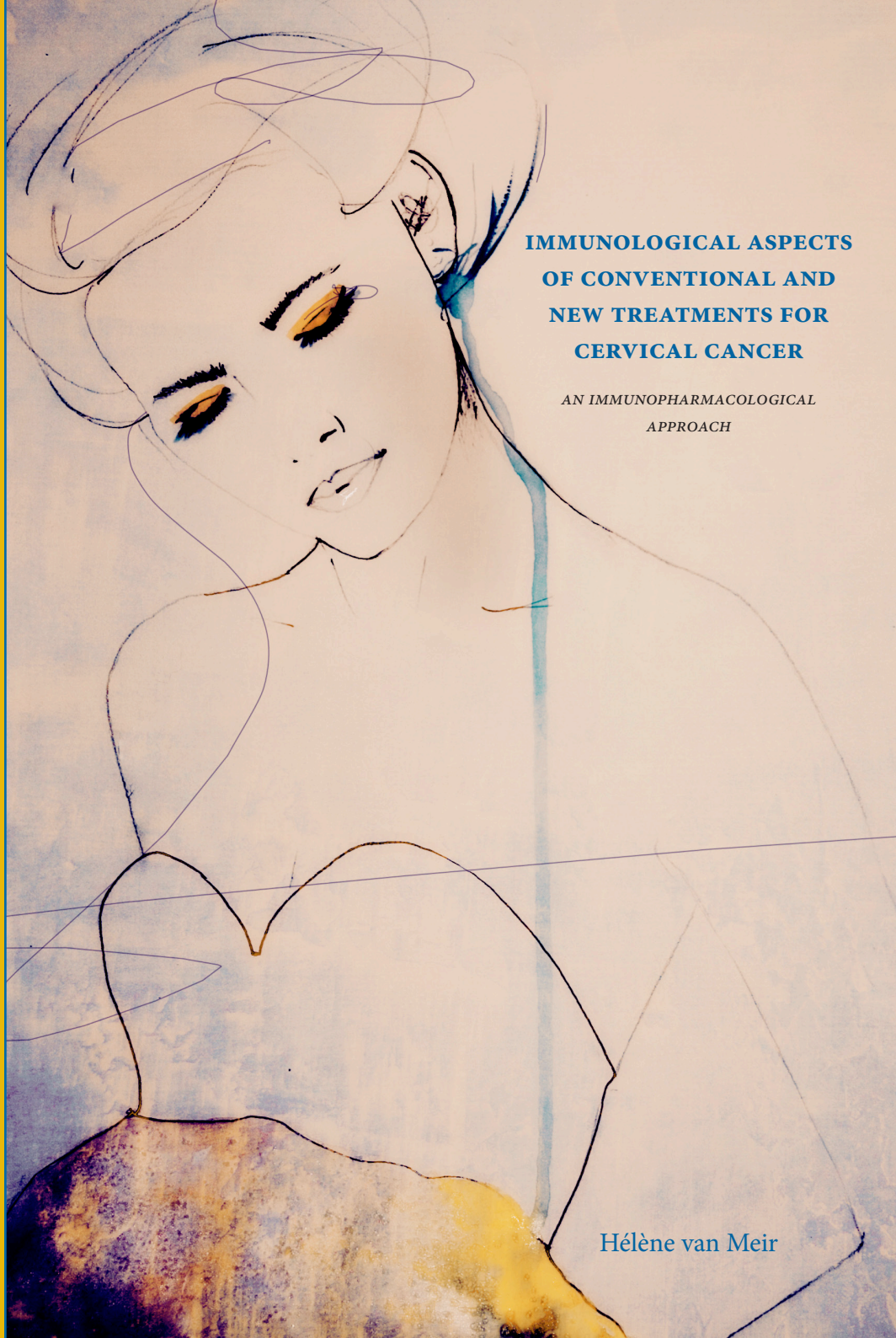

IMMUNOLOGICAL ASPECTS OF CONVENTIONAL AND NEW TREATMENTS FOR CERVICAL CANCER

AN IMMUNOPHARMACOLOGICAL APPROACH

Hélène van Meir

IMMUNOLOGICAL ASPECTS OF CONVENTIONAL AND NEW TREATMENTS
FOR CERVICAL CANCER, AN IMMUNOPHARMACOLOGICAL APPROACH

TO MY PARENTS

TO JORT

IMMUNOLOGICAL ASPECTS OF CONVENTIONAL AND NEW TREATMENTS FOR CERVICAL CANCER, AN IMMUNOPHARMACOLOGICAL APPROACH

PROEFSCHRIFT

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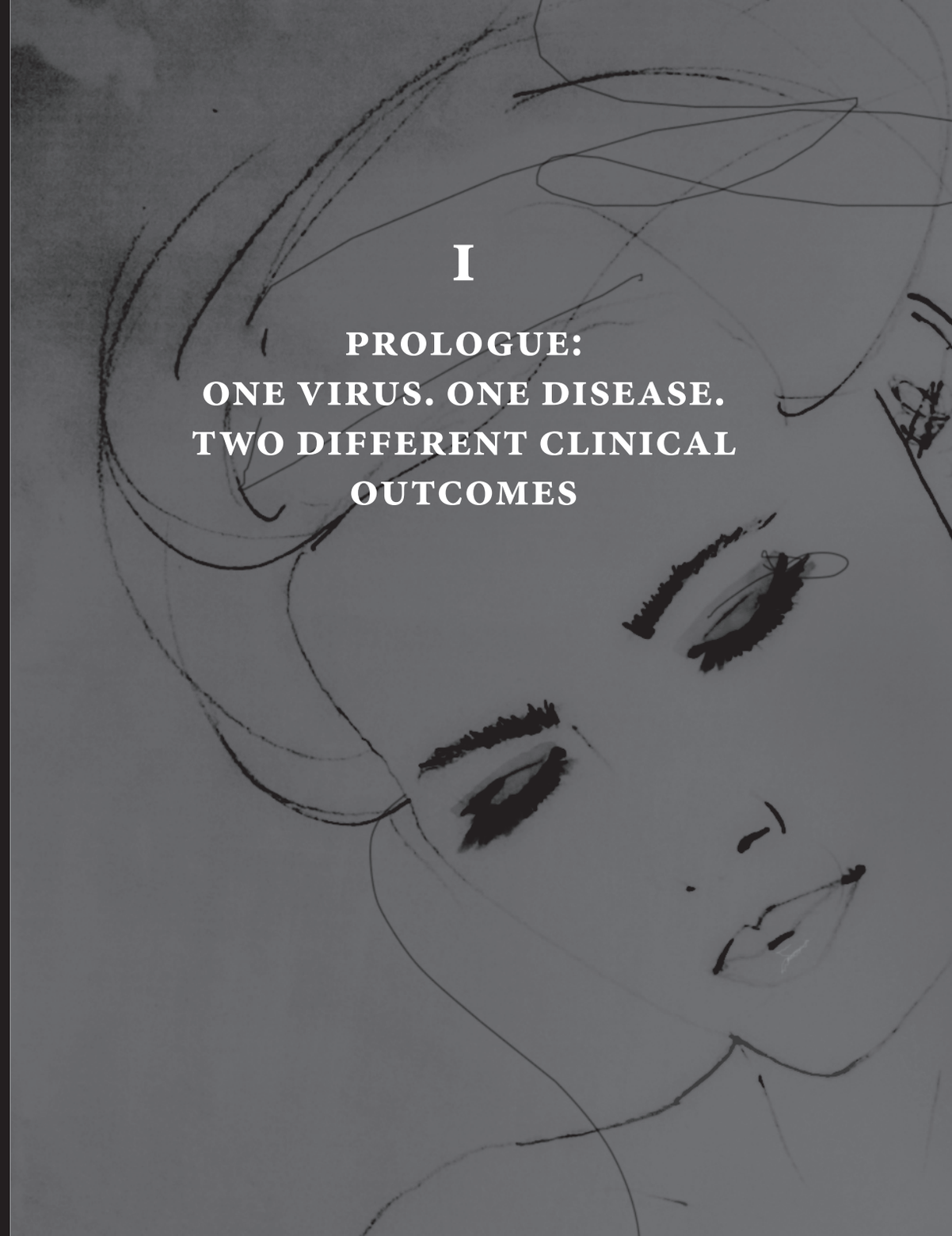
GENERAL INTRODUCTION



I

PROLOGUE: ONE VIRUS. ONE DISEASE. TWO DIFFERENT CLINICAL OUTCOMES

A 43 year old Caucasian woman was referred to the Leiden University Medical Center (LUMC) with FIGO (International Federation of Gynecology and Obstetrics) stage Ib1 cervical cancer. For more than 10 years, she had nonspecific complaints of abdominal pain, for which no medical cause was found. The cervix bled easily at routine gynecological examination. A Papanicolaou (Pap) smear revealed a Pap 5 and biopsies showed a moderately differentiated squamous cell carcinoma of the cervix. The patient was scheduled for a radical hysterectomy with bilateral pelvic lymph node dissection. During surgery, frozen section analysis of a suspicious and enlarged lymph node along the left external iliac artery was found to be tumor positive. Histopathological examination of the surgical specimen showed a non-keratinizing squamous cell carcinoma with a maximal diameter of 27 mm and a maximal infiltration depth of 11 mm. There was extensive lympho-vascular space involvement (LVSI) and the surgical margins at the vaginal cuff were tumor positive. Of the 14 lymph nodes that were removed, 2 turned out to be tumor positive. Human Papilloma Virus (HPV) analysis demonstrated the presence of the high-risk HPV type 16 in the tumor. Because of lymph node metastases and tumor positive surgical margins, it was decided to treat the patient with adjuvant chemo-radiation. She received 46 gray (Gy) external beam radiation therapy (EBRT) with concurrent cisplatin chemotherapy at a dose of 40 mg/m^2 and brachytherapy (BT) boost.

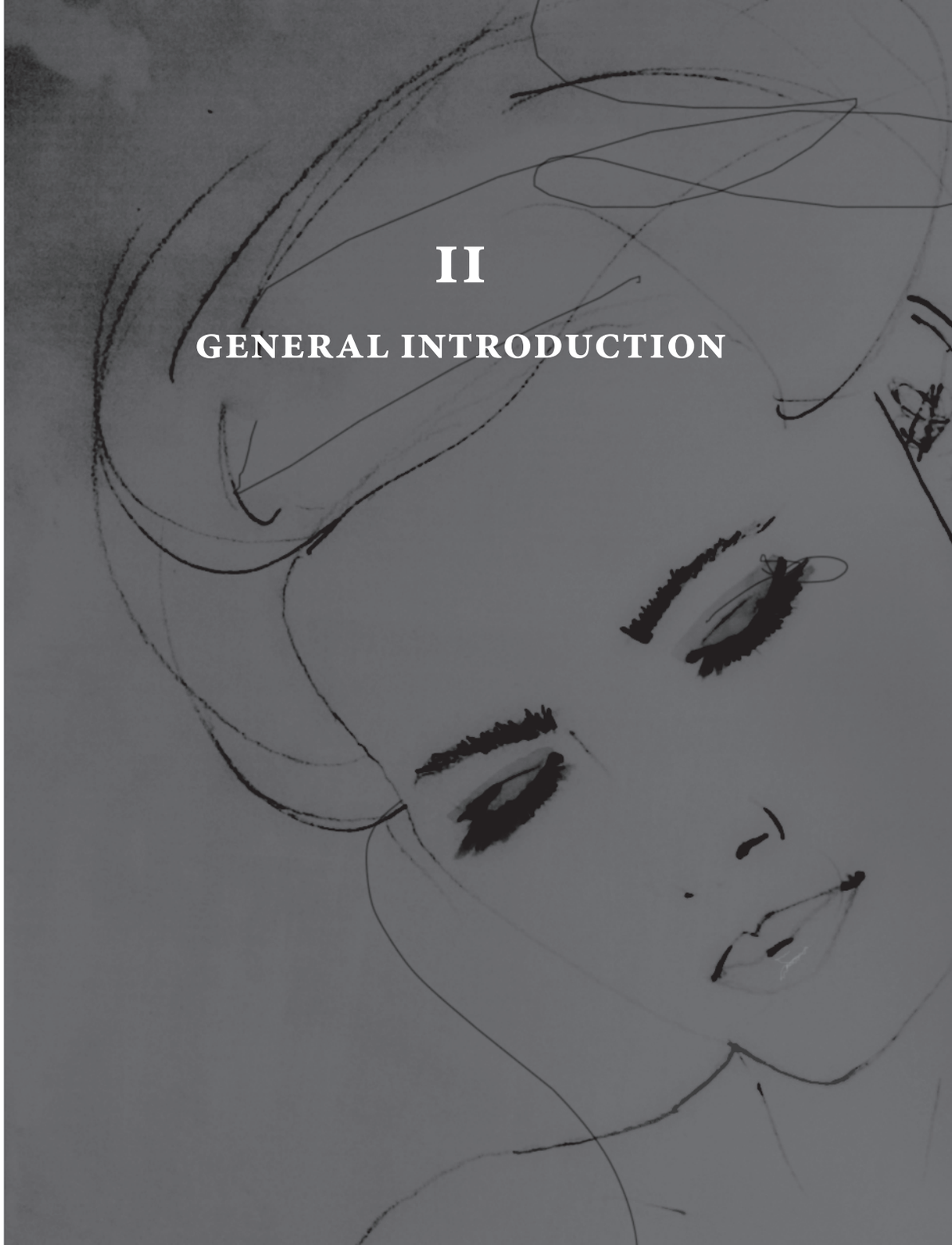
Two years and 4 months after primary treatment, she suffered from severe abdominal pain and dyspnea. Computer tomography (CT) showed extensive metastatic disease with metastases in the liver and bone, enlarged retroperitoneal and mesenteric lymph nodes, carcinomatous pericarditis and pleuritis, and unilateral hydronephrosis. Histopathological examination of pericardial fluid confirmed metastatic cervical cancer. The patient participated in a phase I/II trial and was treated with palliative chemotherapy (6 cycles of carboplatin and paclitaxel) in combination with HPV16 Synthetic Long Peptide (SLP) vaccination which was administered 2 weeks after the 2nd cycle of chemotherapy. The CT scan performed after the 3rd cycle of chemotherapy showed regression of the tumor according to the Response Evaluation Criteria in Solid Tumors (RECIST), and stable disease until 3 months after the last cycle of chemotherapy. However, 5 months after completion of the chemotherapeutic treatment, she developed progressive disease with malignant lymphadenopathy, new liver metastases and extensive pleural and pericardial fluid. Despite experimental treatment with dendritic cell therapy abroad, and local pericardial bleomycine injection, she died at the age of 46, due to progressive cervical cancer.

Around the same time, a 33 year old Caucasian woman was presented at the outpatient clinic of the department of Gynecology with complaints of irregular vaginal blood loss. Standard cervical cancer screening with Pap smears had not been performed previously. At clinical examination, she was found to have a cervical mass, which was biopsied and diagnosed as a squamous cell carcinoma of the cervix, clinically FIGO stage Ib1. Because of her wish to preserve fertility, the patient was scheduled for an abdominal radical trachelectomy with pelvic lymphadenectomy. During surgery, trachelectomy specimens were submitted for frozen section. Unfortunately, the surgical margin was grossly tumor positive and a conversion to radical hysterectomy was performed. Histopathological characteristics revealed a keratinizing squamous cell carcinoma with a maximal tumor diameter of 30 mm, a circumferential growing pattern and a tumor infiltration depth of 17 mm, that reached the serosa. There was no parametrial involvement, no LVSI, and 27 lymph nodes were removed none of which was tumor positive. HPV analysis demonstrated the presence of the high-risk HPV type 16 in the tumor. Because of the transmural growth to the serosal surface at the region of the endocervix and lower uterine segment, the patients was treated with EBRT to the paracervical and parametrial region.

Six months later, magnetic resonance imaging was performed because of complaints of pain in the back and the thigh. This examination revealed a 20 x 17 mm lesion, suspect for metastasis of the cervical tumor, located at the right obturator foramen and with signs of involvement of the sigmoid colon. There were no signs of other distant metastases. Clinical examination showed no signs of local recurrence, and no enlarged lymph nodes. After multidisciplinary consultation, she was surgically treated with complete removal of the tumor and a resection of the adjacent sigmoid colon. Histopathological examination confirmed localization of the squamous cell carcinoma of the cervix, with growth in the connective tissue into the circumferential margin; there was no growth into the colon. Because of the microscopically involved surgical margin, the patient was treated with concurrent chemoradiation to the area of the recurrence and common iliac lymph node regions. Concurrent chemotherapy with 5 cycles of cisplatin at a dose of 40 mg/m^2 was administered. Despite the postoperative and systemic morbidities, the intensive treatments were well tolerated and complete response was achieved. Follow-up was alternately performed by the radiation oncologist and a gynecologic oncologist every 3 months. Until 18 months after first recurrence, consultations showed no signs of recurrent disease.

These cases present 2 patients suffering from the same malignancy, but with a different course of disease and outcome. Differences in clinical presentation, treatment modalities, tumor responses and clinical outcome motivate clinicians and researchers to combine their knowledge from different (bio)medical

specialties as gynecology, oncology, radiotherapy, pathology, immunology and pharmacology. Apparently, a multitude of mechanisms is responsible for either the tumor susceptibility or the escape from aggressive treatment modalities. The cases show that not a single cervical cancer patient is the same, and clinical response depends on more than only the kind of tumor. The burning *'how come'* and *'why'* questions within these cases formed the basis of this thesis.



II

GENERAL INTRODUCTION

The cases presented in the prologue illustrate the unpredictability of the course of disease in patients with cervical cancer, the difficult treatment choices physicians are confronted with especially in case of recurrence, and the diversity in clinical responses that exist to different treatment strategies. Patient histories like these served as an inspiration for several research questions that have been addressed in this thesis.

