Immunological aspects of conventional and new treatments for cervical cancer, an immunopharmacological approach

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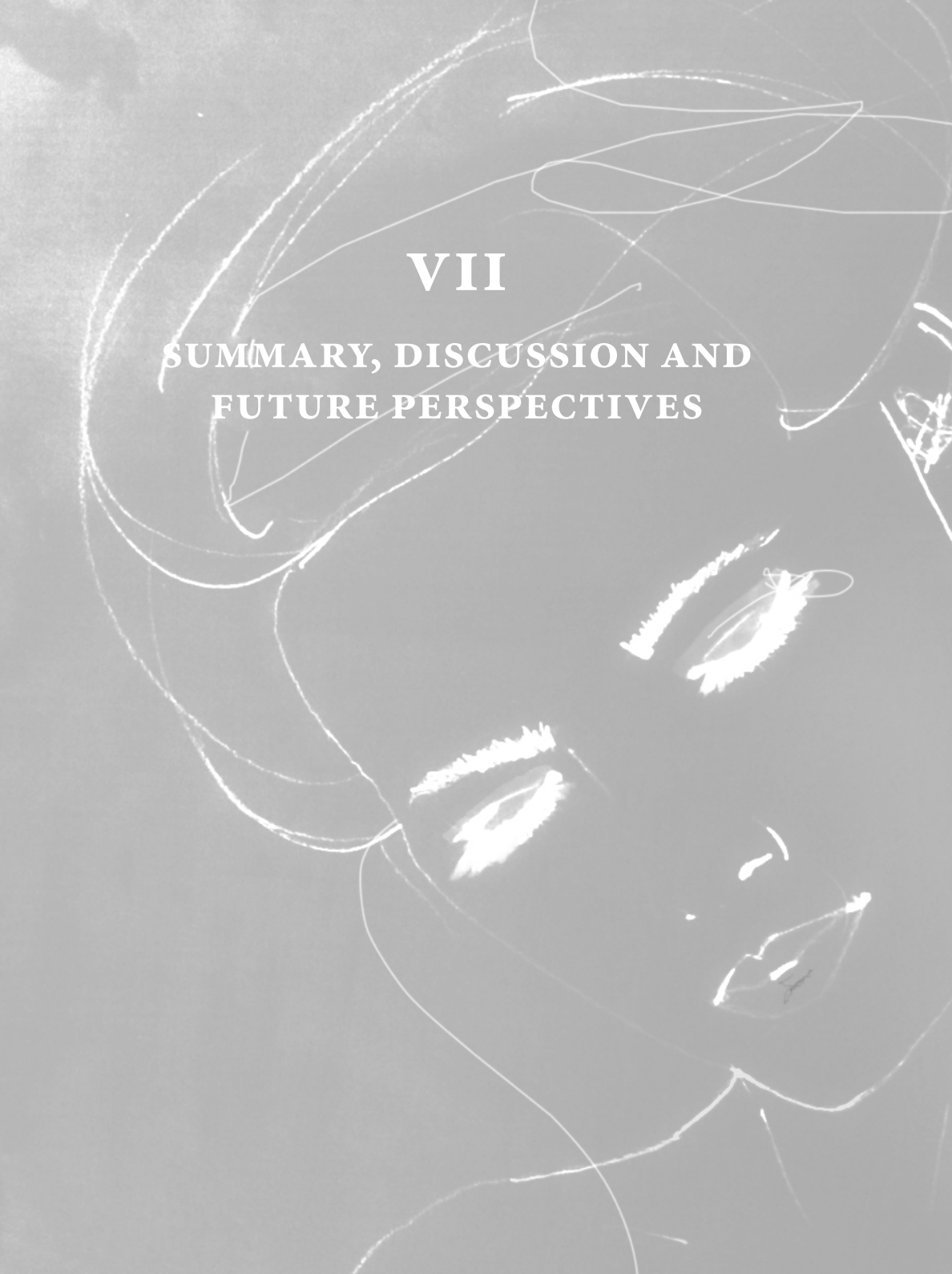

