolizumab or Durvalumab (both anti-PDL1) with autologous tumor cell vaccination or Nivolumab (anti-
PD1) combined with synthetic-Long-peptide (SLP) vaccination targeting HPV16. Furthermore, one study involves combination therapy of ACT, in the form of E7 targeting TCR T cells, with or without ICB Pembrolizumab (anti-PD1).

**Combining immunotherapy with standard-of-care treatment**

Finally, the effects of standard treatment on systemic immunity and the tumor microenvironment are important to consider when proposing combination therapies. In cervical cancer it was shown by Welters et al. that chemotherapy resulted in a systemic decrease of myeloid derived suppressor cells (MDSC).\textsuperscript{64} In chapter 7 we show a similar trend in the depletion/decrease of MDSC in ovarian cancer patients during Carboplatin/Paclitaxel chemotherapy. These results indicate that Carboplatin/Paclitaxel chemotherapy can best be combined with immunotherapy e.g. therapeutic vaccination after 2-3 cycles, when the immune suppressive subset is at its nadir, and the proportion of circulating T cell subsets is not affected.

In line with this data on circulating T cells, we also did not observe T cell depletion after Carboplatin/Paclitaxel chemotherapy in the tumor microenvironment, (chapters 2, 5 and 6). However, there are some pitfalls worth mentioning; we compared tissue samples obtained from either primary surgery (PS) or interval debulking surgery (after three cycles of chemotherapy; NACT), but these cohorts are not matched per patient. Therefore no statements can be made on the direct effect of chemotherapy on T cell infiltration per patient. Furthermore, the cohorts must be approached as two independent cohorts as a selection bias is present due to the decision for treatment regimen by the physician; patients in the NACT cohort generally have a worse prognosis compared to patients in the PS cohort. Lastly, we determined the number of infiltrating T cells per mm\textsuperscript{2}. Assuming chemotherapy diminishes the amount of cancer cells, it could be possible that the effector to target ratio in the NACT cohort is overestimated. In contrast to our data, mouse studies evaluating the effect of different chemotherapeutics on immune infiltration, generally suggest that chemotherapy enhances T cell infiltration into the tumor.\textsuperscript{65} In line with this, it could also be hypothesized that the NACT cohort has lower initial/pretreatment numbers of TIL, and that chemotherapy induces an increase of infiltrating T cells. Ideally, our recommendation would therefore be to evaluate the effects of chemotherapy on the tumor microenvironment in matched tumor samples. However, in ovarian cancer, but also in many other cancer types, it is not standard procedure to collect pre- and post-treatment samples and tumor heterogeneity may still affect outcome of such comparisons.

Our hypothesis that immunotherapy could be combined with MDSC-depleting Carboplatin/Paclitaxel is further supported by two lines of evidence. First, vaccination with an HPV E6/E6 SLP vaccine during Carboplatin/Paclitaxel-chemotherapy in cervical cancer patients significantly augmented the circulating anti-E6/E7 immune response.\textsuperscript{64} Second, depletion of intratumoral MDSCs by Sunitinib, a clinically-applied broad receptor tyrosine kinase inhibitor, augmented
rSFVeE6,7 therapeutic vaccination in our HPV16+ tumor model described in chapter 4. Moreover, combining both low dose irradiation and Sunitinib with therapeutic vaccination even further enhanced the intratumoral ratio of antitumor effector T cells to MDSCs.\textsuperscript{66,67} One note of caution is appropriate as clinically-applied radiotherapy has previously been linked to reduced immune function in cervical- and other cancer patients, although this immune-suppressive effect is likely exacerbated by the high dose of radiation used for eradicating tumor cells. Nevertheless, these results imply that off-label use of clinically used drugs such as Sunitinib should be evaluated to create an optimal immune environment supporting immunotherapeutic activation of tumor-killing T cells.

**CONCLUSION**

Taken together, the data published so far contribute to our understanding of the tumor immune biology of gynecological malignancies and may help guide therapeutic intervention in the near future.
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