Cervical cancer is the most common HPV associated cancer among women with a prevalence of 7 cases per 100.000 women in the Netherlands. In the last decades, the national cervical screening program for (pre)malignant cervical lesions by Pap smear and treatment of premalignant lesions, was expected to have a favorable effect on the incidence rates. Evaluation by the International Agency for Research on Cancer (IARC) in 2005 concluded that there was sufficient evidence that screening for cervical cancer precursors by conventional cytology within screening programs reduced the incidence of invasive cervical cancer by at least 80% among those screened.[1] Nevertheless, in the Dutch screening program the detection of cervical intraepithelial neoplasia (CIN), the precursor of cervical cancer, has increased rapidly within the last 10 years.[2] As a consequence, the number of treatments for CIN lesions has increased and trends in cervical cancer detection rate were not (yet) observed. Recent analysis of the trend of increased CIN detection in the Netherlands showed no relevant influence of demographic factors such as age, screening region, and social economic state.[3] In contrast, implementation of imaging-assisted reading and changes in sexual behavior, smoking and long-term oral contraceptive use (all associated with increased HPV incidence and thus factors that might increase cervical cancer risk[4]) appear to be plausible factors that may explain the increase in CIN detection by cytology within the screening program.[3] The current high quality screening programs are useful for early detection of CIN, but still approximately 700 women are diagnosed with cervical cancer annually in the Netherlands. In addition, data from large meta-analyses indicate that HPV DNA tests have a higher sensitivity than cytology to detect clinically relevant CIN and cervical cancer[5], and several studies showed that HPV screening is preferred as primary test at age 35 and over.[6,7]

The recent introduction of prophylactic HPV vaccines for young adolescents, will hopefully decrease the incidence of high risk HPV infections and the related premalignant lesions.[8,9] As a premalignant lesion is a necessary precursor for a carcinoma of the cervix[10], this decrease should translate into a decreased incidence of cervical cancer in the next decade. However, the prophylactic HPV vaccination program was only introduced in 2009 in the Netherlands and was accepted by only 61% of adolescent girls in 2015.[11] As a result, infections with high risk HPV will still remain highly prevalent, and women having a compromised immune response to the infection with HPV are still at risk to develop premalignant cervical lesions and progressions to invasive carcinoma.[12-15] In addition, the 10 to 15 years latency period between HPV exposure and cervical cancer development[15,16], makes it likely that a significant decrease in cervical cancer will occur many years after implementation of the vaccination programs. As an infection with HPV is highly prevalent in young individuals, with a peak prevalence up to 28% observed in women in their early 20's in Europe[17], cervical cancer can especially occur in young women.

The cases presented in the prologue merely point out that not two patients present alike, and the course of disease and clinical response to primary, additional or recurrence treatment can vary enormously. The aim of this thesis was to increase the knowledge on the role of the immune system in cervical cancer patients treated with chemotherapy and/or radiotherapy, and to accurately explore combinations of therapies within this patient group. This thesis will therefore focus on the selection of patients at risk for recurrent disease, on HPV-based immunotherapy and its potential as an (adjuvant) treatment option in addition to the current standard therapies with chemotherapy and/or radiotherapy.

Standard treatment for cervical cancer

The FIGO staging system is being used for prognosis and planning of primary management of cervical cancer. This staging system specific for gynecological cancers, is based on clinical examination and is outlined in table 1 and figure 1.[18] The FIGO system ranges from early stage disease (stages IA1-IB2) to locally advanced disease (stages IIA1-IIIB) and advanced disease (stage IV). Early stage cervical cancer has a low rate of recurrence (15%) after adequate treatment, while (locally) advanced disease has a high risk of recurrence up to 70%.[19]

Early stage cervical cancer is commonly treated with radical hysterectomy and pelvic lymph node dissection, and has excellent 5-year overall survival (OS) rates, ranging from 85% to 95% in case of tumor negative lymph nodes.[20] Surgical treatment aims to remove all malignant cells, and allows adjuvant therapy to be modified according to histopathological tumor characteristics and the patient's needs. Adjuvant treatment for surgically treated early-stage cervical cancer patients mostly consists of radiotherapy with or without concurrent chemotherapy, depending on the presence or absence of unfavorable tumor characteristics. During the last decade, adjuvant radiotherapy with concurrent

chemotherapy has been introduced, and the addition of cisplatin significantly improved progression-free survival and overall survival among women with high risk early-stage disease after radical hysterectomy and pelvic lymphadenectomy. In case of locally advanced cervical disease, primary management mainly consists of surgery, chemotherapy, radiotherapy and combinations of these treatments.[21,22] Primary radiotherapy with concurrent chemotherapy consisting of cisplatin is generally recommended as treatment, as this increases overall survival and progression-free survival with respectively 10 and 13%.[21]

Patients with advanced, metastatic, or recurrent cervical cancer that is no longer amendable to surgical resection or radiation therapy have a poor survival and less than 20% of these patients survive more than 1 year. These patients are mainly treated by systemic chemotherapy, consisting of platinum-based agents.[19,23,24] A number of single drug and combination regimens have been studied with limited gains in overall survival. Platinum-based chemotherapeutics act by binding to DNA, which results in the activation of different signal-transduction pathways, including those for DNA-damage recognition and repair, cell-cycle arrest and apoptosis. In the last decade, taxanes such as paclitaxel or topotecan have been added to the regimen as the combination of taxanes and platinum-based agents resulted in sporadically improved response rates and short increase in overall survival in patients with advanced cervical cancer.[25,26] Taxanes are mitotic inhibitors which disrupt microtubule polymerization, preventing cancer cells from entering mitosis, and stimulate apoptosis of cancer cells. Unfortunately, many cervical cancer tumors are chemotherapy resistant, the majority of the patients do not respond to chemotherapy and responses are usually limited and brief. With a response rate between 20% and 35% and a median survival of only 8-12 months from recurrence, chemotherapy regimens are seldom curative in these patients and should therefore be considered as a palliative treatment.[23-26] In addition, the adverse effects limit the use of multiple cytotoxic agents.

As the current treatment strategies for recurrent cervical cancer often lack efficacy, novel treatments are necessary and selection of potentially eligible patients that have more benefit seems to be crucial. Many studies have already been conducted to identify other active agents as monoclonal antibodies or immunotherapies.[27-29] Regrettably, only sporadically higher response rates and relatively short increases in overall survival have been reported, which was sometimes even correlated to a high morbidity. So far, new biologicals did not show clinical benefit in end-stage cervical cancer patients with a low performance state, a large tumor burden and/or immunosuppressive conditions.[29,30] Novel combination therapies are being explored for their effectiveness against recurrent cervical cancer, and combined chemo- or radio-immunotherapy might be a potential option. Since recent articles suggest that chemotherapy and radiotherapy partly act through the immune system, these therapies might ultimately be combined with immunotherapy. To understand the rational for the use of immunotherapy in cervical cancer and to understand the mechanisms employed by combined chemo-immunotherapy or radio-immunotherapy, some background on tumor immunology will be provided first.

Immunity

As a defense against foreign pathogens, mammals have evolved a sophisticated system that enables them to distinguish between self and non-self components. The immune system is commonly divided into two components: the innate immune system and the adaptive immune system. The latter is further sub-divided in humoral immunity and cell-mediated immunity (Figure 2).[31] The innate and adaptive immune systems are closely intertwined, through several immune cells and cytokines that are involved in both the innate and adaptive immune response.

The *innate immune response* provides initial and rapid defense against pathogens by epithelial barriers, local inflammation and cytokines, complement system and phagocytic cells (neutrophils, monocytes and macrophages), dendritic cells (DC) and natural killer (NK) cells. These innate immune cells are first-line effectors to damaged cells and cancer cells.[32]

NK-cells recognize tumor cells expressing non major histocompatibility complex (MHC) surface molecules and are responsible for killing these cancer cells directly by releasing the cytotoxic proteins perforin and granzyme that enter the cytoplasm and induce apoptosis.[33] Two functional types of receptors are expressed on the NK-cell surface: stimulatory receptors and inhibitory receptors. Natural killer group 2D (NKG2D) molecule is the best known stimulatory receptor.[34] Binding of stress-related ligands on tumor cells with NKG2D stimulates NK-cells and result in secretion of interferon (IFN)-gamma and perforin, release of inflammatory cytokines and the induction of apoptosis in cancer cells. The inhibitory receptors consist of the killer immunoglobulin-like receptors (KIRs), which recognizes MHC class I molecules on normal cells, and distinguishes healthy host cells from tumor cells.

Macrophages are long-living innate cells which are present in most tissues and display plasticity in their differentiation. These cells can undergo specific differentiation depending on different stimulatory signals within the local

milieu. Macrophages can phenotypically and functionally be categorized into M1-like, pro-inflammatory, tumor-suppressive macrophages (M1) and M2-like anti-inflammatory tumor-promoting (M2) macrophages.[35] M1 macrophages develop in response to bacterial products, acute inflammation and IFNγ, and recognize tumor cells expressing the so-called 'eat-me' molecules at the cell surface. These signals include lipid phosphatidylserine (PS), oxidized PS, oxidized low-density lipoprotein and the multifunctional protein calreticulin[36], which are translocated or redistributed to the tumor cell surface during apoptosis.[37] The interaction between apoptotic tumor cells and these macrophages leads to immune tolerance in a tumor environment. M1 macrophages are also capable of extracellular killing by quick release of cytokines, chemokines and inflammatory mediators, contributing to a local inflammatory response by attracting more immune cells as neutrophils and monocytes. The opposite phenotype, M2 macrophage, plays a role in chronic inflammation. This phenotype has a poor antigen-presenting capacity and dampen effective immune responses by modulation of T-cell responses. In addition, these macrophages produce immunosuppressive cytokines and chemokines that result in alteration of the phenotype and function of local DCs and polarize T-cells to a Th2 phenotype which hampers an effective anti-tumor immune response.[38,39] From these characteristics follows that M2 macrophages render the tumor milieu into an immune suppressive environment benefiting tumor growth.[40]

Myeloid Derived Suppressor Cells (MDSC) are immature myeloid cells that hinder an anti-tumor immune response.[40,41] These myeloid cells are present in tumor micro-environment, as tumors attract myeloid cells and interfere with their differentiation, inducing the suppression of infiltrated effector T-cells. In cancer, the presence of MDSCs and M2 macrophages resemble a state of differentiation and activation of myeloid cells. DCs are highly specialized in antigen presentation to T-cells, and bridge between the innate and the adaptive immune system. After uptake of antigens, DCs travel toward lymph nodes where they can present the antigens and instruct T-cells. The induction of adaptive immune response requires danger signals or maturation of DCs during encountering antigen. Maturation of DCs results in the increased expression of MHC class I and II on the cell surface, in which small antigens are presented as peptides, the production of interleukin-12 (IL-12) and the production of co-stimulatory molecules such as CD86, CD80, CD83 and CD70. MHC class I is expressed by all nucleated cells, but particularly virally infected or tumorous transformed cells. MHC class II molecules are almost exclusively expressed by immune cells, in particular by APCs.

The *adaptive immune response* is highly specific for a particular pathogen, and is characterized by a slowly developing response. However, due to the development

of memory the magnitude of the response increases with each exposure to the same pathogen. The adaptive immune response is mediated by T-lymphocytes and B-lymphocytes. In contrast with the innate immune system, the adaptive immune system has a cellular and a humoral component. The humoral response occurs after uptake of antigen by the B-cell receptor, followed by the production of antigen-specific immunoglobulin (Ig) antibodies.[32] Antibodies neutralize the toxic activity of pathogens by binding to the surface and subsequently prevent interaction with human cells. In addition, inactivation of extracellular pathogens occurs by coating the cell surface with IgG antibodies, which promotes ingestion and destruction by phagocytes. In cancer, tumor-infiltrating B-cells (TIL-Bs) play a key role in the B-cell response. There is increasing evidence that the presence of TIL-Bs is associated with favorable clinical outcomes in cancer. In addition to direct effects through Abs or cytotoxic pathways, B-cells can potentiate the anti-tumor response by producing chemokines and cytokines, by serving as a local APC, and by organizing lymphoid structures in the tumor that sustains the immune response.[42] Whereas B-cells recognize whole molecules and intact pathogens, T-cells possess T-cell receptors (TCR) that recognize small peptide antigens presented by MHC class I or II on the cell surface. Naïve T-cells need to recognize the antigen and receive a co-stimulatory signal to become activated, differentiate and proliferate into effector cells. A large family of co-stimulatory molecules, among which CD80 and CD86 are the best-characterized, provide co-stimulatory signals which are involved in activating and regulating the development antigen-specific T-cells.[43] There are two major T lymphocyte populations, $CD8^+$ and $CD4^+$ T-cells, which recognize distinct fragments of antigens and display distinct effector functions. $CD8^+$ cytotoxic T-cells (CTLs) recognize small peptide antigens that are presented in MHC class I molecules on the cells. After recognition of the abnormally expressed antigen, $CD8^+$ T-cells differentiate into cells that acquire cytolytic capacity, ending with a highly specific mature CTL that can kill the affected cell. $CD4^+$ T-cells recognize antigens presented in MHC class II molecules. In addition to MHC class II expression by immune cells, in particular by APCs, MHC class II expression occurs in activated $CD4^+$ T-cells and $CD8^+$ T-cells, and can be up-regulated in epithelial cells in inflamed tissue or in tumor cells. $CD4^+$ T-cell activation is critical for an optimal $CD8^+$ T-cell-mediated immune response[44], either through the classical helper role of $CD4^+$ T-cells that provide cytokine-support by (IL-2 and IFNγ release) for $CD8^+$ T-cells, or by the activation of CD40 expression on APCs which stimulate $CD8^+$ T-cells.[45,46] $CD4^+$ T-cells can be polarized into multiple different effector T-cell subsets, based on their function and cytokine profile, including type 1 Th (Th1) helper cells, type 2 Th (Th2) helper cells,

type 17 (Th17) helper cells or regulatory T-cells. Th1 cells are characterized by the production of pro-inflammatory cytokines such as IL-2, IFNγ and TNF-β and evoke cell-mediated immunity by the induction of CTLs and phagocyte-dependent inflammation. Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 and evoke strong Ig antibody responses by B-cell stimulation and thus humoral immunity. The Th17 subpopulation of $CD4^+$ cells produce IL-17 and mediate expression of the transcription factors retinoic acid receptor-relate orphan receptor-γt (RORγt) and signal transducer and activator of transcription 3 (STAT3). These Th17 cells mainly play an important role in the induction of auto-immunity, but recent evidence suggests that this effector T-cell subset is also involved in tumor immunology by preparing the tumor environment (by cytokine secretion) and facilitating tumor-infiltrating $CD8^+$ T-cells and NK cells.[47] A specialized subtype of $CD4^+$ T-cells distinguished from the other subpopulations by their role in immune-tolerance, is the regulatory T-cell (Treg) subset. Naturally occurring Tregs are directly derived from the thymus and these highly express CD25 and transcription factor Foxp3. Adaptive Tregs are induced at the periphery and may or may not express Foxp3. Tregs suppress $CD8^+$ CTLs and Th1 mediated responses via various known and unknown mechanisms, including the secretion of immunosuppressive cytokines as IL-10 and TGF-β or the consumption of IL-2, thereby inhibiting other T-cells or APCs.

CANCER IMMUNOLOGY

The immune system plays an important role in the development, maintenance and expansion of cancer. An almost infinite number of immune cells with different subsets, receptors, cytokines, antibodies and chemokines contribute to the elimination or promotion of tumor progression.

Originally, Burnet formulated the *cancer immunosurveillance hypothesis,* postulating that the immune system is able to recognize, inactivate and eventually eliminate potentially malignant cells before they establish themselves and form a tumor mass.[48-50] Malignant cells are ascribed as the result of genetic changes that occur during cell divisions. Genetic changes may result in the expression the so-called tumor antigens on tumor cells, which make malignant cells immunologically distinguishable from normal cells. Boon and coworkers were the first to identify a tumor-associated antigen: the MAGE-1 gene that encodes for antigen expression on metastatic cutaneous melanomas.[51] The expression of tumor antigens gives the opportunity to be recognized by antigen-specific T-cells of the adaptive immune system. To achieve a tumor-specific immune responses, naïve T-cells need to recognize the tumor antigen presented in the context of human leukocyte antigen (HLA), and additionally receive a costimulatory signal in order to become activated, proliferate and differentiate into armed effector cells.[43] Thus tumor antigen presentation is critical for an effective and specific anti-tumor immunity. In the last decade, the cancer immunosurveillance hypothesis has evolved into the more sophisticated *cancer immune-editing concept,* which entails a three step interaction process (including positive and negative effects) between tumor cells and immune cells.[52] These three processes include the elimination, equilibrium and escape phase, representing the fact that the immune system can not only protect the host against tumor development (immunosurveillance – elimination phase), but can also modulate the immunogenic phenotype of malignant cells (equilibrium phase) and thereby facilitating complete tumor escape from immune attack (escape phase) and uncontrolled tumor growth.[52,53] Figure 2 demonstrates the antitumor response and mechanisms used by tumor cells to prevent the activation of specific T-cells. Another major achievement on the field of cancer immunology was the demonstration of the importance of the patients' tumor immune response for their survival. Numerous studies have shown that the nature, quantity, location and functionality of tumor-infiltration T-cells at diagnosis are strongly associated with patient survival in a wide variety of human cancers.[54-58] In addition, there is increasing evidence that the presence of TIL-Bs is associated with favorable clinical outcomes in cancer.[42] The prognostic value of adaptive immune cell infiltration and tumor micro-environment was demonstrated in colorectal cancer, and expressed as an integrated *immunoscore,* which was based on the type, density and location of immune cells.[59,60] This immunoscore represents a standardized, simple, and powerful stratification system, and could ultimately add value to the current prognostic parameters and make the course of disease and response to different therapies less unpredictable. Understanding the importance of cancer immunology for both prevention and promotion of tumor growth is evolving as shown by the updated hallmarks of cancer, described by Hanahan and Weinberg in 2011[61], who added two immunological hallmarks: avoidance of immune destruction and tumor promoting inflammation. The current knowledge about multiple mechanisms of tumor escape and immunological features in case of HPV induced cervical cancer, is discussed below.