VII

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

Cervical cancer is the most common human papilloma virus (HPV) associated cancer among women. Standard treatment for cervical cancer consists of surgery, radiotherapy and chemotherapy, or combinations thereof. In contrast to the early stages of disease, advanced stage cervical cancer has a poor prognosis with high risk of recurrence. A number of single drug and combination regimens have been studied to control advanced stage of disease, however success is limited and recurrent and metastatic cervical cancer remain incurable and eventually fatal. Clearly, the identification of new treatment strategies together with optimal selection of patients in higher risk categories for recurrent cervical cancer that may benefit from a specific treatment, is crucial. In the last decades, novel passive and active immune-based therapies are being explored as a potential alternative or adjuvant treatment for cervical cancer.[1-3] Similar to already available treatments, such new immune-based therapies have not yet shown clinical benefit in end-stage cervical cancer patients suffering from a large tumor burden and/or immunosuppressive conditions.[3,4] Nevertheless, immune-based therapies did show clinical success in patients with pre-malignant lesions.[5,6] Clinical effectiveness depended on patients' immune state or tumor immune microenvironment and subsequently the ability to immunologically respond to a certain immunotherapy.[7,8] Interestingly, findings from pre-clinical and clinical work suggest that conventional therapies such as chemotherapy and radiotherapy partly act through the immune system, and may theoretically be combined with immunotherapy to improve treatment success in patients.[9-11] An additional benefit may be that the combination of immunotherapy with standard of care chemotherapy and/or radiotherapy is more likely to be accepted for treatment of early stage disease, than when immunotherapy is put forward as alternative strategy.

Through the studies in this thesis, we gained more knowledge about the effect of standard chemotherapy and radiation therapy on the immune response in cervical cancer patients. Monitoring of kinetic changes in immune responses during standard treatment for cervical cancer was used to investigate if and how immunotherapy could be combined with existing therapies for optimal treatment effects. This also allowed to determine the best time for additional immunotherapy to be applied. These studies may help to improve combination therapies and may eventually result in individualization of therapy. Indeed, this information was utilized to design a recently initiated clinical trial in which the effects of a combination of chemo- and immunotherapy is investigated (NCT02128126).

Immunotherapy in cervical cancer

Cancer immunotherapy consists of a large and growing number of approaches, including use of antibodies and cytokines, therapeutic vaccination and adoptive cell therapy (ACT). The clinical effectiveness of immunotherapy depends on different issues, varying from patient-specific to tumor- and immune- cell specific conditions. First, patients participating in immunotherapeutic clinical trials frequently have end-stage of disease without any curative options. In these cases, the burden of tumor may be too high to be successfully eradicated by an activated immune response. Better results might be obtained when immunotherapy is applied in patients suffering from pre-malignancies or in situations of minimal disease (e.g. shortly after successful primary therapy). In patients with HPV induced (pre)malignancies, several therapeutic vaccination strategies with different delivery systems have been explored clinically. These trials included recombinant viral vector-, peptide- or protein-, nucleic acid-, and cell-based therapeutic vaccines targeting the HPV16 E6 and/or E7 antigens.[12] A therapeutic synthetic DNA vaccine VGX-3100 targeting HPV 16 and 18 E6 and E7 proteins, showed clinical efficacy in patients with grade 2/3 cervical intraepithelial neoplasia lesions.[6] Vaccination of patients with HPV16-induced premalignant vulvar lesions with a therapeutic HPV16 overlapping synthetic long peptide (HPV16-SLP) elicited a strong and broad HPV-specific $CD4^+$ and $CD8^+$ T-cell response[7], and partial or complete lesion regression.[5,8] When the HPV16-SLP vaccine was administered in patients with recurrent or advanced cervical cancer, it showed fair immunogenicity but no clinical benefit.[1,3] This absence of clinical effects may reflect strong immune suppression which is often associated with large tumor burden and makes an extended immune response hardly possible nor clinically effective. Hence, vaccine therapy might also be of value in patients with minimal residual disease. As described in *chapter 3*, more than 50% of patients with recurrent disease after surgical treatment for early stage of disease develop distant metastases, suggesting that a substantial number of high-risk patients have residual micro-metastases after what was thought to be a successful primary treatment. Because of its poor prognosis, the reduction of recurrent disease is important, and immunotherapy might be an interesting adjuvant approach to achieve this. In comparison, immunotherapeutic options have emerged as a potential adjuvant treatment option in high-risk surgically treated melanoma patients.[13] It is conceivable that this paradigm to control tumor metastasis and kill remaining cancer cells also applies to high risk cervical cancer patients treated with immunotherapy concurrent with or after primary treatment. This requires that identification of patients at high risk of

recurrence should not be based on histopathological characteristics only, but should include assessment of the status of the immune system. When this is systematically and uniformly done, it may result in stratification of patients that allows to prospectively predict the course of disease and the response to (immuno)therapy.[14]