IMMUNE RESPONSE TO HPV AND CERVICAL CANCER

In gynecological cancers, the causal role of HPV infections in the development of cervical (pre)malignancies has been unambiguously recognized.[62] Genital infections with high-risk HPV, in particular HPV type 16 (HPV16), is highly

prevalent in young individuals (with a lifetime incidence of 80%[10,12,63]), and the virus is mainly acquired through sexual activity.[64] Despite the ability of HPV to evade the host immune system and down regulate innate immunity, the majority of immune competent individuals infected with the virus are able to control and eventually eliminate the viral infection. In most women, an HPV infection is asymptomatic, transient and cleared within 2 years. Persistent infections with HPV occur in less than 10% of the infected women, but substantially increase the risk of development of (pre)malignant cervical lesions.[8,62]

HPV is a non-lytic, circular double stranded DNA which encodes for six early non-structural or regulatory genes (E1, E2 and E4-E7) and two late structural proteins (L1 and L2).[65] These proteins exert specific functions during the different stages of HPV replication, and contribute to the development and progression of HPV associated lesions. The replication of HPV takes place in the supra-basal layer, where early genes E1, E2 and E5 are expressed. The oncoproteins E6 and E7 are consistently expressed in the basal cells of the epithelium layer and play an essential role in the viral lifecycle by modifying the cellular environment and allow viral genome amplification, mainly by driving S-phase re-entry in the upper epithelial layers.[66,67] Late structural proteins L1 and L2 encode for viral capsid proteins, with functions in viral transcription, replication and genome partitioning.[68] In case of persistent infection with high risk HPV, integration of the HPV DNA into the host cell genome might occur and is accompanied with overexpression of E6 and E7 oncoproteins. Persistent high level expression of E6 and E7 accumulates genetic errors in the host genome, causing dysplastic cells which can progress to high grade intra-epithelial lesions or micro invasive carcinoma.[69]

The important role of the adaptive immune system is supported by the fact that immune deficient humans often develop tumors, in particular virally-induced tumors. More specifically, immune suppressed individuals are known to be at high risk for persistent HPV infections, HPV-associated malignancies and progression of disease.[70,71]

INNATE IMMUNITY TO HPV AND ESCAPE MECHANISMS The undifferentiated keratinocytes at the *stratum basale* of the epithelium are the primary target for HPV. Keratinocytes express pathogen recognition receptors (PRRs), including the Toll-like receptors (TLRs), nucleotide oligomerization domain-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), which recognize pathogen-associated molecular patterns (PAMPs) on microbes and viruses.[72] TLR1-3, TLR5, TLR6, TLR10, RIG-I, protein kinase R (PKR) and MDA5 are expressed irrespective of the differentiation state of keratinocytes, while the

expression of TLR9, the PPR that can recognize viral DNA of HPV, is only induced after terminal differentiation.[73] HPV infects undifferentiated keratinocytes of the basal layer, and replicates inside the cell during differentiation of these cells.[69] Genome-wide expression profiling of HPV infected keratinocytes versus non-infected keratinocytes have shown that the presence of HPV suppresses the downstream signaling of the PRRs in infected cells. The suppressed downstream signaling of several PRRs that might recognize parts of HPV is provoked by the upregulation of ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), a cellular protein which dampens the production of interferon's, cytokines and chemokines. In addition, HPV infection down regulates a network of genes encoding for the production and secretion of anti-virals (such as type I interferon) and chemotactic and pro-inflammatory cytokines, including IL-1β which is crucial for the attraction and activation of adaptive immunity.[73,74] HPV also attenuates the effector cytokine reaction of infected cells to the exposure to IFN-γ and/or TNF-α, allowing transient escape from immune response.[75] These mechanisms may also play a role in cancer development. Further, HPV has the ability to manipulate Langerhans cells (LCs) residing in infected epithelia, and turn them into inappropriately activated APCs. The functional and phenotypic maturation of LCs, as well as the decrease in number of LCs occurs in the HPV-infected epidermis and disturbs antigen-presentation to T-cells.[76-79] The accumulation of tolerogenic APCs in the microenvironment can be the result of HPV affecting the extent of the CD40 signaling in the infected cells, and consequently the production of cytokines and pro-inflammatory signals.[80,81] Apparently, HPV is able to regulate the activation and migration of APC, resulting in a failure to augment immune cell migration toward the HPV infected epithelial cells.

Taken together, these mechanisms show that HPV can efficiently hamper the innate immune system soon after infecting the keratinocytes at the basal layer. Furthermore, HPV disturbs the production of cytokines and suppresses the antigen presenting pathway, delaying the activation of the adaptive immune system.

ADAPTIVE IMMUNITY TO HPV AND ESCAPE MECHANISMS Memory B-cells may release HPV capsid type specific antibodies that can opsonize the virus and protect against subsequent infection with the same HPV type. After natural infection with HPV the serum-neutralizing antibody levels are low or weak as the infection is located intraepithelially and barely systemically. Seroconversion is generally detected within 18 months after infection, but the level of Ig antibodies directed against the viral HPV capsids L1 and L2 is low and even undetectable in 30-50% of the patients.[82,83] Control of HPV is achieved by activation of the HPV-specific, interferon-γ (IFNγ)-producing $CD4^+$ and $CD8^+$ type 1 T-cell responses to

the viral proteins E2, E6 and E7. These responses have been extensively studied, and were detected in the peripheral blood mononuclear cells (PBMCs) of healthy, HPV-negative but exposed subjects (e.g. after clearance), and in women with regression of their HPV-associated cervical lesions. In the majority of these individuals circulating proliferating, IFNγ and IL-5 producing T-cells against E2, E6 and E7 were found.[84,85] Results from a cross-sectional cohort study showed that the infiltration of low-grade squamous intraepithelial lesions by $CD8^+$ cytotoxic cells is related with regression of the lesions, whereas the number of CTLs is substantially lower in patients with progressive or persistent low grade cervical lesions.[86] In patients with progressive HPV-induced disease this type of immunity is weak and often a systemic HPV-specific response against E6 and E7 is not detectable in the blood, or consists of Th2 cells, non-polarized T-cells or Tregs.[87-91] At the site of progressive high grade squamous intraepithelial and (micro) invasive lesions, the number of infiltrating $CD4^+$ and $CD8^+$ T-cells is reduced as well, and T-cells lose their ability to produce IFNγ.[86,92]

These data demonstrate that in patients with progressive HPV-associated cervical disease, proper activation of the HPV-specific T-cells fails or is not sustained resulting in chronic infections and undisturbed progression to high grade squamous lesions or cervical cancer. In addition to impaired APC function and suppressed PPR signaling in keratinocytes, mechanisms playing a role in this escape from the immune system include resistance to apoptosis, HLA loss, co-inhibitory expression, local immune suppression by myeloid cells and Tregs, and T-cell exhaustion. The down-regulation of HLA class I and class II molecules on HPV transformed cells makes the infected cells less visible to the adaptive immune system and evades host immunity. This was demonstrated in patients with cervical dysplasia where allelic loss of HLA-B44 expression showed progression of the lesions, while no down-regulation was seen in non-progressive lesions.[93] These data are consistent with the loss of HLA class I and HLA-A expression in cervical carcinomas.[94,95] Non-classical HLA types HLA-G, HLA-E and MHC class I chain related molecule A (MICA) are addressed to induce the pertinacity of HPV infections and lesions, as the expression of HLA-G and HLA-E is associated with progression of cervical intraepithelial neoplasia's to invasive squamous cell carcinoma[96,97] and low expression of MICA is associated with impaired survival in patients with cervical tumors.[95] The non-classical HLA types HLA-G and HLA-E are found to inhibit the function of NK cells and CTLs by their interaction with different inhibitory receptors (e.g. CD94/NKG2A)[96], while MICA that interacts with the stimulating receptor NKG2D is downregulated in cervical tumors.[95]

Th cells and CTLs may be rendered dysfunctional through tumor expressed molecules. The expression of such inhibitory molecules may result in suppression of the effector function of T-cells and may counteract migration of these cells to the infected lesions. This was demonstrated in different studies which showed that activated T-cells express inhibitory molecules such as Cytotoxic T-lymphocyte Antigen 4 (CTLA-4), program death 1 (PD-1) and T-cell immunoglobulin mucin-3 (TIM-3). Upon interaction with their ligands (CTLA-4 ligand, PD ligand 1 and/or 2 and Galactin-9), induction of apoptosis of Th1 cells and inhibition of functional CTLs and Th1 cells occur.[98-100] In addition, tumor associated (M2) macrophages and Tregs are attracted to the tumor site, where they form an immunosuppressive environment.[38,39,101] In high grade lesions or tumors, the proliferation and function of effector T-cells are thus suppressed by Tregs, and it was shown that the ratio of tumor-infiltrating $CD4^+/CD8^+$ T-cells and the presence of Tregs in tumors is strongly associated with the prognosis and survival of patients with cervical cancer.[95,101,102] One can imagine that in case of advanced or recurrent cervical cancer patients often suffer from a large tumor burden, which is associated with local immune suppression that can hamper T-cells to exert their full effector function. This was demonstrated by Piersma *et al* who showed that cervical tumor tissue was strongly infiltrated by Tregs compared to healthy cervices. In addition, the infiltration of $CD8^+$ T-cells in cervical carcinoma showed to be associated with a lack of pelvic lymph node spread and thus a favorable prognosis.[58] The quantification of the number of invading immune cells in cervical tumors revealed that a strong intraepithelial infiltration of M1 macrophages, was associated with a large influx of intraepithelial T lymphocytes, improving disease-specific survival.[103]

IMMUNOLOGIC APPROACHES FOR THE TREATMENT OF CERVICAL CANCER

Since an infection with HPV is necessary for the development of cervical cancer, vaccination to prevent HPV infection and subsequently preclude HPV related disease is of high importance. Prophylactic vaccines aim to prevent an HPV infection by antibodies or humoral immune responses. These prophylactic HPV vaccines have no therapeutic effects as they do not increase viral clearance in subjects already infected with HPV.[104]

For patients with progressive disease, multiple therapeutic immuno-therapeutic modalities have been developed, of which therapeutic vaccination, non-specific immune stimulation with cytokines and antibodies and adoptive

cell therapy (ACT) are best-known. Monoclonal antibodies or recombinant cytokines directly activate the immune system or mitigate the tumor-induced immunosuppressive conditions. The blockade of immune inhibitory pathways by targeting CTLA-4 (ipilimumab) and PD-1/PDL-1 (nivolumab), has demonstrated to be successful in pre-clinical studies and melanoma patients.[105-108] For the treatment of virus-induced malignancies and cancer, various therapeutic immunotherapies have been investigated with the goal to induce robust cell-mediated immunity.[109] Specificity is required to prevent destruction of healthy host tissue and memory is required to prevent recurrences of primary tumors. Therefore, studies on immunotherapy mostly focused on reinforcement of antigen-specific T lymphocytes.[110] Therapeutic vaccines aim at regression or control of HPV induced (pre) malignancies by specific stimulation of the host's own immune system to reject and destroy tumor cells. Based on the different mechanisms of tumor cells to elude from the immune system, the success of therapeutic immunotherapy could probably rely in the reinforcement of the tumor specific immune responses, as well as reversion of the immune suppressive state.

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.[111] A subunit vaccine comprising a recombinant HPV16 E6E7L2 fusion protein (tissue antigen-cervical intraepithelial neoplasia) showed clinical responses in patients with vulvar intraepithelial neoplasia when this vaccine was combined with the local treatment with topical immunomodulator imiquimod.[112] Another promising vaccination strategy is VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins, which showed clinical efficacy against cervical intraepithelial neoplasia 2/3 lesions.[113] Instalment of a robust HPV16-specific immunity by the use of a therapeutic HPV16 overlapping synthetic long peptide (HPV16-SLP) vaccine, developed at the Leiden University Medical Center, resulted in partial or complete regression of HPV16-induced premalignant lesions of the vulva.[114,115] Clinical response was associated the induction of a strong and broad HPV-specific $CD4^+$ and $CD8^+$ T-cell response. Notably, however, non-responsive patients had larger lesions at inclusion, mounted weaker effector T-cell responses and showed an increased infiltration of HPV-specific Tregs at the lesion. When the HPV16-SLP vaccine was administered in patients with advanced cervical cancer, it showed fair immunogenicity but no overt clinical benefit.[27,29] These minimal clinical effects may reflect strong immune suppression which is often associated with large tumor burden.

As discussed above, a multitude of mechanisms can be responsible for the impaired tumor immune responses and immune suppressive conditions in these cancer patients. These conditions may not only frustrate the effector phase of tumor-specific T-cells but may also disable the capacity of patients to mount a T-cell response to the vaccine. In patients with advanced cervical cancer, the repression of the immune suppressive conditions is obviously necessary to create an optimal circumstance to implement successful immunotherapy.

CHEMOTHERAPY AND RADIOTHERAPY: A WAY TO REINFORCE THE IMMUNE SYSTEM?

Chemotherapy is frequently used for the treatment of metastatic solid cancer. It was originally considered as a treatment whose efficacy was exclusively attributed to interferences with cellular division and mainly affects dividing cancer cells as they begin to proliferate. The therapeutic goal of radiotherapy was explained by its radiobiology; maximizing the anticancer effects while minimizing the toxic effect on the surrounding healthy tissue.[116] Although the main goal of chemotherapy and radiotherapy is obviously to kill tumor cells, these treatments are also reported to require the immune system for optimal efficacy. Murine tumor models have shown that chemotherapy is more effective when administered to immunocompentent mice compared to immunocompromised animals.[117-121] The important role of the immune system in response to chemotherapy in humans was also suggested by Ray-Coquard *et al*, who reported that cancer patients suffering from lymphopenia before the start of chemotherapeutic treatment, are less likely to respond.[122] Indeed, many of the available cytotoxic anticancer drugs have shown to influence the immune system and thus contribute to tumor regression and therapeutic response.[123-125] Through different cellular and molecular interactions, chemotherapy and radiotherapy can apparently positively influence the immune system.[126,127] The immunostimulatory effects can be explained by mechanisms as dendritic cell activation by apoptotic tumor cells, direct activation and stimulation of tumor-specific immunity and depletion of immunosuppressive cells which converts the tumor milieu into a site permissive for T-cells.[123,128] These mechanisms are discussed in further detail in chapter 3 of this thesis. For the cytotoxic drugs most frequently used in cervical cancer, platinum-based chemotherapeutics and taxanes, the positive immune-related effects have been extensively studied. As an example, immune effects were shown in a mouse model, where oxaliplatin induced immunogenic cell death via calreticulin (CRT) exposure on tumor cells, thereby stimulating the induction of a tumor-specific T-cell response.

In addition, oxaliplatin and cisplatin induce the release of HMGB-1 and ATP in dying tumor cells, which activates APCs via TLR-4 stimulation. APC activation contributed to a shift in the local tumor environment and boosts tumor specific T-cell responses.[121,129] Ramakrishnan *et al* showed that cisplatin and paclitaxel enhanced sensitivity for granzyme B-mediated tumor cell death by intratumoral T-cells. Recent work from van der Sluis *et al* showed that upon vaccination combined with cisplatin in mice, the tumor environment was highly infiltrated with leukocytes, including HPV-specific cytokine-producing anti-tumor T-cells. Together with the production of TNFα by the abundant T-cells in the tumor, cisplatin enhanced tumor cell death and caused decreased tumor cell proliferation.[130] This effect was mediated via upregulation of mannose-6-phosphate receptors on the surface of tumor cells, observed in mice and human cells.[131]

In patients with advanced cervical cancer, the immune cell composition of the tumor draining lymph nodes was modified by the treatment with chemoradiation. A low (39.6 Gy) dose of radiotherapy in combination with cisplatin induced a Th1 type anti-tumor immune response and reduced the amount of potent regulatory T-cells.[132] Interestingly, the higher dose of neoadjuvant chemoradiation (50 Gy) resulted in a decrease of $CD4^+$ T-cells in the tumor-draining lymph nodes, which may be considered unfavorable for the immune potential. As $CD4^+$ T-cells are the most radio-resistant cells among human cells in vitro[133], this decrease in $CD4^+$ T-cells was explained by the direct detrimental effect of radiation on naïve T-cells, disturbing $CD4^+$ T-cell maturation.[132]

The above mentioned studies demonstrate and sometimes explain the positive influences of several chemotherapeutic agents and radiotherapy on the immune system, which makes it reasonable to assume that these treatments could attribute to a successful application of immune modulators in the treatment of advanced stages of (cervical) cancer. To improve the poor outcome in patients with advanced, metastatic or recurrent disease, the exploration of novel treatment paradigms is needed. In an area of personalized and molecular medicine, the development of immunological compounds to be used alone or in conjunction with cytotoxic chemotherapy or radiotherapy should be a priority. With the current knowledge of chemo- and radiotherapy positively affecting the immune system, the combination of these therapies with immunotherapy could be a serious option to achieve better clinical responses. Several studies in mice showed synergy between platinum treatment or radiotherapy with immunotherapy, indicating that these therapies act synergistically in tumor eradication by influencing the immune regulatory activity and making the tumors more prone for immune attack.[134,135] Recent work from our group showed that many chemotherapeutic treatments did not negatively influence immunotherapy and a number of them even synergized with immunotherapy. This research also showed that chemotherapy could be applied at lower doses, thereby reducing chemotherapy-associated toxicity.[130] Accurate exploration of immunotherapy application in addition to chemotherapy or radiotherapy is crucial, in order to optimally utilize the immunostimulatory effects of chemotherapy and/or radiotherapy, and establish an synergistic immunological and clinical effect between different therapies.

Scope of the thesis

This thesis includes the overlapping areas of immunology, pharmacology and immunotherapy. Similarly to pharmacology, immunology also deals with receptors, agonists and antagonists. Immunopharmacology focuses especially on the mechanism of action of pharmacologic compounds that regulate immune responses and the physiologic, pathologic and pharmacological role of the aspects of the immune system.

In this thesis different translational research projects are described with the aim to characterize the pharmacological effect of chemotherapy and radiotherapy on the immune system when used in patients with cervical cancer. Monitoring the immune effect provides the opportunity to determine whether combination therapies with immunotherapy could be performed in cervical cancer, and whether or not there is an optimal time-window in which different treatment strategies could be combined to treat this devastating disease.

This introduction chapter provides an overview of the involvement of the immune system in the development and expansion of cervical cancer. It summarizes the use of chemotherapy and radiotherapy in advanced, metastatic or recurrent stages of disease. The underlying mechanism of traditional therapies positively affecting the immune system, must be clarified in order to optimally apply synergistic approaches.