Secondly, cancer is often associated with immune escape and suppression[15,16] and these conditions might also affect the efficacy of immunotherapy in (cervical) cancer. Success of immunotherapy against cervical cancer depends on different immunological conditions in which immunotherapy needs to operate. These conditions mainly include the induction of strong tumor-specific T-cell responses at the tumor site, control over the regulatory mechanisms (including immunosuppressive cells as Tregs, M2 macrophages and myeloid derived suppressor cells and immunosuppressive substances as IDO, IL-10 and TGF-β) and the creation of a pro-inflammatory micro-environment.[12,17]

Different immunotherapeutic strategies act on parts of these conditions. The challenge of immunotherapy is to induce long-lasting protective anti-tumor immune responses, counteract tumor-induced immune suppression and suppress tumor escape from immune recognition. Non-specific immune stimulation with cytokines and antibodies, ACT and therapeutic vaccination are the best-known immunotherapeutic modalities to achieve this. Monoclonal antibodies or recombinant cytokines can directly activate the immune system or abrogate immunosuppressive mechanisms. Blockade of immune inhibitory pathways has widely been investigated and seems to be a promising strategy for patients with a pre-existing immunological antitumor response and/or patients of whom the tumor expresses foreign antigens such as mutated antigens, viral antigens or translocations. In human cervical cancer samples the inhibitory molecule PD-1 was expressed by more than half of the infiltrating $CD8^+$ T-cells, suggesting that blocking of PD-1 or its ligand PD-L1 could be a rational therapeutic option in the treatment of recurrent and/or metastatic cervical cancer.[18,19] Ipilimumab, the human monoclonal antibody directed against CTLA-4, was FDA approved in 2011 for the treatment of metastatic melanoma, is currently tested in a Phase II trial study in patients with metastatic or recurrent cervical cancer (NCT01693783). Another promising potential strategy is the stimulation of co-stimulatory receptors by agonistic antibodies. Heusinkveld *et al* showed in cervical carcinoma cells that CD40 activation tumor-induced shift M2 macrophages to the pro-inflammatory M1-like macrophages in the presence of IFNγ.[20] This suggests that a monoclonal antibody to CD40 could be a potential therapy for combination with traditional treatments or other immunotherapies for cervical cancer. This needs to be further investigated, but it seems promising

as in patients with pancreatic ductal adenocarcinoma combination therapy of CD40 gemcitabine showed positive results.[21-23] Another important mechanism to achieve beneficial immune response is recruitment of a sufficient number of tumor specific type 1 $CD4^+$ and $CD8^+$ T-cells into the tumor. In the light of the multiple immune modulation strategies, it appears that the key to success lies in combining immunotherapy with therapies that target immune-escape mechanisms.

The effects of standard treatments for cervical cancer on the immune system

In *chapter 4*, the effects of standard treatments were described in further detail and it was concluded that cytotoxic drugs can influence the complex network of tumor cells, cancer growth stimulating immune cells and tumor reducing immune cells.[24] Treatment of cervical cancer commonly consists of cisplatin, a combination of carboplatin and paclitaxel, and/or radiation therapy. Evaluation of their immunological effects is crucial for potential combinations of immunotherapy with these standard treatments.

CHEMOTHERAPEUTIC AGENTS INDUCE IMMUNOLOGICAL EFFECTS

Originally, chemotherapy was considered as a treatment whose efficacy was attributed to the direct cytotoxic effect on dividing cancer cells. However, accumulating evidence showed that chemotherapeutic agents also mediate their effects through immune mechanisms. The underlying mechanisms include dendritic cell activation by apoptotic tumor cells, direct activation and stimulation of tumor-specific immunity and depletion of immunosuppressive cells which converts the tumor environment into a T-cell permissive site.[25-29] We described the unexpected long term survival that was observed in 5 patients with end-stage cervical cancer treated with the HPV16-SLP vaccine in a phase I trial *(chapter 4)*. It was carefully evaluated if patients were treated with chemotherapy within 3 months before or after vaccination. A post-hoc analysis suggested that the application of vaccination within 3 months before or after chemotherapy was associated with a favorable clinical outcome, compared to standalone chemo- or immunotherapy. The heterogeneity in disease stage, previous therapies and clinical conditions, made it difficult to delineate the contribution of each treatment on survival rates. Therefore, we further investigated the impact of conventional therapies on the immune system taking into account treatment

schedules, timing and dosing of the different treatment modalities with the aim to design optimal combinations of these treatments with therapeutic vaccination in cervical cancer.