In *chapter 3* tumor characteristics and clinical factors in patients who developed recurrent disease after primary surgery for early-stage cervical cancer are investigated. Prognostic markers that can be used for selection of patients at high risk of recurrence, and therefore those most in need of alternative therapies, are identified. This chapter highlights the importance of the use of predictive models and adaptive study design to identify the potential eligible patients that might have more benefit from alternative treatment modalities.

The long-term clinical outcome of patients with advanced cervical cancer treated with HPV16 E6/E7 SLP vaccine in a phase I trial was evaluated with respect to the timing of immunotherapy given closely before or after chemotherapy and clinical outcome in comparison with isolated immunotherapy or isolated chemotherapy. The data were discussed in the context of the effects of chemotherapy on the immune responses as observed in pre-clinical and clinical trials with an emphasis on challenges such as optimal dosing schedule and the identification of immune-specific biomarkers as reviewed in *chapter 4*.

In the face of optimal use of immunotherapy in addition to standard chemotherapy, it is of vital importance to monitor immunological changes and responses during treatment. *Chapter 5* explores the optimal time-window to start immunotherapy with the HPV16 Synthetic Long Peptide (HPV16-SLP) vaccine, in combination with chemotherapy. The combination of chemotherapy with HPV16-SLP vaccination is first tested in a HPV16 E6/E7-expressing tumor mouse model. Subsequently, it was investigated if these animal data could be translated to the clinic by performing timed vaccination in patients with advanced, metastatic or recurrent cervical cancer patients.

In *chapter 6*, the effect of pelvic radiation therapy on the immune system in cervical cancer patients has been investigated. The study which is described in this chapter, focuses on the influence of pelvic radiation on immune responses in patients with cervical cancer during and after their treatment. Changes in immunological status and antitumoral responses in these patients were intensively monitored over time to determine whether radiotherapy could be combined with immunotherapy in the future. The results are important for the timing of combination therapies in this context.

Chapter 7 is the general discussion in which the results and conclusions from the previous chapters are combined and placed in a broader perspective. The role of immunotherapeutic strategies for the treatment of cervical cancer is being discussed and suggestions for future research are being given. The general discussion is followed by a summary in Dutch.

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Table 1 Carcinoma of the cervix uteri: FIGO nomenclature for staging classification.
(Adapted with permission from Quinn et al[18])

STAGE 0	Carcinoma in situ, cervical intraepithelial neoplasia (CIN) grade III.	
STAGE I	The carcinoma is strictly confined to the cervix (extension to the corpus would be disregarded).	
	IA	Invasive carcinoma which can be diagnosed only by microscopy. All macroscopically visible lesions – even with superficial invasion – are allotted to Stage IB carcinomas. Invasion is limited to a measured stromal invasion with a maximal depth of 5.0 mm and a horizontal extension of not > 7.0 mm. Depth of invasion should not be > 5.0 mm taken from the base of the epithelium of the original tissue – superficial or glandular. The involvement of vascular spaces – venous or lymphatic – should not change the stage allotment.
		IA1 Measured stromal invasion of not > 3.0 mm in depth and extension of not > 7.0 mm.
		IA2 Measured stromal invasion of >3.0 mm, but not >5.0 mm and extension of not >7.0 mm.
	IB	Clinically visible lesions limited to the cervix uteri or preclinical cancers greater than Stage IA.
		IB1 Clinically visible lesions not > 4.0 cm.
		IB2 Clinically visible lesions > 4.0 cm.
STAGE II	Cervical carcinoma invades beyond the uterus, but not the pelvic wall or the lower third of the vagina.	
	IIA	No obvious parametrial involvement.
	IIB	Obvious parametrial involvement.
STAGE III	The carcinoma has extended to the pelvic wall. On rectal examination, there is no cancer-free space between the tumor and the pelvic wall. The tumor involves the lower third of the vagina. All cases with hydronephrosis or nonfunctioning kidneys are included, unless they are known to be due to another cause.	
	IIIA	Tumor involves lower third of the vagina, with no extension to the pelvic wall.
	IIIB	Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney.
STAGE IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous edema, as such, does not permit a case to be allotted to Stage IV.	
	IVA	Spread of the growth to adjacent organs.
	IVB	Spread to distant organs.

Figure 1 Carcinoma of the cervix uteri. Staging cervical cancer: primary tumor and metastases (FIGO and TNM). (Adapted with permission from Quinn et al[18])

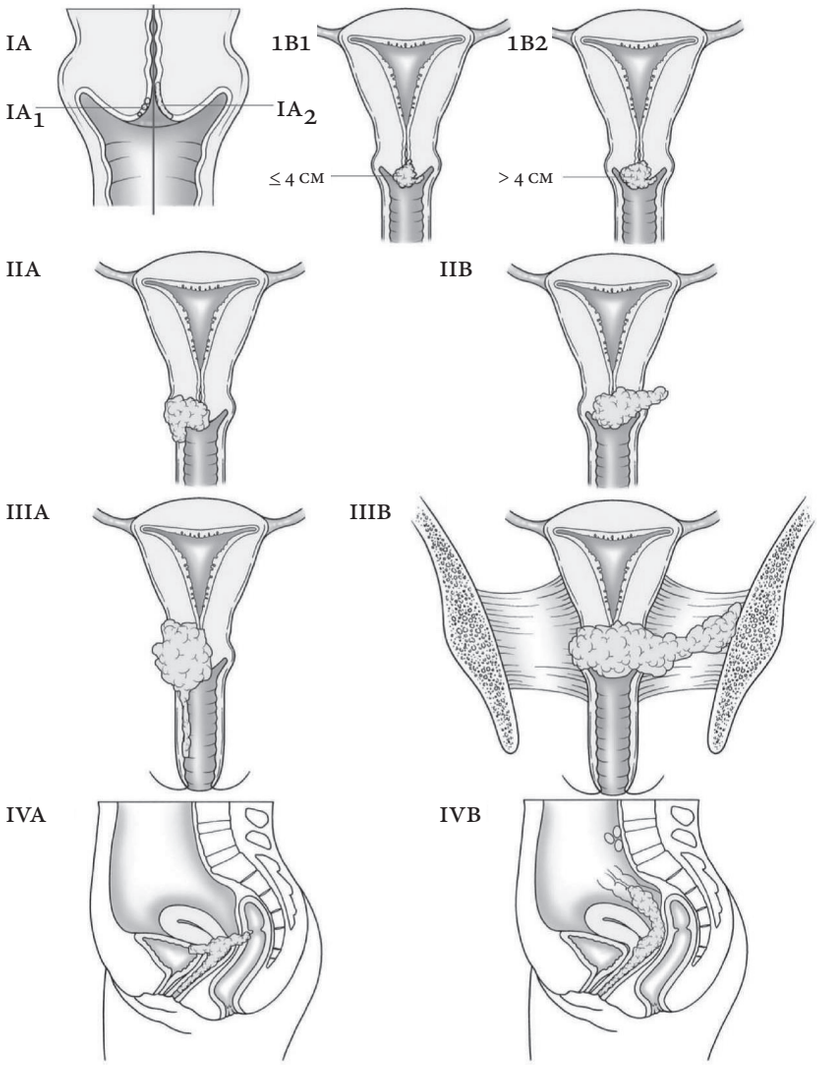
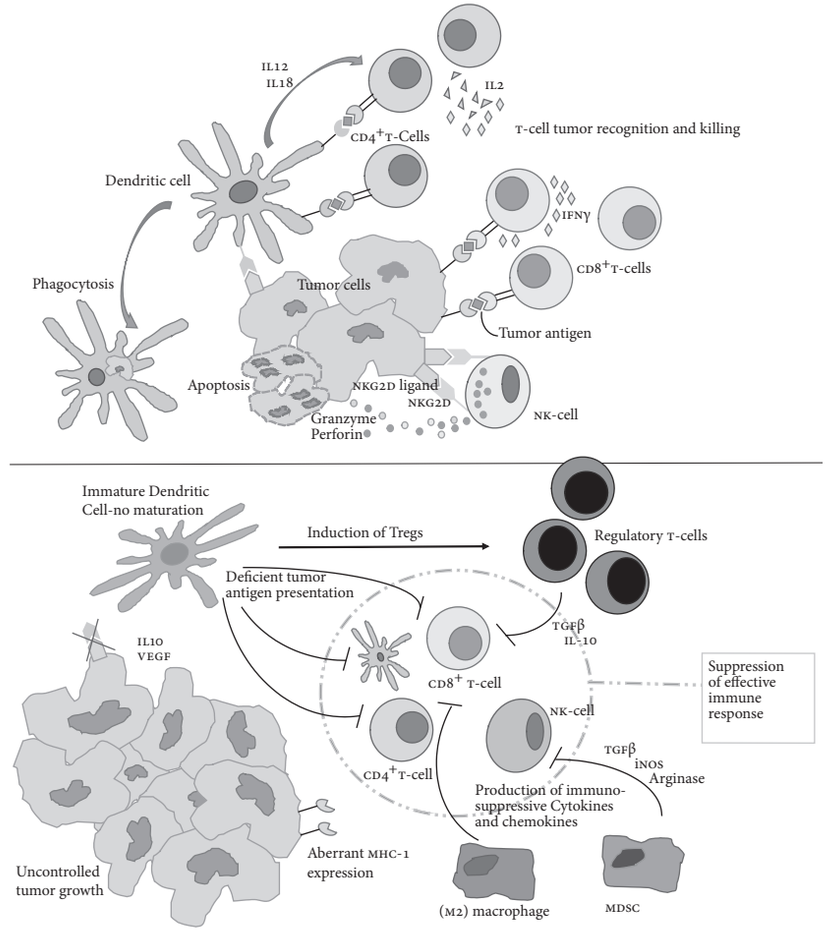


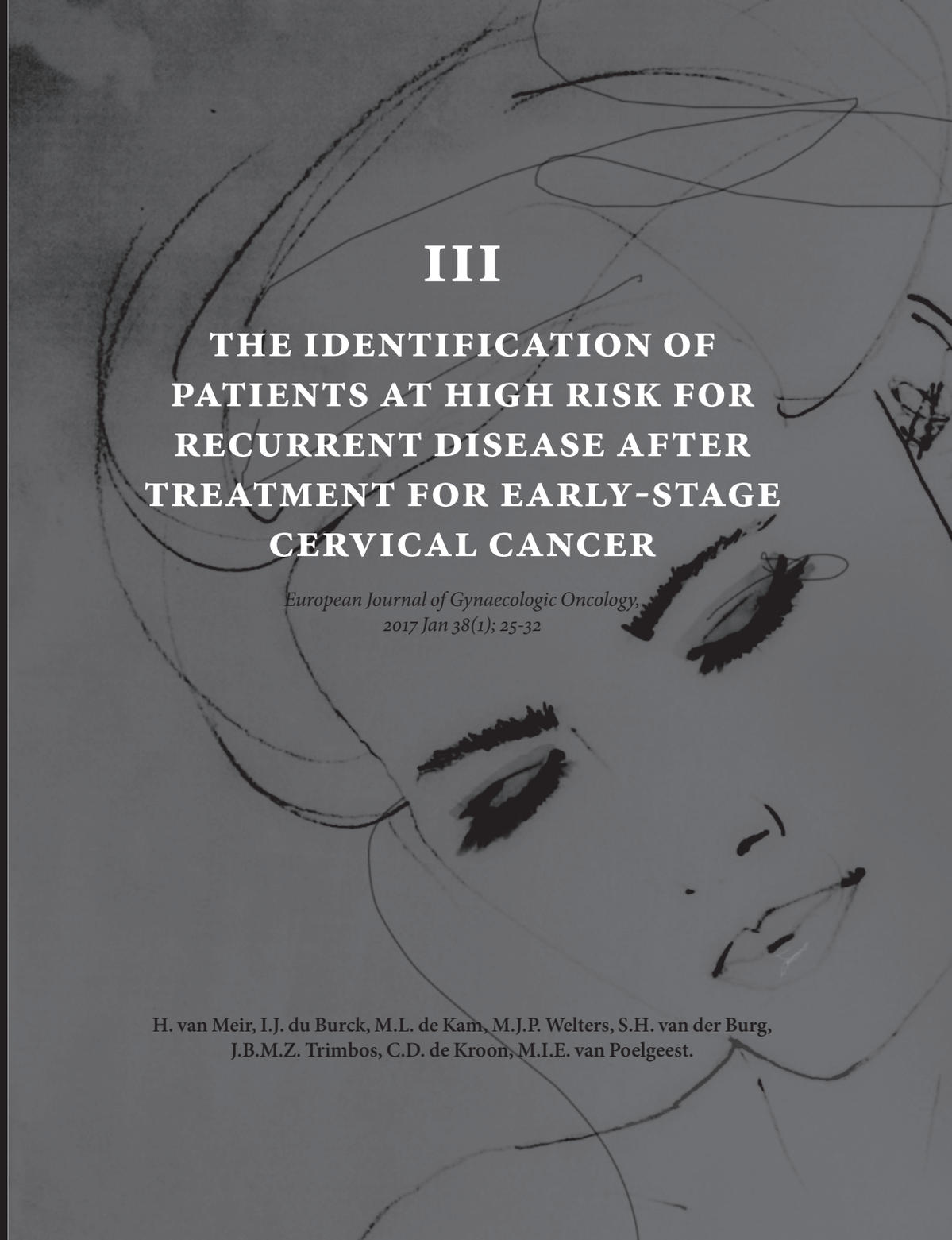
Figure 2 Tumor elimination phase.



Up: The expression of tumor antigens, the release of co-stimulatory signals and the activation and proliferation of armed effector cells, contribute to an effective tumor-specific immune responses against (potentially malignant) cells. **Down**: Malignant cells escape from immune attack, as an antitumor response is suppressed. Together with M2 macrophages, regulatory T-cells, MDSCs, and immunosuppressive cytokines and molecules, tumor cells form an immunosuppressive micro-environment.

PART 1

STANDARD TREATMENT OPTIONS FOR CERVICAL CANCER



III

THE IDENTIFICATION OF PATIENTS AT HIGH RISK FOR RECURRENT DISEASE AFTER TREATMENT FOR EARLY-STAGE CERVICAL CANCER

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ABSTRACT

OBJECTIVE To investigate prognostic factors in patients with recurrent cervical cancer after treatment for early-stage disease in order to identify high-risk patients who might benefit from alternative treatment strategies.

STUDY DESIGN We retrospectively analyzed clinical and pathology data from 130 recurrent cervical cancer patients after surgical treatment for early-stage disease. Patients were compared with a recurrence-free control group matched for age, FIGO stage, and adjuvant treatment. Univariate and multivariate Cox regression analyses were performed to determine prognostic factors for recurrence and survival.

RESULTS Of 889 patients, 130 (14.6%) developed recurrent disease after primary treatment for early-stage cervical cancer. Local or loco-regional metastasis was observed in 45%, distant metastasis in 31%, and combined pelvic and distant metastasis in 24%. Median survival after recurrence was 12 months (range 1-107 months). Median 5-year survival was 96% in the control group and 29% in the recurrence group. Tumor size ≥ 40 mm and lymph node metastasis were independent unfavorable prognostic factors for overall and disease-free survival. The number of positive lymph nodes (≥ 1) and bilateral occurrence of pelvic lymph node metastasis were associated with adverse clinical outcome.

CONCLUSIONS Tumor size ≥ 40 mm and lymph node metastasis were independent unfavorable prognostic factors in surgically treated, early-stage cervical cancer patients. The combination of these factors was particularly associated with recurrence. Future trials should focus on the role of alternative adjuvant treatment strategies in patients at high risk of recurrent disease (e.g., by chemotherapy, immunotherapy or combinations thereof).

Introduction

Cervical cancer is the fourth most common cancer in women worldwide[1], and is staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging system, based on clinical evaluation.[2] Most patients with cervical cancer present with early-stage disease (I-IIA), which generally has a good prognosis after primary treatment with either surgery or (chemo) radiation therapy.[3] In young patients, the surgical approach has advantages over radiotherapy because ovarian function is preserved and less sexual dysfunction occurs.[4,5] Radical hysterectomy with pelvic lymph node dissection results in excellent 5-year overall survival (OS) rates, ranging from 75% to 95%.[6]

Patients with lymph node metastasis, parametrial involvement, and tumor-positive surgical margins are treated with pelvic radiotherapy after surgery as this has shown to reduce the risk of recurrences with 47%.[7,8] More recently, adjuvant radiation in the context of unfavourable tumor factors has been suggested to be beneficial. These unfavourable factors include: tumor diameter ≥ 40 mm, tumor infiltration depth ≥ 15 mm, and the presence of lymphovascular space involvement (LVSI).[9-12] During last decade, adjuvant radiotherapy with concurrent chemotherapy was introduced, the addition of cisplatin improved progression-free survival and OS among women with high risk early-stage disease after radical hysterectomy and pelvic lymphadenectomy.[13,14]

Recurrent cervical cancer is associated with poor outcomes, with a reported 1-year survival rate of 15-20%[15], and median survival rates of 7-36 months after recurrence treatment.[16] For patients with metastatic disease, chemotherapy is the standard treatment, although it is neither curative nor associated with long-term disease control: response rates are between 20% and 35%, and median survival is only 8-13 months.[17,18] As chemotherapy has poor outcome and results in significant morbidity, alternative adjuvant treatment strategies in patients at risk for recurrent disease are crucial.We investigated tumor characteristics and clinicopathologic factors in patients who developed recurrence after primary surgery for early-stage cervical cancer. The aim of this study was to identify prognostic markers that can be used to stratify patients regarding the increasing risk of recurrence, and therefore those most in need of alternative therapies.

Materials and Methods

Between 1984 and 2009, 889 patients were surgically treated for FIGO stage I-IIA cervical cancer and underwent radical hysterectomy with pelvic lymphadenec-

tomy at the Leiden University Medical Center (LUMC). From 2001 on, the nerve-sparing Swift radical hysterectomy was performed.[19] Lymph nodes were divided into high nodes along the common iliac artery, superficial nodes along the external iliac artery and vein, and deep nodes from beneath the level of the external iliac vein in the obturator fossa. Histopathological characteristics were documented for each patient: tumor size, histological tumor type, parametrial involvement, tumor-positive lymph nodes, and surgical margins. Depth of invasion was measured in millimeters from the basement membrane of the surface epithelium. LVSI was considered positive when cancer cells were present within endothelium-lined spaces.