Our study on the immune effects of chemotherapeutic treatment with carboplatin and paclitaxel (carbo-taxol) showed improved T-cell reactivity 1-2 weeks after the second and subsequent cycles of chemotherapy, without changes in absolute lymphocyte counts or strong alterations in frequencies and phenotype of $CD4^+$ and $CD8^+$ T-cells *(chapter 5)*. These findings seem to corroborate earlier results reported by Coleman *et al* and Wu *et al* in ovarian cancer, were it was found that $CD8^+$ T-cell function is not permanently suppressed in advanced cancer patients during systemic chemotherapy and displayed the highest level of activity 12-14 days after chemotherapy.[30,31] Together with the increase in T-cell reactivity, we found a strong decrease in the numbers of circulating myeloid cells upon chemotherapeutic treatment. Notably, the number of circulating myeloid cells before chemotherapeutic treatment was much higher in patients with cervical cancer than in the blood samples from healthy donors and is thought to be caused by a high tumor burden, similar as in our mouse model, and reported by others.[32-34] Carbo-taxol treatment reduced the numbers of myeloid cells to almost normal levels. In depth analysis of the affected myeloid cell subsets revealed that the decrease in myeloid cells was found across all circulating myeloid subpopulations, including tumor growth suppressing myeloid cells (M1 macrophages), tumor-promoting myeloid cell populations (M2c macrophages) and MDSCs ($CD45^+CD3^-CD19^-CD1a^-HLA\text{-}DR^{low}$). This suggests that carbo-taxol normalizes the abnormal levels of myeloid cells in cervical cancer patients. In line with our observations, paclitaxel has previously shown to deplete tumor-infiltrating MDSCs, both in mouse tumor models and in melanoma, resulting in therapeutically relevant restoration of CTL activity.[35,36] In addition, we found a slight decrease in circulating Tregs during carbo-taxol treatment as was also reported earlier in patients with advanced ovarian cancer.[31]

It needs to be emphasized that our analyses were limited to systemic immunity of cervical cancer patients, rather than direct examination of the tumor and its micro-environment. As the clinical trial included patients with advanced stage of disease with a moderate clinical condition, it was not possible to obtain tumor tissue during or after chemotherapeutic treatment. While many immune processes are anticipated to be regulated similarly in the tumor and the circulation, and systemic immunity has shown relevance to clinical response, long-term immune surveillance and risk of recurrence, we anticipated that many of our findings would be even more striking in the tumor micro environment. This is supported by the finding that depletion of intra-tumoral myeloid

populations in mice upon carbo-taxol treatment results in predominance of Gr-1^{int}CD11b^{hi} cells in the tumor, together with markedly reduced circulating Gr-1^{hi}CD11b^{hi} cells *(chapter 5)*. The remaining intra-tumoral Gr-1^{int}CD11b^{hi} cells have a high expression of the macrophage marker F4/80, CD11c, CD80, CD86 and MHC class II, but not Ly6G (granulocytic marker), suggesting a relative greater loss of myeloid cell-associated immune suppression in tumor. Furthermore, the association between myeloid depletion and improved T-cell reactivity was further explored *in vitro*. When CD14^{+} myeloid cells were depleted from PBMCs, an improved T-cell reactivity against recall antigens, and a more efficient boost of the HPV16-specific immune response was observed. The association between high myeloid cell frequencies and immune suppression was earlier found in pulmonary adenocarcinoma patients, whereas a high circulating CD14^{+} myeloid cell concentration was observed, accompanied with absent proliferation response and low cytokine production upon XAGE-1b (a cancer antigen aberrantly expressed in pulmonary adenocarcinoma) stimulation. When CD14^{+} cells were removed from the PBMCs, and the remaining cells were stimulated with a XAGE-1b peptide mix, cell proliferation and cytokine production did occur.[37] Together with our experiments, these data indicate that T-cell reactivity can be impaired by myeloid cell populations, and restored by the depletion of these cells. It is well known that myeloid cells suppress immune responses by inhibition of T-cell activation, and intratumoral myeloid cell counts are therefore considered as a valuable prognostic factor in ovarian and cervical cancer.[38,39] Our data provide evidence for combinatorial therapies targeting myeloid cell populations, directly or through the pathways that regulate their recruitment, in combination with cytotoxic therapy.