Indications for adjuvant radiotherapy included lymph node metastasis, parametrial involvement, and tumor-positive surgical margins. Since 1997, patients with tumor-negative lymph nodes but 2 or 3 unfavourable tumor parameters also received adjuvant radiotherapy. From the year 2000 on, patients with ≥ 2 tumor-positive lymph nodes, parametrial infiltration, or tumor-positive surgical margins were offered chemo-radiation therapy.

Follow-up by a gynecologic oncologist took place every 3 months for the first 2 years after surgery, every 6 months for the next 3 years, and annually thereafter. Patients receiving postoperative (chemo)radiation therapy were followed with 3 monthly appointments, alternately by a radiation oncologist and a gynecologic oncologist.

Recurrence of disease was defined as any new lesion diagnosed with physical examination, radiology, and histopathology. Local recurrence was defined as recurrent disease involving the vagina. Recurrence was loco-regional if it was located in the vagina as well as in the bladder, rectum, side wall of the pelvis, or inside the pelvis. Recurrence at other sites, including lungs, bones, supraclavicular lymph nodes, and various abdominal sites, was classified as distant. In our surgically treated cervical cancer group, 130 patients (14.6%) were diagnosed with recurrent disease. These patients were matched for age, FIGO stage and primary treatment with a control group without recurrent disease.

Statistical analysis was performed using SAS version 9.1.3. Fisher's exact test was used to analyze patient characteristics for categorical variables or factors. Continuous data was summarized by recurrence and compared with an unpaired *t*-test. The correlation between characteristics was analyzed using Spearman's correlation. Survival analysis was performed using Kaplan-Meier curves and Cox proportional hazards model. Independent prognostic factors were determined through multivariate analysis using the Cox proportional hazards model. The significant prognostic factors determined in the multivariate analysis, were used to stratify patients into risk groups. The frequency of lymph node metastasis and location of recurrence were compared using Fisher's exact test. The statistical significance level was set at $p \leq 0.05$.

Results

Clinical and histological characteristics of patients with recurrent cervical cancer and the matched control subjects are outlined in Table 1. Notably, of 260 patients, only 38% had a documented FIGO stage IB1 or less; 38% had deep infiltrating tumors (≥ 15mm), 55% had LVSI, 15% had parametrial involvement, and 38% had lymph node metastasis.

The respective 5-year OS for the recurrence and control groups were 29% and 96% (figure 1A). In 67% of cases, recurrent disease occurred within 2 years after primary treatment, with a mean of 23.7 months (median 14; range 1-134). Survival after the diagnosis of recurrence was 28% after 24 months and 10.7% after 5 years, with a median survival of 12 months (95% CI 10-15; range,1-107). A disease free survival (DFS) of < 12 months was significantly associated with poor survival, compared with a DFS between 12 and 24 months (HR 0.55, p = 0.0105) and a DFS of > 24 months (HR 0.23, p < 0.0001) (Figure 1B).

Median survival was 58 months (95% CI 20-74) in cases of local recurrence, 24 months (95% CI 17-42) for loco-regional disease, and 39 months (95% CI 26-58) for patients with distant metastases (Figure 1C). Treatments for recurrent disease are listed in Table 2. Figure 1D depicts the OS for patients with recurrent disease, categorized by treatment modality.

TUMOR SIZE AND LYMPH NODE METASTASIS STRONGLY PREDICT SURVIVAL

Univariate analysis revealed that tumor infiltration depth ≥ 15 mm, tumor diameter ≥ 40 mm, and the presence of lymph node metastasis were significantly associated with impaired survival. On multivariate analysis, lymph node metastasis and tumor size ≥ 40 mm were significant predictors for OS (p = 0.0046 and p = 0.0588, respectively) and DFS (p = 0.0015 and p = 0.0007, respectively) (Table 3).

Patients with a tumor diameter ≥ 40 mm were at risk for the development of recurrent disease; a 62% incidence of recurrence was noted in this group, compared with 40% in the patient group with a tumor < 40 mm (p = 0.0014). Furthermore, mean time to recurrence was significantly shorter among patients with a tumor ≥ 40 mm than among patients with a tumor < 40 mm (12.3 months [95% CI 10.0-15.3] vs. 20.2 months [95% CI 15.9-25.7]; p = 0.0027).

Further analysis revealed that tumor size was associated with infiltration depth, LVSI, and lymph node metastasis. Tumor size correlated strongly with infiltration depth (Spearman correlation 0.61, $p < 0.0001$). Regarding LVSI, mean tumor infiltration depth was 14.8 mm (95% CI 13.5-16.1), compared with 10.9 mm (95% CI 9.4-12.3) in tumors without LVSI (unpaired *t*-test, $p < 0.0001$). Patients with a tumor ≥ 40 mm exhibited more frequent presence of lymph node metastasis than patients with a tumor < 40 mm ($p = 0.0295$).

Based on the presence or absence of these two prognostic factors, the 260 patients were stratified into the following risk groups: low (patients without risk factors), medium (patients with a tumor ≥ 40 mm *or* lymph node metastasis), and high (patients with a tumor ≥ 40 mm *and* lymph node metastasis). OS was significantly better in the low-risk group (hazard ratio 1.7, 95% CI 1.16-2.59) than in the medium and high-risk groups (hazard ratio 3.0, 95% CI 1.98-4.97) (Figures 2 and 3). Seventy-one percent of the 31 patients in the high-risk group that developed recurrent disease also had distant recurrence, which demonstrates that this group might experience impaired survival due to more distant tumor metastasis.

PROGNOSTIC IMPACT OF THE NUMBER AND SITE OF LYMPH NODE METASTASES

Sixty-two (48%) of 130 patients with recurrent cervical cancer had tumor-positive lymph nodes at surgery. For the recurrence and control groups, the 5-year survival rate was 70% for patients without tumor-positive lymph nodes, compared with 46% for patients with tumor-positive lymph node(s) (HR 2.17; 95% CI 1.53-3.09, $p < 0.0001$). Survival was significantly lower with an increasing number of tumor-positive lymph nodes ($p = 0.0001$) (Figures 4A, B). OS and DFS were especially poor in patients with ≥ 2 tumor-positive lymph nodes, with an OS of only 34% and a high risk of developing recurrent disease; 69% incidence of recurrence was noted in this group, compared with 48% in the patient group with 1 tumor-positive lymph node and 40% in patients without lymph node metastasis ($p < 0.0001$) (Figure 4B).

Of 98 patients with lymph node metastasis, 42 patients (43%) developed distant metastasis, and 21 patients (21%) had local or loco-regional recurrences. Bilateral occurrence of lymph node metastasis was associated with recurrent disease ($p = 0.034$). The number of lymph nodes removed during surgery and the extension of lymph node metastases to the common iliac or para-aortic nodes were not associated with the site of recurrence. Kaplan-Meier curves for OS and DFS for patients with positive lymph node metastases, by the site of positive lymph nodes, are depicted in Figures 4C, D. Although not statistically significant, extension of lymph node metastases to the common iliac or para-aortic lymph nodes exhibited a trend toward impaired survival and higher risk of recurrent disease.

Comment

The aim of this study was to evaluate tumor characteristics of surgically treated early-stage cervical cancer patients in order to evaluate whether a subgroup at high risk of recurrent disease could be identified. The retrospective analysis was performed on a period of 25 years. The standard care has obviously been changed in this period of time with the introduction of Magnetic Resonance Imaging (MRI), the addition of FIGO stage IB1 and IB2, and the use of radiotherapy and subsequently chemotherapy.

After primary treatment for early-stage cervical cancer, recurrence occurred in 14.6% of the patients, indicating a good overall prognosis for the majority of patients. In this study, 65% of the patients who developed recurrent disease had undergone adjuvant (chemo)radiation therapy after radical surgery; therefore, recurrences occurred despite aggressive and combination primary treatment. This suggests that either the adjuvant treatment had nothing further to add after radical surgery, or it was not sufficient to provide any survival benefit.

Our study confirms results from other studies demonstrating that large tumor size is a prognostic factor in cervical cancer patients.[20,21] Since 1995, stage IB cervical cancer has been divided into subgroups based on clinical tumor size: stage IB1 indicates a tumor diameter < 40 mm, and stage IB2 a tumor diameter ≥ 40 mm (bulky tumor).[22] This sub-classification recognizes that tumors > 40 mm require different treatment approaches. With regard to our results, it states that appropriate selection of patients upfront for chemoradiation rather than surgery is crucial. Moreover, recent studies have shown that a morphologic characteristic, the Barrel Index (BI), the ratio of tumor width to tumor length, is also an independent prognostic factor for recurrence and survival in bulky cervical cancer.[23,24] This suggests that in addition to tumor diameter, tumor morphology might be helpful in identifying a subgroup of high-risk patients with a worse prognosis. Although we did not make this division in stage IB in barrel-shaped versus exophytic tumors in the present study, the division might also have been associated with clinical outcomes. As there are no clear guidelines regarding the best treatment approach for bulky cervical tumors, different treatment approaches are explored. Neo-adjuvant chemotherapy

followed by radical surgery offers the potential to reduce tumor volume, thereby facilitating primary surgery and positively effecting microscopic disease.[25] A meta-analysis of 6 randomized controlled trials[26] and the results of a phase III study by the Gynecologic Oncology Group (GOG)[27] demonstrated advantages in neo-adjuvant chemotherapy reducing the rate of lymph node metastasis and parametrial infiltration, thereby improving OS and DFS. In addition, there is promising evidence for enhanced activity of weekly platinum/taxane regimens to improve prognosis of patients with locally advanced cervical cancer.[28] EORTC trial 55994 is currently ongoing and aims to investigate whether neo-adjuvant chemotherapy followed by surgery confers a survival advantage compared with concomitant radiotherapy and chemotherapy in patients with stage IB2, IIA ≥ 40 mm, and IIB cervical cancer.

The present study demonstrated that the number of tumor-positive lymph nodes is a relevant prognostic factor for OS and DFS. Other studies have shown that the number of metastatic lymph nodes,[29-31] common iliac or para-aortic lymph node involvement,[31] and bilaterality of lymph node metastasis[32] are associated with clinical outcome. In general, patients with para-aortic lymph node metastasis are treated with extended-field radiotherapy, which is associated with significant morbidity.[33] Unfortunately, the heterogeneity in trial results and use of different treatment regimens result in a lack of consensus concerning treatment choice. Alternative treatment strategies have not been studied extensively. A small retrospective analysis showed that adjuvant chemotherapy in high-risk patients did not result in better outcomes compared with patients after adjuvant radiotherapy. However, a subgroup of patients with common iliac and > 2 lymph node metastases exhibited improved survival.[34] The choice for and type of adjuvant treatment in patients at high risk for recurrence should be examined further. The GOG-9926 study started in 2011 and examines the role of adjuvant paclitaxel and carboplatin in women with para-aortic lymph node metastasis after extended field radiation therapy.

Immunotherapy is as another alternative and promising treatment strategy for patients with cervical cancer. Because of the viral etiology and the expression of the viral oncoproteins E6 and E7, cervical cancer is regarded as highly immunogenic. Immunotherapy has been shown to lack clinical efficacy in end-stage patients with large tumor burden and immunosuppressive conditions,[35,36] but results from vaccination trials in patients with HPV16-induced pre-malignant vulvar lesions have shown that smaller lesions are more likely to regress in response to vaccine-induced HPV16-specific immunity.[37] Hence, activating the immune system by immunotherapy might be of value in patients with minimal residual disease. In our study, 72 of 130 patients with recurrent cervical cancer developed distant metastases, suggesting that a substantial number of high-risk patients have residual micrometastases after primary treatment. Alternative systemic therapies that attempt to reduce the risk for recurrence are mandatory rather than a change of primary local treatment. Immunotherapy may well be an alternative adjuvant systemic approach in these patients, particularly because effective alternative therapies are identified for the clinical management of other malignancies. For example, immunotherapeutic options have emerged as a potential adjuvant treatment option in the context of high-risk surgically treated melanoma patients.[38] Future studies could introduce targeted therapies in an adjuvant setting for high-risk cervical cancer patients.

In summary, this study demonstrates that positive lymph node status and tumor size are prognostic factors associated with poor survival in patients surgically treated for early-stage cervical cancer. In particular, patients with both factors are at high risk for recurrent disease and might benefit from alternative adjuvant treatment strategies. New therapeutic approaches should be explored in these high-risk patients, such as (neo)adjuvant chemotherapy, immunotherapy, or combinations of these treatments.

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Table 1 Patient characteristics. Data are presented as n (%) unless otherwise specified. FIGO, International Federation of Gynecology and Obstetrics; EBRT, external beam radiation therapy; LVSI, lymphovascular space involvement.

Factors	Recurrence (n=130)	Control (n=130)	p
Median follow-up (range)	30.5 (5 - 203)	72.5 (12 - 287)	
AGE			
<40	45 (34.6%)	49 (37.7)	
40-70	75 (57.7%)	69 (53.1%)	0.78
>70	10 (7.7%)	12 (9.2%)	
FIGO STAGE			
IA	1 (0.8%)	1 (0.8%)	
IB	109 (84%)	109 (84%)	1
IB1	48 (44%)	49 (45%)	
IB2	25 (23%)	16 (15%)	
N/A or unknown*	36 (33%)	44 (40%)	
IIA	20 (15.4%)	20 (15.4%)	
PRIMARY TREATMENT			
Surgery	45 (34.6%)	45 (34.6%)	
Surgery + adjuvant EBRT	71 (54.6%)	71 (54.6%)	1
Surgery + adjuvant Chemo-EBRT	14 (10.7%)	14 (10.7%)	
HISTOLOGICAL TYPE			
Squamous cell carcinoma	91 (70%)	99 (76.1%)	
Adenosquamous carcinoma	9 (6.9%)	9 (6.9%)	
Adenocarcinoma	28 (21.5%)	21 (16.2%)	0.51
Undifferentiated	1 (0.8%)	0	
Unknown	1 (0.8%)	1 (0.8%)	
SITE OF RECURRENCE			
Local	29 (22%)		
Loco-regional	29 (22%)		
Distant	41 (32%)		
Loco-regional and distant	31 (24%)		
DEPTH OF INVASION			
<15 mm	65 (50%)	74 (56.9%)	0.6
≥15 mm	51 (39.2%)	49 (37.7%)	
Unknown	14 (10.8%)	7 (5.4%)	
TUMOR DIAMETER			
<40 mm	58 (44.6%)	86 (66.1%)	0.0014
≥40 mm	59 (45.4%)	36 (27.7%)	
Unknown	13 (10%)	8 (6.2%)	
LVSI			
Yes	78 (60%)	65 (50%)	0.06
No	41 (31.5%)	56 (43.1%)	
Unknown	11 (8.5%)	9 (6.9%)	
PARAMETRIAL EXTENSION			
Yes	24 (18.5%)	16 (12.3%)	0.2
No	104 (80%)	112 (86.2%)	
Unknown	2 (1.5%)	2 (1.5%)	
LYMPH NODE METASTASIS			
Yes	63 (48.5%)	35 (26.9%)	<0.001
No	67 (51.5%)	95 (73.1%)	
PATIENT STATUS			
Alive	13 (10%)	122 (94%)	<0.001
Dead	116 (89.2%)	8 (6%)	
Disease-free	7 (5.4%)	119 (91.5%)	
Stable disease	4 (3%)	0	
Progressive disease	2 (1.5%)	0	
Dead due to cervical cancer	116 (89.2%)	0	
Dead due to other causes	0	8 (6.2%)	
Lost to follow-up	1 (0.8%)	3 (2.3%)	

** There is no information on FIGO stage IB1 or IB2 cervical cancer (based on a tumor diameter of 40 mm) in patients treated and operated before 1995.*

Table 2 Treatments for recurrent disease.

Treatment	Patients, n=130
MONOTHERAPY	60 (46.2%)
SUR	6 (4.6%)
RT	24 (18.5%)
CH	28 (21.5%)
VAC	2 (1.6%)
COMBINATION THERAPY	54 (41.5%)
SUR + CH	4 (3.1%)
SUR + CH + RT	5 (3.8)
SUR + RT (+/- HT)	8 (6.2%)
SUR + VAC	1 (0.8%)
RT + HT	5 (3.8%)
RT + VAC	1 (0.8%)
CHRT (+/- HT)	21 (16.1%)
CH + HT	6 (4.6%)
CH + VAC	3 (2.3%)
SUPPORTIVE TREATMENT	16 (12.3%)

SUR = surgery; RT = radiotherapy; CH = chemotherapy; VAC = vaccination; HT = hyperthermia

Table 3 Regression analyses of predictors for overall and disease-free survival in cervical cancer patients after surgical treatment for early-stage disease.