In addition to the effects described by us, platinum anticancer drugs may also act on the inhibitory pathways such as the PD/PD-L pathway. Lesterhuis *et al* showed that both cisplatin and carboplatin cause down regulation of the inhibitory molecule PD-L2 in a STAT6 dependent manner, both on DCs and tumor cells, resulting in enhanced antigen-specific T-cell proliferation with Th1 cytokine secretion and increased sensitivity for tumor lysis by cytotoxic T-cells.[40] Cisplatin was shown to enhance cell death and causes decreased proliferation of tumor cells in the presence of tumor necrosis factor-α (TNF-α). Combination of cisplatin with peptide-based anticancer vaccines that stimulated efficient tumor infiltration by TNFα-producing T-cells resulted in improved cure of tumor-bearing mice.[41] The ability to increase the susceptibility of tumor cells to CTL lysis has also been shown for docetaxel and involves calreticulin exposure on the cell surface.[42] This indicates that the platinum-based cytotoxic drugs have an immune stimulatory potential that operates via several distinct mechanisms.

It has been proposed that the most effective chemotherapeutic compounds trigger cancer cell death while inducing DC maturation and subsequent immune responses against the tumor. This chemotherapy-induced immunogenic cell death has thus far been restricted to selected agents, including doxorubicin, oxaliplatin, cyclophosphamide and mitoxantrone.[43] For the treatment of cervical cancer the exact mechanism of synergy between chemotherapy and immunotherapy is not fully elucidated yet, and apparent differences between chemotherapeutics exist. Importantly, none of the chemotherapeutic compounds impairs the impact of HPV16-SLP vaccination on tumor growth, as shown in a pre-clinical tumor model.[41] It was found that combined treatment with oxaliplatin, doxorubicin or paclitaxel with HPV16-SLP vaccination did not enhance overall survival compared to vaccination alone, while combination therapy with the same vaccine and cisplatin, topotecan, carboplatin or gemcitabine showed clear synergy in terms of survival. Thus a failure of chemotherapeutic compounds to stimulate immunogenic cell death should not pose a problem when additional vaccine therapy is given to stimulate T-cell immunity.[41] Cisplatin displayed the strongest synergy in combination with therapeutic SLP vaccination. This cytotoxic agent, as well as carboplatin and gemcitabine are known to affect myeloid cell populations, a mechanism that might explain part of the synergism with HPV16-SLP vaccination.

Together with our findings on depletion of myeloid cells upon carbo-taxol treatment, these examples indicate that chemotherapeutic compounds, with limited immunogenic cell death stimulatory potential on their own, may synergize with immunotherapy when combined appropriately. Indeed HPV16-SLP vaccination administered to advanced cervical cancer patients within the best immunological window, 2 weeks after the second cycle of carbo-taxol, resulted in strong HPV16-specific proliferative T-cell responses. These responses were retained beyond the last cycle of chemotherapy, and had a greater magnitude compared to those observed in a previous trial where recurrent cervical cancer patients were treated with the HPV16-SLP vaccine after chemotherapy.[3] In clinical trials with colorectal and ovarian cancer patients it was shown that the immune response can be further increased by the addition of IFNα. A combination of a p53 SLP vaccine with IFNα resulted in enhanced inflammation, a stronger type 1 cytokine polarized p53-specific T-cell responses, and a better p53-specific $CD8^+$ T-cell response.[44,45] This confirms IFNα's ability to induce full maturation of DCs, to improve cross-presentation of tumor antigens, to generate CTLs, and enhance proliferation and survival of T-cells, thereby enhancing an anti-tumor response.[46–48]

Based on these results, a multicenter phase I/II trial (NCT02128126) is currently executed in which a multimodality approach consisting of carbo-taxol chemotherapy, HPV16-SLP vaccination and IFNα cytokine therapy is applied for the treatment of advanced cervical cancer.

IMPACT OF RADIOTHERAPY ON LYMPHOCYTE SUBPOPULATIONS AND IMMUNE FUNCTION

Traditionally, radiation therapy was thought to cause direct cytotoxic and cytostatic effects on malignant cells. However, experimental data from multiple cancer models indicate that the additional therapeutic potential of radiation therapy may reside in its immunological effects, although other mechanisms cannot be excluded. Pre-clinical data suggest that immunological effects of radiotherapy includes (re)activation of an antitumor immune response as well as counteracting the tumor-induced immune suppressive conditions.[49-51] This pre-clinical evidence is sporadically observed by clinical observations in patients with different cancer types at advanced stage of disease. As an example, metastatic tumors outside the radiation treated field may respond to treatment, suggesting an abscopal effect of radiotherapy which may be related to induction of antitumor immunity.[52-54] Such objective clinical immune-modulated abscopal effects are uncommon and optimal radiation regimens for a given tumor type to harness the pro-immunogenic effects of radiation remain to be defined. It is further unclear if standard radiation treatment regimens can be modified to restore effective immunity and overcome dominant immunosuppressive pathways.[51]

The characterization of radiotherapy effect on systemic and local immune responses in clinical trials for a given tumor type, is however especially important for future trials that aim to incorporate immunotherapy with (chemo)radiation therapy. The optimal sequencing (including dose and fractions applied) of radiation therapy would be invaluable to choose the type of immunotherapy to be part of the combination.[51] The clinical study described in *chapter 6* investigated the effect of standard-of-care (chemo)radiation therapy on the immune function in cervical cancer patients . The impact of radiation therapy on different lymphocyte subpopulations was determined. Our results provided more detail on radiation-induced lymphopenia as previously reported in patients with cervical cancer and treated with similar doses (45-50 Gy) of radiation therapy.[55-57] We demonstrated that radiation of the pelvis for different stages of cervical cancer causes substantial and long-lasting

immune suppression, regardless of the tumor-load and concurrent cisplatin. Radiotherapy induced a significant and prolonged suppression of lymphoid cells and an increase in myeloid cells. In addition, PD-1 expression on $CD4^+$ T-cells was strongly up-regulated upon radiotherapy. This radiation effect was accompanied with severe impairment of the circulating T-cell response to common pathogens. Similar immune suppressive effects were found in patients treated with chemo-radiation therapy for HPV-related oropharyngeal cancer; immunophenotyping of peripheral immune cells showed a decrease in $CD4^+$ and $CD8^+$ T-cells, an increase of MDSCs and an unfavorable $CD8^+$/Treg ratio.[58] Of note, up-regulation of PD-1 expression on $CD4^+$ T-cells occurred at 3 weeks after completion of therapy. It this study, it was suggested that interventions that enhance radiation resistance of $CD8^+$ T-cells or that deplete Tregs and myeloid suppressor cells could potentially restore immune homeostasis.[58]

Takaya *et al* described the first clinical case of abscopal effect in cervical cancer. Para-aortic lymph node metastases outside the irradiated field disappeared. Due to patient's economic status, radiation was in this case not performed according to the planned schedule but applied in 2 sessions, with an interval of 41 non-treatment days (first session: 16 fractions of 1.8 Gy, total dose 28.8 Gy; second session: 11 fractions of 2 Gy, total dose 22 Gy).[53] The question rises whether modified sequencing of radiotherapy could have influenced the induction of the abscopal effect, if radiotherapy can be optimized to a dose and schedule with retained local cytotoxic tumor effect and but simultaneous (re) activation of anti-tumor immunity. Based on empirical experience, the use of multiple daily doses around 2 Gy to a total dose of approximately 46-50 Gy (23-25 fractions), has evolved as a standard approach to control disease for most tumor types, including cervical tumors. It has been speculated that this conventional fractionated radiotherapy with multiple fractions is immunosuppressive, while ablative radiotherapy generates systemic immuno-activation by increases of $CD8^+$ T-cell priming in draining lymph nodes.[59] However, Battaglia *et al* showed an immune enhancing effect in the tumor draining lymph nodes of cervical cancer patients undergoing fractionated low-dose radiation (total dose 39.6 Gy), but a more immunosuppressive and tumor-friendlier effect of fractionated high-dose radiation (total dose 50 Gy). Although these dose differences were only minor, lower-dose radiation was associated with an increase in the antitumor Th1 and Tc1 subsets and a decrease in Tregs when compared to high-dose radiation therapy.[60] In our clinical study a standard approach (high dose) radiation therapy with 46-50 Gy was applied. We showed that upon radiotherapeutic treatment the numbers of Tregs remained stable with a simultaneous unfavorable reduction in $CD4^+$ and $CD8^+$ cells. As the pelvic bone marrow is extremely radiosensitive,[61]

studies on improvement of delivery and efficacy of bone marrow sparing radiotherapy should be investigated. This could include further characterization of systemic and local immune responses in cervical cancer. This monitoring is of great value to determine whether alternative treatments as immunotherapy could synergistically improve immune responses and patient outcomes.