Prognostic factors	Univariate analysis						Multivariate analysis					
	Overall Survival			Disease Free Survival			Overall Survival			Disease Free Survival		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
RECURRENT DISEASE												
yes vs no	26.3	12.82-53.97	< 0.0001									
AGE												
< 40 yrs vs 40-70 yrs	0.82	0.56-1.20	0.3	0.91	0.63-1.32	0.6						
> 70 yrs vs 40-70 yrs	0.85	0.44-1.64	0.6	0.86	0.44-1.66	0.6						
SMOKING												
yes vs no	1.07	0.73-1.58	0.7	0.99	0.68-1.44	0.9						
DEPTH OF INVASION												
<15 mm vs ≥ 15 mm	1.51	1.04-2.20	0.03	1.22	0.84-1.75	0.3	1.09	0.68-1.74	0.72	1.13	0.72-1.77	0.6
<10 mm vs ≥ 10mm	1.45	0.96-2.19	0.08	1.42	0.95-2.13	0.09						
TUMOR SIZE												
< 40mm vs ≥ 40 mm	1.63	1.12-2.37	0.01	1.96	1.36-2.82	0.0003	1.52	0.98-2.34	0.0588	2.12	1.38-3.27	0.0007
< 30 mm vs ≥ 30 mm	2.02	1.32-3.11	0.0013	2.16	1.42-3.29	0.0003	1.77	1.07-2.91	0.025	2.56	1.56-4.21	0.0002
LYMPH NODE METASTASIS												
yes vs no	2.17	1.53-3.09	< 0.0001	1.91	1.35-2.70	0.0002	2.20	1.28-3.75	0.004	2.46	1.41-4.29	0.0015
PARAMETRIAL EXTENSION												
yes vs no	1.5	0.96-2.34	0.08	1.36	0.87-2.11	0.18	1.12	0.63-2.01	0.7	1.06	0.59-1.9	0.85
LVSI												
yes vs no	1.42	0.96-2.10	0.08	1.36	0.93-1.98	0.11	1.12	0.69-1.82	0.63	1.17	0.73-1.86	0.5

HR, hazard ratio; CI, confidence interval; LVSI, lymphovascular space involvement

Figure 1 (A) Five-year overall survival (OS) for surgically treated FIGO IA-IIA cervical cancer patients. (B) Five-year OS for patients with recurrent disease, categorized with respect to disease-free survival (DFS). DFS < 12 months vs DFS 12-24 months: HR 1.82, p = 0.0105. DFS < 12 months vs DFS > 24 months: HR 4.35, p < 0.0001. (C) Five-year OS of patients with recurrent disease, categorized with respect to site of recurrence (local, loco-regional, distant and combined loco-regional and distant). (D) Five-YEAR OS for patients with recurrent disease, categorized with respect to treatment modality. Types of monotherapy include chemotherapy, radiotherapy, surgery, or vaccination; combination therapy includes combinations of chemotherapy, radiotherapy, hyperthermia, surgery and/or vaccination; supportive therapy indicates no active treatment (palliative treatment only). Median OS was 17 months (range 1 – 91 months) for supportive therapy, 29 months (range 8 – 98 months) for monotherapy, and 51 months (range 28 – 102 months) for combination therapy. Combination therapy was associated with survival benefit vs monotherapy (p = 0.007) and supportive (palliative) therapy (p = 0.0017).

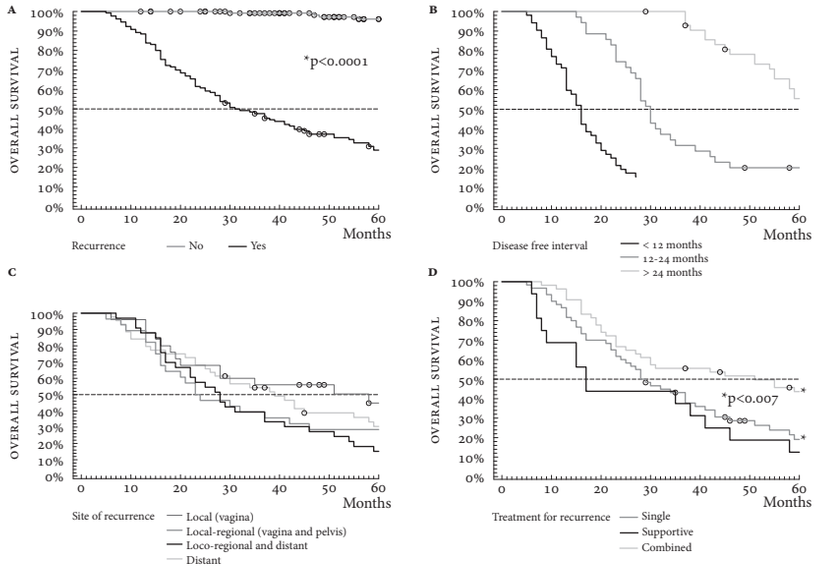


Figure 2 Overall survival based on prognostic factors. Low-risk: tumor size < 40 mm without lymph node metastasis; medium-risk: tumor size ≥ 40 mm or lymph node metastasis; high-risk: tumor size ≥ 40 mm and lymph node metastasis. Low-risk group vs medium-risk group: p = 0.01; low-risk vs high-risk group: p < 0 .0001.

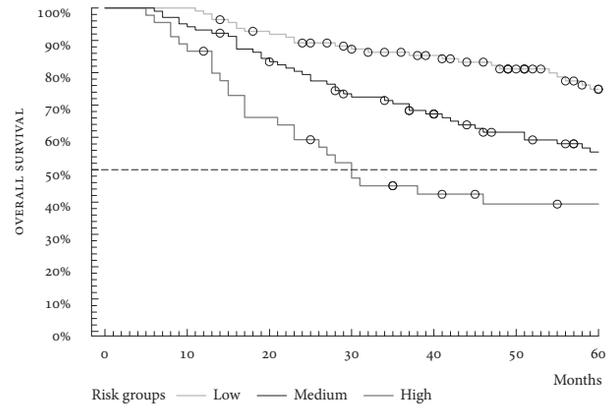


Figure 3 Disease-free survival based on prognostic factors. Low-risk: tumor size < 40 mm without lymph node metastasis; medium-risk: tumor size ≥ 40 mm or lymph node metastasis; high-risk: tumor size ≥ 40 mm and lymph node metastasis. Low-risk group vs medium-risk group: p = 0.007; low-risk vs high-risk group: p < 0.0001.

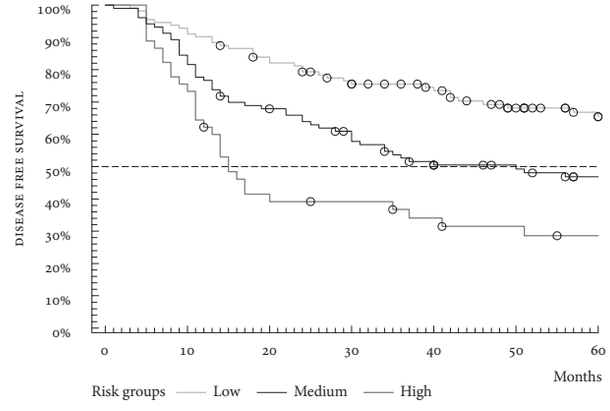
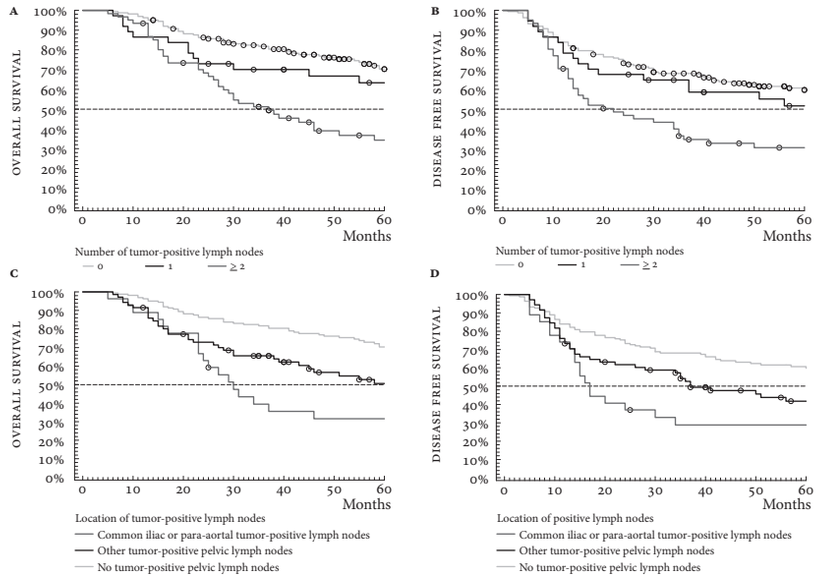


Figure 4 Overall survival (OS) and disease-free survival (DFS) for surgically treated patients with early-stage cervical cancer, categorized by number of tumor-positive lymph nodes (A, B) and by the site of tumor-positive lymph nodes (C, D). No lymph node metastasis, n = 162; 1 tumor-positive lymph node, n = 37; ≥ 2 tumor-positive lymph nodes, n = 61. (**A**) OS: None vs 1 tumor-positive lymph node: p = 0.056; None vs ≥ 2 tumor-positive lymph nodes: p < 0.0001. (**B**) DFS: None vs 1 tumor-positive lymph node: p = 0.26; None vs ≥ 2 tumor-positive lymph nodes: p < 0.0001. (**C**) OS: none vs common iliac or para-aortic tumor-positive lymph nodes: p < 0.0001; common iliac or para-aortic tumor-positive lymph nodes vs other tumor-positive pelvic nodes: p = 0.1. (**D**) DFS: none vs common iliac or para-aortic tumor-positive lymph nodes: p = 0.0002; common iliac or para-aortic tumor-positive lymph nodes vs other tumor-positive pelvic nodes: p = 0.1



IV

THE NEED FOR IMPROVEMENT OF THE TREATMENT OF ADVANCED AND METASTATIC CERVICAL CANCER, THE RATIONALE FOR COMBINED CHEMO-IMMUNOTHERAPY

Anticancer Agents in Medicinal Chemistry.
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ABSTRACT

The prognosis of patients with metastatic cervical cancer is poor with a median survival of 8-13 months. Despite the potency of chemotherapeutic drugs, this treatment is rarely curative and should be considered palliative only. The last decades, targeted therapies such as immunotherapy have emerged as an attractive option for the treatment of these patients. Immunotherapy can consist of different modalities such as monoclonal antibodies, adoptive lymphocyte transfer and vaccines, which all are intended to augment the antitumor immune responses in cancer patients. The available evidence indicates that both active and adoptive immunotherapeutical strategies are quite effective against small tumor burdens, but are usually insufficient to eradicate the disease in patients with advanced stages of different kinds of cancer, despite strong induction of tumor-specific immune responses. Although chemotherapy and immunotherapy have not shown to be curative as single modalities, accumulating evidence suggests that combinations of these treatments hold potential for improved clinical outcomes in advanced stages of cancer. Therefore, the combination of chemotherapy and immunotherapy is no longer considered incompatible, because of the emerging insight that certain chemotherapy-based cancer treatments may activate the immune system against the tumor through several molecular and cellular mechanisms. Chemotherapeutic agents and immunotherapy may thus be synergistic and enhance the clinical response.

In this review, we show the rationale for combined chemo-immunotherapeutic strategies, and summarize recent data from clinical trials performed in patients with different types of cancer. Challenges such as the selection of the optimal dose and treatment schedule, will be discussed as well as the identification of immune-specific biomarkers. Furthermore, we evaluated the long-term clinical outcomes of patients with advanced cervical cancer treated with HPV16 E6/E7 SLP vaccination with or without chemotherapy. Finally, the future of vaccination therapy in combination with chemotherapy for the treatment of cervical cancer is discussed.

Introduction

Recurrent cervical cancer has a poor prognosis with a reported 1-year survival rate between 15% and 20%.[1,2] Most women suffering from local recurrence, including those with International Federation of Gynaecology and Obstetrics (FIGO) stage IVB or metastatic disease, are not amendable to curative surgery or radiotherapy but are mainly treated by palliative systemic chemotherapy.[3] The clinical outcome with current chemotherapy is disappointing with response rates of 20-35% and a median survival of only 8-13 months.[4-7] To improve the poor prognosis of these patients, new therapeutic approaches are needed. Various studies have been conducted to identify other active agents, such as tyrosine kinase inhibitors, to be used as monotherapy or in combination with currently available chemotherapeutics (reviewed in[8,9]).

Cervical cancer is caused by a persistent infection with a high risk type Human Papilloma Virus (HPV) infection, predominantly by HPV type 16 (HPV16), which is detected in 60% of all cervical cancers worldwide.[10] A number of observations suggest that the cellular arm of the immune system may be protective against HPV-induced disease (reviewed in[11]). HPV16 specific T-cell immunity targeting the early proteins of HPV is frequently detected in peripheral blood mononuclear cell (PBMC) cultures of healthy individuals but not in specimens obtained from patients.[12,13] In addition, a high incidence of cervical HPV-infections and resulting lesions is observed in transplant patients receiving immunosuppressive medication.[14] Further, the presence of a relatively high number of tumor-infiltrating $CD8^+$ T-cells over regulatory T-cells in HPV-induced cervical cancer is associated with a better overall survival in surgically treated patients, suggesting that a successful immune mediated regression of a neoplasm requires the induction of a strong tumor-specific Th1/Cytotoxic T-cell (CTL) response, the control over regulatory mechanisms and an immune stimulating microenvironment.[15-17] These findings thus indicate that the immune system plays an important role in the protection against the development, maintenance and expansion of cervical cancer and suggest that specific stimulation of the host's own immune system against cancer – referred to as immunotherapy - may be a beneficial treatment modality for cervical cancer patients. A number of immunotherapeutic successes have been achieved in the treatment of other cancers and have led to U.S. Food and Drug Administration (FDA) – market approvals for immunological compounds for the treatment of human cancers.[18] Among these, antibody-based therapies are most widely available. In most cases the antibodies are directed against antigens at the cell surface of tumor cells or to soluble antigens produced by these tumors. For cervical cancer, no treatment with monoclonal antibodies has been authorized, but encouraging results with

bevacizumab, directed against the Vascular Endothelial Growth Factor (VEGF), have been reported.[19,20] Catumaxomab, a trifunctional monoclonal antibody consisting of a mouse IgG2a chain binding to human Epithelial Cell Adhesion Molecule (EpCAM) and a rat IgG2b chain that binds to human CD3, received in 2009 an EU marketing authorization for the intraperitoneal treatment of malignant ascites in patients with EpCAM-positive carcinomas when standard therapy is not available or no longer feasible.[21] Recently, the FDA approved the CTLA-4 inhibiting monoclonal antibody Ipilimumab, which is known to release the brake on T-cell proliferation and activation, as a treatment for unresectable or metastatic melanoma, thus enhancing the spontaneous T-cell response against the patient's tumor.[22]

Treatment with ex-vivo stimulated immune effector cells (adoptive cell transfer), is another approach that has shown to mediate tumor regression in patients with metastatic cancer.[23] Finally, the vaccine Sipuleucel-T has been approved by the FDA as an immunotherapeutic agent for the treatment of patients with asymptomatic or minimally symptomatic castration-resistant prostate cancer[24], indicating that therapeutic vaccination may induce clinical benefit in metastatic disease. However, in all cases the effects of single therapy are modest when expressed as the percentage of patients exhibiting a clinical response. Hence, new combinations of different treatment modalities are explored which focus on enhancing the tumor-specific T-cell response while reducing the immune regulatory pathways formed by regulatory T-cells, tumor-promoting myeloid cells and immune-suppressive cytokines.[25,26]

In patients with cervical cancer, several therapeutic vaccination strategies with different delivery systems have been explored clinically. These trials included vector-, peptide- or protein-, nucleic acid-based, and cell-based therapeutic vaccines targeting the HPV16 E6 and/or E7 antigens recombinant viral vectors. The common aim of these therapies was to increase the magnitude and quality of the HPV16-specific immune responses to treat HPV16-driven cervical cancer.[27] Although initial results were promising, patients with advanced cervical cancer had minimal benefit from these therapies probably because of a large tumor burden, which is often associated with immune suppression.[28,29] These immune suppressive conditions could disable the HPV16 vaccines to exert an effective therapeutic action by itself.[27,30]

Although the main mode of action of chemotherapy is to reduce tumor burden, accumulating evidence shows that chemotherapy may have immunostimulatory effects in addition to its direct cytotoxic effect.[31] Mechanisms to explain this include dendritic cell activation by apoptotic tumor cells, direct stimulation of immune effectors and depletion of immunosuppressive cells.[32-34]

In this manuscript, we review the effects of chemotherapy on the immune system as observed in clinical trials. Specifically, the immunological effects of chemotherapeutic compounds used in cervical cancer and promising immune-based therapies in combination with chemotherapy will be discussed with emphasis on challenges such as optimal dosing schedule and the identification of immune-specific biomarkers. Finally, we will outline strategies that could refine these treatment approaches to enhance potential benefits in cancer patients.

Effects of chemotherapy on the immune system

Chemotherapy is frequently being used for the treatment of metastatic solid cancer, and was originally considered as a treatment whose efficacy was exclusively attributed to interferences with cellular division and mainly affects dividing cancer cells as they begin to proliferate. However, many cytotoxic anticancer drugs have additional impact on the immune system that might contribute to tumor regression and therapeutic response.[34-38] Murine tumor models have shown that chemotherapy is more effective when administered to immunocompetent mice compared to immunodeficient animals, indicating that an intact immune system enhances the therapeutic effect of cytotoxic drugs.[39-43] This requirement for the immune system has recently received more attention and has led to the identification of a number of potential mechanisms through which cytotoxic agents might act to positively influence the immune response to cancer.

IMMUNOGENIC CELL DEATH VIA APOPTOSIS

A well-studied cellular mechanism in animals is the immune-mediated tumor cell death induced by tumor cell apoptosis caused by cytotoxic drugs. Platinum-based chemotherapeutics, which cause DNA-damage by cross-linking DNA, may activate p53-independent and p53-dependent pathways that result in the exposure of stress signals (such as natural killer group 2 member D (NKG2D) ligands, MHC class I related chain A (MICA) and MHC class I related chain B (MICB) antigens), the upregulation of major histocompatibility molecules (MHC) class I and the increased expression of death receptors (particularly TNF-related apoptosis-inducing ligand (TRAIL) receptors). The cytotoxic-induced oncogenic stress can activate tumor suppression, causing apoptotic cell death, and leads to the production of pro-inflammatory cytokines, which induce cell cycle arrest.[44,45] Apoptotic tumor cells can provoke an anti-tumor response by providing tumor antigens to dendritic cells (DCs) and induce their activation.[46] Apoptotic tumor

cell death is caused by cytotoxic drugs such as doxorubicin, paclitaxel, gemcitabine and oxaliplatin (reviewed in [31] and [47]). This chemotherapy-induced tumor antigen loading and activation of DCs are provoked by different molecular pathways that have been investigated extensively in vitro. It has been shown that tumor damage associated with the action of anthracyclines and oxaliplatin, is characterized by rapid translocation of calreticulin to the dying tumor cell surface where it acts as a mandatory eat-me signal for DCs.[48] Beyond the exocytosis of calreticulin, dying tumor cells secrete additional signals such as extracellular nucleotide adenosine 5'-triphosphate (ATP) and high-mobility group box 1 protein (HMGB1). ATP has a high affinity with the P2X7 purinergic receptors on the surface of DCs, thereby activating the inflammasone in these cells and the production of interleukin 1β, which in turn polarize $CD8^+$ T-cells towards the production of IFN-γ.[49-51] The nuclear protein HMGB1 is a danger signaling protein which interacts with the toll-like receptor 4 (TLR4) on DCs.[40] It was found, both in vitro and in vivo, that release of HMGB1 by tumor cells (and its effect on TLR4) was required for immunogenic cell death of the tumor. However, subsequent research showed that neither HMGB1 nor calreticulin could promote complete DC maturation and tumor eradication.[40, 42] In most instances the mechanism of enhanced cross-presentation of tumor cells by DCs after chemotherapy is indeed not enough to induce a sufficiently robust T-cell response for tumor destruction, especially not in advanced or metastatic tumors.[31, 52]

It needs to be emphasized that the cellular mechanisms were mainly studied in vitro, while the effects of different cytotoxic compounds in vivo can be substantially different. Nevertheless, chemotherapy-induced apoptosis may potentially yield benefit when appropriate loading and maturation of DCs occur under conditions which allow a subsequently increased tumor-specific immune response. This concept may be particularly relevant in a setting of minimal residual disease where control of tumor outgrowth is critical.