The number of clinical studies on combinatorial or sequential administration of an immunotherapeutic agent plus radiation therapy is growing exponentially. Nevertheless, the panel of radiotherapeutic and immunotherapeutic regimens is rather heterogeneous for the treatment of a variety of malignancies.[50] Rationales for combinatorial radiation-immunotherapy approaches are based on the widely reported immunomodulatory effects of radiation therapy. On one hand, radiotherapy was reported to prime the immune system against cancer through immunogenic cell death, the recruitment of circulating immune cells and increased antigen exposure and presentation.[62,63] On the other hand, the immune system remains potentially suppressed under radiation therapy, because of enhanced activity of inhibitory immune cells, and relative increases in the number of locally suppressive immune cells (MDSCs, Tregs and TAMs) upon radiation therapy.[64-66] Apparently, these immunosuppressive cell types are less radiosensitive than other lymphocyte subsets. In pre-clinical models, several combinations of local radiation and immunotherapy suggest to induce powerful anti-tumor immunity, but the optimal strategy to achieve this effect remains to be defined. The regimen of radiation therapy revealed to be a critical determinant of the success of combined radiation-immunotherapy. For example, in combination with anti-CTLA-4, different dose fractionation radiation strategies in two carcinoma models growing in syngeneic mice were compared. Each of the radiotherapy regimen had similar effect on the growth delay of primary tumors. The addition of anti-CTLA-4 caused enhanced tumor response at the primary site, and an abscopal effect in mice treated fractionated radiotherapy (3 x 8 Gy), but not in mice receiving a single dose of 20 Gy. Mice treated with 5 fractions of 6 Gy, showed intermediate results, suggesting that a specific therapeutic window may exist for optimal use of (fractionated) radiotherapy in combination with immunotherapy.[67] This is in contrast with the above mentioned study showing that conventional fractionated radiotherapy with multiple fractions (4 fractions of 5 Gy) is immunosuppressive, while ablative radiotherapy (1 fraction of 20 Gy) generates systemic immunity in mice, by the increases of $CD8^+$ T-cell priming in draining lymph nodes.[59] It can be speculated that anti-CTLA-4 has reversed the radiotherapy-induced immunosuppressive effect. Likewise, it is likely that induction of optimal immune responses depends on a threshold of fractionation and dosage of radiation therapy. For cervical cancer, there is currently a

paucity of data on the exact immunogenic demise of cancer cells as induced by radiation therapy, which hinders the design of effective combinatorial radio-immunotherapeutic strategies. An ongoing Phase I clinical trial (NCT01711515) examines the effect of ipilimumab (CTLA-4 targeting) after chemoradiation in patients with stage IB2/IIA cervical cancer with positive para-aortic lymph nodes only or those with stage IIB, IIIB or IVA disease with positive lymph nodes. In this study, patients receive standard cisplatin-based chemoradiation followed by brachytherapy and intravenous ipilimumab within 2 weeks of finishing brachytherapy. As the objectives of this study include progression free survival and HPV-specific T-cell responses, it appears that this study can benefit from measuring changes in circulating and tumor infiltrating immune cell populations relating to dose, delivery and schedule of radiotherapy. With the results from our exploratory study described in *chapter 6*, it appears that altering myeloid and lymphoid cell populations and PD-1 up-regulation are relevant mechanisms in radiotherapy-induced immune suppression. These mechanisms should be taken into account when considering combination of radiotherapy and immune-based modalities. In addition, before combination radiotherapy-immunotherapy can successfully be applied in cervical cancer, a considerable challenge is to overcome the long-lasting suppression of immune responses and thereby optimizing dosing strategies of both therapies. For the identification of the optimal dose and schedule of delivering local radiation therapy to the host, the cytotoxic effects for tumor eradication should remain equal, while lymphocyte populations and immune responses are barely affected. Advances in radiotherapy technology, such as daily (MRI) guided EBRT or proton therapy may allow radiation oncologists to deliver radiation more precisely, thereby reducing tumor burden, boosting protective immunity and inducing disease control.

Challenges facing immunotherapy for cervical cancer

As a number of cytostatics modulate the immune system, combinatorial anti-cancer therapy with novel immunotherapeutic compounds is promising because of potential synergistic effects which may result in improved clinical response rates.[10,50,51,68] This may also apply for combined treatments consisting of radiotherapy and immunotherapeutic compounds. Indeed, many immuno-stimulatory agents are investigated to be used in combination with each other or with conventional therapies to boost tumor-specific immunity and improve clinical response rates.[69-71] For these trials to be successful, a couple of questions need to be answered. These include, among others, the following:

- Which therapies show synergistic effects with immunotherapy in the treatment of (cervical) cancer?
- Are there optimal settings – in terms of dosing, timing and interaction - to combine these old and new treatments?
- Which patients would benefit most, and eventually show improved clinical outcome with minimal side effects?
- How do we optimally monitor these immunological and/or clinical responses?

The selection of clinical effective combinatorial therapeutic regimens should be based on the immunological effects of the anticancer agents. When such immunological side-effects of a cytotoxic compound are characterized in further detail, a combinatorial regimen with immunotherapy can carefully be explored, based on whether the cytotoxic agent stimulates an anticancer immune responses, or depletes immunosuppressive conditions. These considerations aim at the accurate implementation of synergistic approaches in those patients that benefit most.

As patients suffering from advanced, recurrent or cervical cancer currently have limited and non-curative treatment options, this patient group is often used to study multimodality approach including chemotherapy, radiotherapy and immunotherapy. Nevertheless, these patients are not ensured to have clinical benefit in terms of survival, as the tumor burden is high, the immune state severely suppressed, and clinical performance state generally worsening and not able to undergo – at least – 2 cycles of chemotherapy. One must consider carefully, and individually for each patient, whether a combination of specific modalities is beneficial in terms clinical response, disease-free survival and quality of life. For patients suffering from early or locally advanced cervical cancer, standard-of-care treatments are relatively effective, and the major concern relies in preventing recurrent disease, especially in high risk patients. High risk patients should ideally be evaluated for number, function and location of infiltrating immune cells in the tumor micro-environment. The identification and implementation of such immunological parameters could enable the selection of a population of patients that is most likely to respond to additional immunotherapy, and could make the course of disease and response to different therapies more predictable.[14] For example, clear links between immune response and clinical outcome in patients with vulvar lesions have been found, and patients who develop an immunological response are more likely to benefit from the treatment than those who do not generate an immune response.[7] For cervical cancer, strong intra-epithelial infiltration of fully matured M1 macrophages and a high $CD8^+$/Treg ratio were strong prognostic factors for disease free survival.[38]

The identification and validation of such prognostic immunological factors is crucial, especially in clinical trials. In addition, an effort should be made to identify reliable, predictive immune-specific biomarkers to accurately predict efficacy and toxicity of immunotherapeutic agents when used in combination with conventional therapies.

No matter in which patients combinational immunotherapy is considered, monitoring of immune- and clinical responses is of major importance. Chemotherapy and radiation therapy have proven value as neo-adjuvant, adjuvant or palliative interventions against a majority of malignancies, and a significant number of clinical trials were a priori not envisioned on a chemo- and/or radio-immunotherapeutic approach. Many studies did not specifically aim at evaluating the clinical effectiveness of combinatorial therapies, but regarded the treatment (most often radiation therapy) as part of the conventional therapeutic regimens. A major limitation of the immense number of clinical trials on combination therapies, is the lack of uniformity in trial set-up, clinical and immunological response definitions and data interpretation, which hampers to conclusively compare immunological and clinical effectiveness. The use of immunological and clinical response parameters are crucial to investigate immunological and clinical effectiveness of combination therapies. Moreover, the unique characteristics of immunotherapeutic compounds are able to induce a tumor-specific immune response well before clinical response in terms of tumor growth or survival can be detected. This implies that immune related Response Criteria (irRC) should be used hand in hand with the more traditional Response Evaluation Criteria In Solid Tumors (RECIST) criteria.[72]

In conclusion, conventional therapies as chemotherapy and radiotherapy impact immune populations and immune responses in cervical cancer patients. This led to new perspectives into the important role of the immune system and possibilities to optimally implement immunotherapy in the treatment of cervical cancer. Immunotherapy with HPV16-SLP vaccination is a suitable candidate for combined therapy with chemotherapy, when administered within the optimal time window, as it maximizes vaccination efficacy while tumor-induced immune suppression is tackled. To eventually improve clinical outcome in cervical cancer patients, multimodality treatment approaches need further exploration. Within that approach, the assignment of treatment dose, timing and route of administration of both immunotherapy and the classic conventional therapies are important. In addition, individualization of patients therapy based on immune markers prior to treatment should be a goal to optimize combination therapy, minimize side effects and improve clinical outcomes.

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