INCREASED SUSCEPTIBILITY OF CANCER CELLS TO IMMUNE ATTACK

Another mechanism, by which chemotherapy may influence the immune system, is the property of anticancer agents to stress tumor cells, making them immunogenic and prone to lysis by immune effectors. This has been demonstrated for chemotherapy with DNA-damaging agents that upregulate the expression of death-receptors and tumor antigens on tumor cells thereby favoring CTL attack.[53] Platinum-based chemotherapeutics have been shown to enhance the immunostimulatory potential of DCs and decrease the immunosuppressive capability of cancer cells by the inhibition of signal transducer and activator

of transcription 6 (STAT6)-regulated expression of programmed death ligand 2 (PD-L2).[54] Programmed death (PD)-ligands are expressed in different human cancers, and the PD-pathway is of pivotal importance in regulating the immune balance between T-cell activation and inhibition.[55] Downregulation, in a STAT6-dependent manner, of the inhibitory molecule PD-L2, results in tumor-specific T-cell expansion and activation with a concomitant sensitivity of tumor cells for lysis via increased cytotoxic T-cells.[54,56] Apparently, platinum compounds via their action on tumor cells, modulate the expression of tumor antigens that results in better recognition of cancer cells by the immune system and decreased immunosuppression by tumor cells. Another example of chemotherapeutic-mediated increased susceptibility of tumor cells to the cytotoxic effects of cytotoxic T-cell lymphocytes (CTLs) was reported in murine and human tumor cells for cisplatin and paclitaxel. These agents, when administered as single agents and in combination, were shown to increase the permeability of tumor cells to granzyme B.[57] The serine protease granzyme B is a main member of the granzyme family and cleaves target cell proteins at specific aspartate residues and triggers caspase activation.[58] The uptake of granzyme B by tumor cells plays a major role in sensitization of tumor cells to CTLs. Remarkably, the increased permeability of the cell membrane to granzyme B was also measured in neighboring tumor cells that did not express the recognized antigen. This 'bystander effect' was due to upregulation of mannose-6-phospate surface receptors upon challenge with chemotherapeutics.[59] Due to the substantial increase in this receptor expression on tumor cells, the activated CTLs interacting with antigen-expressing tumor cells enables greater release of granzyme B that can penetrate into the neighboring tumor cells without cell-cell contact.[57] These mechanistic examples suggest that chemotherapy has close interactions with the immune system which may be synergistic. Hence, it can be envisaged that combinations of chemotherapy and immunotherapy may have beneficial effects for cancer patients, on the condition that optimal combinations are identified.

Various forms of combined immunotherapy and chemotherapy and their effects on tumor growth and/or survival have been investigated. Cisplatin and paclitaxel have frequently been combined with different types of vaccine strategies in murine tumor models and have shown enhanced tumor control and regression of the established tumors in breast cancer, HPV-16-induced cervical cancer, colorectal cancer and lung carcinoma.[60-62] In pre-clinical models, the platinum-based cytotoxic drugs indeed enhanced anti-tumor immune responses when co-administered with a vaccine.[63,64] A dramatic therapeutic synergy between cisplatin-based chemotherapy and the HPV E7 subunit vaccine-based immunotherapy was observed in treating established E7 expressing TC-1 tumors

in mice. Animals treated with the combined therapy displayed improved cure and recurrence rates and long-term antitumor immunity when compared to the animals treated with cisplatin or the E7 subunit vaccine alone. Furthermore, mice treated with combination therapy showed increased numbers of tumor infiltrating lymphocytes and a reduced tumor cell density.[63] The underlying immune potentiating mechanism was proved to have increased sensitivity of cisplatin-exposed tumors to CTL-mediated killing.[63]

Similarly, paclitaxel has also been reported to sensitize tumor cells to CTLs.[59] Furthermore, paclitaxel was shown to have an immune stimulatory effect on the priming of immune cells to tumor antigen in a murine mammary carcinoma model. This is most likely due to the enhancement of the phagocytic activity of antigen presenting cells (APCs) by paclitaxel which then potentiates the capacity of a vaccine to induce antigen-specific $CD8^+$ T-cell responses. As a result, improved antitumor efficacy by enhanced inhibition of tumor growth was observed.[60] When paclitaxel was combined with a granulocyte/macrophage-colony stimulating factor-secreting, HER-2/neu-expressing whole-cell vaccine in the same model, macrophages were activated, resulting in augmented antitumor effector function and induction of secretion of cytokines such as tumor necrosis factor, IL-12, and granulocyte-macrophage colony-stimulating factor.[65] Finally, paclitaxel appeared to amplify the antigen specific T-cell response.[65]

DIRECT EFFECT ON IMMUNE CELLS

Some chemotherapeutic agents are known to have a direct effect on immune cells, a tumor-cell extrinsic immune mechanism that may contribute to an improved anti-tumor immune response. These favorable effects on immune cells include the activation of immune effector cells (such as cytotoxic $CD8^+$ T-cells), but also depletion and/or inhibition of immunosuppressive cells such as regulatory T-cells (Treg) and tumor-promoting myeloid cells.[66] The direct effects of the cytotoxic drugs cyclophosphamide, gemcitabine and the immunotoxin dinileukin diftitox (Ontak) on the immune system have been investigated most intensively. These antitumor agents exert several immunosuppressive actions such as depletion of $CD4^+CD25^+$ Treg, down-regulation of FoxP3 expression and glucocorticoid-induced TNF-receptor related protein, and reduction of $CD11b^+GR1^+$ myeloid-derived suppressor cells, which all have immunosuppressive properties.[67-71] A decrease of Treg cells was shown also in tumor-draining lymph nodes of cervical cancer patients following pre-operative platinum based chemo-radiation therapy.[72] This Treg cell drop correlated with the reduction of primary tumor mass. It has been previously proposed that a decreased Treg

frequency and a concomitant recruitment of effector T-cells and natural killer cells to the tumor draining lymph nodes, contributed to the reduction of tumor mass in preoperative chemoradiation-treated cervical cancer patients.[73] It is however unclear whether the reduction of Tregs and tumor mass contributed to a better clinical outcome in terms of recurrence-free and overall survival in these patients. Different mechanisms may explain the association between the reduction in tumor mass and the drop of Treg frequency. Chemotherapy kills tumor cells and their tumor-derived factors as immunosuppressive cytokines. The elimination of suppressor cells may have facilitated the generation of T-cells mediating the destruction of tumor cells left behind after chemotherapeutic treatment.[72] On the other hand, the complete or near complete destruction of the tumor mass, induced by pre-operative chemo-radiation therapy, may prevent the attraction of Tregs to the lymph nodes and might hinder T-cells – via apoptotic tumor cell uptake of DCS – to undergo differentiation towards a suppressive phenotype. Paclitaxel has also shown to improve cancer immune responses by its direct effects on the immune system. For example, paclitaxel has been reported in mice to decrease the percentage of Tregs and specifically impair the viability and cytokine production of Treg cells, without injuring $CD4^+$ effector T-cells.[74] Additionally, high T-cell blastogenesis and increased natural killer cell lytic activity were reported in response to paclitaxel administration in breast cancer patients, supportive for a positive effect of taxane on T-cell proliferation and NK cytolysis which could favor the development of an antitumor immune response.[75]

Cyclophosphamide has been shown to act synergistically with adoptively transferred wild-type p53-specific CTL in controlling the growth of an aggressive mutant p53-induced and overexpressing tumor in mice.[76] The expression of the target antigen (p53) was influenced by the chemotherapy, since p53 responds to DNA damage induced by the mutagenic agent cyclophosphamide.[77] Pre-treatment with a cyclophosphamide showed efficacy in terms of tumor growth when followed by subsequent adoptive transfer of immune cells.[76]

Synergism between chemotherapy and adoptive T-cell immunotherapy was also shown in another animal model, with chemotherapeutic drugs causing the release of antigen to sensitize stromal cells for tumor destruction by adoptively transferred cytotoxic T-cells. This tumor-reducing synergism appeared to be dependent on the involvement of the tumor microenvironment.[78] Of interest, it was shown in treated mice that the synergism of chemotherapy and adoptive immunotherapy was dependent on $CD4^+$ T-cells and on the cooperation of transferred cells with the host immune system.[79] Optimal therapeutic responses to the adoptive transfer of immune cells were found to be associated with the chemotherapy-mediated induction of a 'cytokine storm' occurring during the

rebound phase after drug-induced myelo-lymphodepletion.[79] Combinations of various monoclonal antibodies (MoAbs) with chemotherapeutic agents such as cisplatin, carboplatin and paclitaxel have pre-clinically shown to be associated with significantly greater anti-tumor effects compared to either therapy alone even in the case of established tumors.[80-83] Many tumor-expressed targets for therapeutic antibodies are growth factors, which show an increased expression during tumor growth. Well known target receptors are epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), which are frequently overexpressed in solid tumors and therefore the target of widely used MoAbs.[84] By the normalization of growth factor receptors, MoAbs might sensitize tumor cells to the cytotoxic effects of chemotherapy.[85] For example, vascular endothelial growth factor (VEGF)-targeted therapy blocks binding of VEGF to its receptor on the vascular endothelium, and prevents from angiogenesis. In combination with cytotoxic chemotherapy, the VEGF-specific humanized monoclonal antibody (bevacizumab, avastin) has been registered for breast, colorectal and ovarian cancer.[86-89] In addition, the favorable, immunomodulatory effect of chemotherapy on immune cells could enhance the antitumor efficacy of therapeutic antibodies when used in combination. Clinically, combinations of MoAbs with chemotherapy have been registered for various tumor types, e.g. trastuzumab for the treatment of breast cancer[90,91] and bevacizumab for breast, colorectal and ovarian cancer.[86-89]

These examples illustrate that different mechanisms exist by which cytotoxic drugs can influence the complex network of tumor cells, cancer growth stimulating immune cells and tumor reducing immune cells (Figure 1). As for platinum-based (cisplatin or carboplatin) chemotherapy and taxanes like paclitaxel, there is accumulating evidence that these drugs are not immunosuppressive but stimulate antitumor immune responses by several cancer cell-exogenous and off-target immune modulatory mechanisms. This has resulted in an increasing number of studies investigating whether the combination of active specific immunotherapy, MoAbs or adoptive lymphocyte immunotherapy with chemotherapy, not only increases anti-tumor effects but ultimately results in favorable clinical outcomes.

Clinical reports of combined chemo-immunotherapy

A growing number of publications report promising results of treating patients with different types of cancer by combination of chemotherapy and immunotherapy.[86-90,92-99] Interpretation and comparison of the results of clinical trials with chemo-immunotherapy is difficult because of heterogeneity in study design, patient eligibility criteria including the type of malignancy, therapeutic approach used and immune endpoints measured. For instance, most studies included patients with recurrent or advanced disease, who may have had poor clinical conditions because of low performance status, extensive pre-treatments and a large tumor burden. Immunotherapy is considered to be less effective in patients with a large tumor burden, and the classic volumetric response criteria have been shown to be inadequate for the evaluation of the efficacy of immunotherapy or combined chemo-immunotherapy.[27,30,100]

Also, the studies were generally not powered statistically to test the synergistic effects of immunotherapy and chemotherapeutic agents, were retrospective and compared the results with data of an historical control group of patients. As most clinical trials did not reach phase III yet, effects on primary endpoints such as overall survival and progression-free survival are not available, while these are important in the development of a new therapeutic approach. Furthermore, a variety of immune effects were used as surrogate endpoints, in particular immune infiltrate parameters and serologic antitumor immune markers, that suggested to have a positive prognostic and predictive impact on the clinical benefit for cancer patients (reviewed in[101]). It was additionally reported that the optimal sequence of combined chemo-immunotherapy remains to be established and more knowledge on schedules and doses are required to optimally combine cytotoxic chemotherapy with immune stimuli.

IMMUNOLOGICAL OUTCOMES OF CHEMO-IMMUNOTHERAPY TRIALS IN PATIENTS WITH ADVANCED CANCER

Monoclonal antibodies are widely used for the treatment of cancer, and combinations of MoAbs with chemotherapy have been registered for various tumor types, eg trastuzumab for the treatment of breast cancer [90,91] and bevacizumab for breast, colorectal and ovarian cancer.[86-89] The combination of trastuzumab and paclitaxel induced humoral and cellular HER2-specific immune responses in 27 patients with advanced breast cancer. In this small study, it was suggested that by the induction of HER2-specific $CD4^+$ cells and humoral immunity, therapeutic antibodies could promote active immunity when combined with chemotherapy.[102]

Data from studies in cancer patients have shown that the induction of tumor-specific T-cells is not impaired by chemotherapeutic treatment. In a pilot study, the effects of dacarbazine on the immune response were evaluated in ten HLA-$A2^+$ disease-free melanoma patients, who received anti-cancer vaccination

either as mono-therapy or one day after chemotherapy. In the chemotherapy-pretreated patients, a marked expansion of blood-derived peptide-specific CD8+ T-cells displaying a long-lasting effector memory phenotype was observed.[103] In a small cohort of colon cancer patients, the effect of treatment with oxaliplatin and capecitabine on non-specific and specific DC vaccine-induced adaptive immune responses showed that platinum-based therapy did not affect DC vaccination and the proliferative capacity of T-cells upon stimulation with phytohemaglutinin (PHA) even increased upon treatment.[104] This effect has not been reported before for platinum-based compounds. The fact that platinum-based chemotherapy induces an immunogenic type of tumor cell death resulting in enhanced DC activation[105-107], supports the strategy to combine platinum-based chemotherapy with immunotherapy.

In another trial, eleven patients with advanced gastric or colorectal carcinoma received six peptide vaccinations every two weeks in combination with daily oral administration of low or standard dose of 5-fluorouracil-based chemotherapy during 4 weeks. This combination therapy was associated with an increase in peptide-specific antibodies, i.e. immunoglobulin G (IgG) in the vast majority of the patients. An increase in peptide-specific interferon-gamma (IFNγ) production by CD8+ T-cells was detected in patients treated with the highest dose of chemotherapy.[92] A recent phase 2 single-arm study in ten patients with recurrent ovarian cancer, administration of p53 synthetic long peptide (SLP) vaccine was preceded by the administration of low-dose intravenous cyclophosphamide (300mg/m^2) in attempt to improve immunogenicity by effects on the number of regulatory T-cells.[108] Although in this study no quantitative reduction of Tregs nor a demonstrable qualitative difference of Treg function in vitro was induced by cyclophosphamide, the number of vaccine-induced p53-specific IFNγ-producing T-cells was higher in the cyclophosphamide pre-treated patients compared to findings of a previous study in which a similar patient group was treated with p53-SLP mono-therapy.[108,109] Similarly Audia et al reported a failure of cyclophosphamide to modulate significantly Treg numbers or function in humans.[110] There are, however contrasting, reports describing that the same low dose of cyclophosphamide (300mg/m^2) in combination with immunotherapy decreased the number of Tregs and did impair their function.[111] Cyclophosphamide is also reported as an inducer of a profound and systemic type I interferon release, resulting in enhanced activation and expansion of DCs and T-cells, which partly explains the immunomodulatory effects of cyclophosphamide.[112] When combined with specific vaccinations, tumor-specific immune responses were induced. This suggests that targeting function and frequency of Tregs by cyclophosphamide enhances tumor-specific T-cell responses.[113] Cyclophosphamide

administration can enhance tumor-specific immunity in a variety of ways and does not impair the induction of vaccine-induced tumor-specific effector T-cell responses at these doses or in the specific treatment schedules used.

COMBINATION OF CHEMO- AND IMMUNOTHERAPY; SYNERGY AND OPTIMAL TIMING

Different studies have suggested that the timing of chemotherapy administration relative to immunotherapy plays a crucial role in patient's outcome.[31,96,114] In some clinical trials, immunotherapy and chemotherapy were given within the same time frame which allows immunotherapy to be present at the earliest phase of chemotherapy-induced antitumor and immunomodulatory effects.[94] In addition, chemotherapy might modulate immunosuppressive cells and improve immunotherapy-induced immune responses. The timing of chemotherapy may differ per immunotherapeutic regimens. For instance, a randomized study on the timing of ipilimumab in extensive small-cell lung cancer revealed that ipilimumab should be given best after a first round of chemotherapy.[114] One can envisage that it is first needed to activate T-cells via immunogenic cell death of the tumor before one increases T-cell expansion by ipilimumab. On the other hand, when vaccines are used to drive the tumor-specific T-cell response one might opt for a schedule where immunotherapy precedes chemotherapy, if such a therapy causes apoptotic tumor cell death, stress signal release and upregulation of recognition molecules on tumor cells.[96,115]

Currently, there is no systematic assessment of the order in which cytotoxic therapies and tumor vaccines are administered, but it is clear that the different mechanisms that may cause possible synergy between chemotherapy and immunotherapy strongly depend on both the chemotherapeutic compound and the immunotherapeutic approach. Below, the outcomes of some clinical trials in which different sequences of combined chemo-immunotherapy are outlined.

SIMULTANEOUS CHEMO-IMMUNOTHERAPY Studies investigating combined chemo-immunotherapy have employed different designs to explore the additional effect of chemotherapy and immunotherapy. For example, combined chemo-immunotherapy with gemcitabine and personalized peptide vaccination administrated simultaneously (both weekly and at the same day), was performed in 13 patients with advanced pancreatic cancer, and showed a reduction of tumor size in 85% of the patients and an augmentation of peptide-specific CTL activity against pancreatic cancer cells in all patients.[94] This translated into a median time to progression of 7 months and a median overall survival of 9 months.[116]

In a controlled phase 2B trial, it was investigated whether administration of a therapeutic vaccine could improve the clinical outcome of non-small cell lung cancer patients (n = 148) receiving simultaneous first-line chemotherapy.[93] Chemotherapy consisting of cisplatin (day 1) and gemcitabine (day 1 and 8) was administrated every 3 weeks for up to 6 cycles, while the vaccine was given weekly during 6 weeks, and subsequently 3-weekly during chemotherapy. A higher response rate was noticed in the combination therapy group, compared to patients treated with chemotherapy alone. The 6-months progression free survival was 43.2% in the combination therapy group, compared to 35.1% in the chemotherapy alone group, but median overall survival was similar in both groups.[93]

Ipilimumab, the moAb which blocks CTLA-4 and thereby expands T-cell activation and proliferation[22], was combined with the chemotherapeutic agent dacarbazine in a phase III study with metastatic melanoma patients.[117] Patients were assigned to receive ipilimumab plus dacarbazine or dacarbazine plus placebo every 3 weeks for 4 cycles. This treatment was followed by 4 cycles of dacarbazine every 3 weeks. A significant improvement in overall survival was noted among patients treated with ipilimumab plus dacarbazine, compared to the dacarbazine plus placebo group. In addition, survival rates were higher for the ipilimumab-dacarbazin group at 1 year (47% versus 36%), 2 years (28% versus 18%) and 3 years (21% versus 12%).[117]

CHEMOTHERAPY AFTER IMMUNOTHERAPY In a small trial with 29 extensive stage small cell lung cancer patients, patients first received p53-pulsed DCs followed after 3-4 weeks by chemotherapy with paclitaxel or carboplatin. It was shown that surprisingly high rates of objective clinical response (complete or partial response) occurred when chemotherapy was administered after immunotherapy with p53-pulsed DCs (61.9%).[96] It was reported that up to 38% of the patients receiving immunotherapy followed by chemotherapy, survived at one year following vaccination. This is surprisingly high when compared to historical data showing objective response rates to a second-line chemotherapeutic of 6-16% and less than 20% of the patients alive after one year.[118] The objective clinical responses in the combination treatment group were closely associated with the induction of an immunologic response to vaccination, as 9 out of 12 patients who had a positive immunologic response to immunization, developed a complete or partial clinical response.[96] This suggests that the presence of anti-p53 cellular immunity synergizes with subsequent chemotherapy to provide potent anti-tumor immunity responses or to improve chemotherapeutic target effects in these patients.[96] These data are consistent

with observations made in a phase I study in which 17 patients with different types of advanced stage cancer were treated with cytochrome P450 1B1 (CYP1B1)-directed vaccination, followed by salvage chemotherapy.[98] The carcinogen activator cytochrome P450 1B1 is expressed on almost all human tumors and it was suggested that it could function as a 'universal' tumor antigen.[119] While 10 from the 11 patients, who did not develop an anti-CYP1B1-specific T-cell response, failed to respond to subsequent salvage therapy, 5 out of 6 patients showing immunity against CYP1B1 demonstrated clinical benefit to salvage therapy. It was hypothesized that immunity to CYP1B1 primes for response to salvage therapy.[98] Wheeler et al[95] retrospectively analyzed the overall survival of 25 vaccinated (13 with and 12 without subsequent chemotherapy) patients versus 13 non-vaccinated patients suffering from de novo glioblastoma subsequently receiving chemotherapy. The survival of patients receiving vaccination and chemotherapy was significantly higher compared to the survival in the isolated chemotherapy group and the vaccine alone group. Three patients exhibited objective (> 50%) tumor regression, two of which had an overall survival of more than 2 years.[95] In another randomized study, 57 patients with castration resistant prostate cancer were treated with chemotherapy or chemo-immunotherapy. Twenty-eight patients received personalized peptide vaccination plus low-dose estramustine phosphate and 29 patients received standard-dose estramustine phosphate.[99] The combination therapy was associated with increased immunological responses, resulting in significantly longer median progression-free survival of 8.5 months compared to standard-dose estramustine phophate treatment (2.8 months). It is thus plausible to suggest a potential clinical benefit of first line personalized peptide vaccination plus low-dose estramustine phosphate as compared to standard-dose estramustine phosphate. However, follow-up periods were short, hampering to draw conclusions on the real clinical efficacy of adding peptide vaccination to chemotherapy. Arlen et al reported on a phase II study in patients with metastatic androgen resistant prostate cancer who were randomized to receive a prostate-specific antigen vaccine either alone or in combination with weekly low-dose docetaxel. In this trial it was demonstrated that docetaxel did not inhibit vaccine-specific T-cell responses.[97] In addition, patients who were previously vaccinated with the anti-cancer vaccine, responded longer to docetaxel (progression free survival of 6.1 months) compared to a historical patient control group receiving only docetaxel (3.7 months). Based on these results, the authors hypothesized that cancer patients treated with an anticancer vaccine may respond longer to a cytotoxic agent as docetaxel.[97]

Taken together, these studies suggest that immunotherapy followed by chemotherapy has higher clinical efficacy than what is found for historical

or randomized patient control groups treated with chemotherapy alone. In addition, these studies show that clinical responses were associated with an immunologic response to vaccination. These observations not only suggest that the immunostimulatory functions of conventional chemotherapeutics may be beneficial in combination with immunotherapy, but enhanced anti-tumor immune responses might be predictive for the success of chemotherapy and eventually for the clinical benefit. As most studies were small and non-randomized, confirmation of the role of the immune status of the patients in the prediction of clinical success is warranted. It is uncertain whether clinical responses are caused by combined chemo-immunotherapy or whether patients with enhanced anti-tumor responses simply respond better to chemotherapeutic treatment due to their positive immune status before treatment initiation. The previously beneficial immune and clinical effects of combined of immune-chemotherapy is a new and promising field in clinical research. Nevertheless, given the nature of the adjuvant treatment, the clinical state of patients, the short follow-up times and the limited number of patients and non-controlled trials, it is too early to draw robust conclusions on the clinical efficacy of this treatment modality.

CHEMO-IMMUNOTHERAPY IN THE TREATMENT OF CERVICAL CANCER

In recurrent or metastatic cervical cancer, chemotherapy regimens with cisplatin or carboplatin and paclitaxel are most commonly used.[5,6,120] The cisplatin-paclitaxel combination has demonstrated favorable trends in response rates, progression free survival and overall survival compared to combinations of cisplatin with vinorelbine, gemcitabine or topotecan in advanced and recurrent cervical cancer patients.[6] Therefore, the platinum-based doublet combination with paclitaxel is currently the most frequently used treatment. Historically, cisplatin is the most extensively studied cytotoxic agent in cervical cancer, but this may change in favor of carboplatin which has similar efficacy both as a single agent and in combination with paclitaxel and a more favorable non-hematologic toxicity profile.[121,122]

As previous clinical studies showed no encouraging results on the use of MoAb monotherapy, new studies evaluate whether the addition of MoAbs to standard cytotoxic treatment for cervical carcinoma could result in better in outcomes in terms of progression-free survival and overall survival (reviewed in[123]). The use of passive immunotherapy with EGFR and VEGF MoAbs in combination with standard chemotherapy in cervical malignancies is investigated in ongoing trials, but data have not been reported yet.

Recent studies have shown that in patients with high grade premalignant lesions of the vulva therapeutic vaccination with a vaccine consisting of the HPV16 E6 and E7 synthetic long peptides is highly immunogenic in patients with HPV16-induced (pre)malignant disease and resulted in clinical success.[124-127] In a phase I study with advanced cervical cancer patients, HPV16 E6/E7 SLP vaccination showed limited overall clinical efficacy, despite a robust HPV16 E6- and E7-specific T-cell mediated IFNγ-production.[124,128] This was probably due to the immunosuppressive micro-environment of the tumor and other immune escape mechanisms.[129] Interestingly, unexpected long term survival was observed in a small number of patients: 5 of the 43 vaccinated patients had stable disease for at least one year, and 1 patient had a complete remission. These 5 patients mounted robust T-cell responses to E6 and E7 at follow-up, 3 weeks after last vaccination. Anecdotally, the patient with a complete remission was treated with chemotherapy before vaccination, and four of the five others received platinum-based chemotherapy after immunotherapy. We therefore retrospectively evaluated the long-term clinical outcomes of the patients from this study.[124] We analyzed if immunotherapy given closely before or after chemotherapy ('combined' chemo-immunotherapy) was associated with a more favorable outcome, compared to isolated immunotherapy or isolated chemotherapy. We also obtained follow-up data of an historical control group. This historical control group consisted of 24 recurrent or advanced cervical cancer patients treated with chemotherapy between October 1987 and December 2007 at Leiden University Medical Center from whom clinico-pathological and follow-up data were available. Clinical parameters of the patient groups were collected, including data on age, FIGO-stage, histology of the tumor, primary treatment, time to recurrence, site of recurrence, treatment of recurrence, interval between different treatments and date of death or last follow up (Table 1). Baseline characteristics were not different between the 3 treatment groups at the time of diagnosis of recurrence (Table 1). All vaccinated patients (with and without chemotherapy pre- or post-treatment) had advanced or recurrent carcinoma of the cervix and met the same eligibility criteria, as they participated in the same phase I clinical trial. Recurrence treatment was defined as combined chemo-immunotherapy if the interval between chemotherapy and HPV16 E6/E7 SLP vaccination was less than 3 months. This interval was based on previous clinical studies that retrospectively examined the impact of therapeutic vaccination of the efficacy of conventional chemotherapy in cancer patients.[95,96]

The majority of the patients treated with chemotherapy before vaccination-study participation had not responded or disease progression. Patients without previous chemotherapy were patients who were ineligible for further standard treatment. All patients had a life expectancy of more than 3 months.

A post-hoc analysis of the clinical outcomes of the patients revealed a mean survival time since recurrence of 26.4 months for patients treated with the combination of chemotherapy and HPV16 E6/E7 SLP vaccination compared to 9.4 months for patients treated with chemotherapy alone (p = 0.03, log-rank test) and 17.4 months for patients treated with HPV16 E6/E7 SLP vaccination alone (p = 0.2, log-rank test). Although statistically not significant for all comparisons, these data suggest that therapeutic vaccination in this category of patients has limited therapeutic action by itself, but might exert improved therapeutic activity if combined with chemotherapy. Kaplan - Meier curves are shown in Figure 2. Patients were comparable at the time of the treatment, but post-hoc analyses showed that patients treated with isolated chemotherapy more often had an early stage of disease at the first diagnosis of cervical cancer, while in the immunotherapy groups more advanced FIGO stage occurred (Table 1), suggesting that the group receiving chemotherapy only was not more likely to display lower survival than the vaccinated patients.

This phase I study was not designed to test the presence of synergistic effects between chemotherapy and vaccination and may be prone to several sources of bias. The heterogeneity in disease stage at presentation, previous therapies, stage and treatment of disease makes it difficult to exactly delineate the contribution of cytotoxic, vaccine treatment or a combination thereof on survival rates. It might have been that patients eligible for immunotherapy had a favorable clinical status at baseline and that this explains the observation of a trend in survival difference between the combination group and immunotherapy group only. Nevertheless, the observations of clinical responses, prolonged survival times and induced anti-tumor responses are encouraging and support the investigation of combined chemo-immunotherapy using carefully designed trials. All combined chemo-immunotherapy studies, including ours, suffer from small sample size, several sources of bias, and possibly patient selection. The lack of consensus regarding optimal timing and dosing and the possibility that different tumor types may require different chemo-immunotherapy combinations further complicates interpretation of the available data. The optimal schedule of chemo-immunotherapy for cancer patients remains to be established, and additional clinical studies are necessary to ultimately determine this optimal combination regimens schedule. Within such clinical studies, it is highly important to ensure inclusion of carefully selected patients, their stage of disease and tumor type but also the selection of the kind, dose and sequence of both the cytotoxic compound(s) and the immunotherapy. An important focus should be the kinetics of the immune response in relation to the chemotherapy schedule, since this is likely to be critical for a successful clinical response.

Concluding remarks and future perspectives

Despite some advances during the last decade in the field of active cancer immunotherapy, clinical efficacy and progress in cancer patients has been slow. Nevertheless, combinations of chemotherapy and immunotherapy have shown more encouraging clinical outcomes than either of these treatment modalities alone. Experimental and clinical studies have suggested that several chemotherapeutic agents may facilitate an enhanced immune-mediated anti-tumor response, and may synergize with immunotherapy. For the implementation of effective combinatorial treatments, an elicited long lasting protective T-cell response appears to be required within an appropriate therapeutic regimen. It must be emphasized that chemotherapeutic compounds showing immune effects, and thus the preferred compounds to be used in combination with immunotherapy, must be scrutinized further with regard to optimal timing, dosing and scheduling of the two therapies. This research therefore needs comparison of different, non-standard treatment schemes to obtain synergy between both treatments.[115]

To achieve such optimal designs, the immunological effects of chemotherapy on the tumor itself and on the effector lymphocytes and antigen presenting cells of the immune system in vivo in cancer patients should be monitored and evaluated. For instance, it would be a challenge to perform prospective trials in which peripheral blood samples, performed before, during and after treatment with standard doses of chemotherapy, are evaluated for both general and specific anti-tumor immune responses. Ideally, the evaluation of systemic immune effects in blood in combination with the evaluation of local immune effects in tumor samples, would allow an even more complete understanding of the immunological effects of chemotherapy in cancer patients. The additional challenge is to understand the final outcome of the changes in various stimulatory and regulatory immune factors in cancer patients under chemotherapy and being able to manipulate these mechanisms effectively to enhance anti-tumor responses. If such dynamic immunopharmacological effects can be monitored in time, it might be possible to determine exactly if chemotherapy can enhance (vaccine induced) immunity in cases of cancer. These types of studies are just emerging. Currently, we are investigating the effects of standard chemotherapy with carboplatin and paclitaxel on the immune system in cervical cancer patients. Eventually this should lead to controlled, clinical trials with patients allocated to chemotherapy in combination with immunotherapy. This pharmacology- driven approach could give a true insight in the effect of immunotherapy on chemotherapy, immune responses and eventually survival

rates. In addition, the identification of immune-specific biomarkers and further elucidation of immunotherapeutic mechanisms of action will be essential to determine at which moment patients will have the greatest benefit of combined chemo-immunotherapy. Surrogate endpoints such as immune responses can be helpful in the prediction of the clinical outcomes.[130] To optimally capture the effects of combined chemo-immunotherapy, response profiles of both chemotherapy and immunotherapy should be investigated. Notably, the unique characteristics of immunotherapeutic agents may induce cancer-specific immune responses far before affecting tumor growth or patient (progression-free) survival.[131] Therefore, recently established endpoints as immune related Response Criteria (irRC) could offer an additional tool, as these criteria appear to more comprehensively capture all observed response patterns compared to those of cytotoxic agents.[100] Frequently, there is a delayed detection of clinical activity after immunotherapeutic treatment, and the RECIST criteria may not offer a complete description of the response to immunotherapeutic agents. As chemotherapy has shown to ultimately influence the immune system, these new immune-related response criteria could additionally be used in a concept for the clinical investigation of combined chemo-immunotherapy.

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Figure 1 Interaction of tumor cells and immune system of cytotoxic chemotherapeutics used for cervical cancer. Some of the anticipated positive effects cytotoxic chemotherapeutics used for cervical cancer on the immune system of include: the rapid translocation of calreticulin (CRT) to the cell surface and the release of adenosine 5'-triphosphate (ATP) and high mobility group protein box 1 (HMGB1) inducing immunogenic cell death and activation of dendritic cells through calreticuline receptor (CRTR), P2X purinoceptor (P2RX7) and Toll-like receptor 4 (TLR4); depletion of suppressive immune cells as regulatory T-cells (Tregs) and myeloid derived suppressor cells (MDSCs); inhibition of signal transducer and activator of transcription 6 (STAT6) and down regulation of programmed death ligand 2 (PD-L2), increasing the sensitivity of tumor cells for lysis by cytotoxic T-cells and triggering tumor-specific T-cell expansion and activation; direct activation of dendritic cells; increasing the permeability of tumor cells to Granzyme B by mannose 6 phosphate (M6P) upregulation.

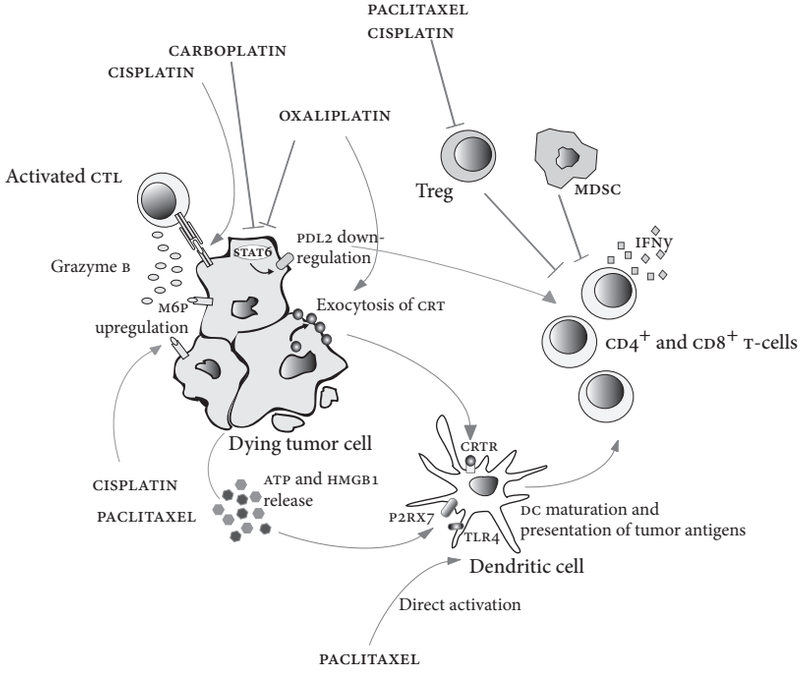

Figure 2 Kaplan-Meier curves suggesting that therapeutic vaccination in patients with advanced cervical cancer has limited clinical effects by itself, but might exert improved therapeutic action if combined with chemotherapy

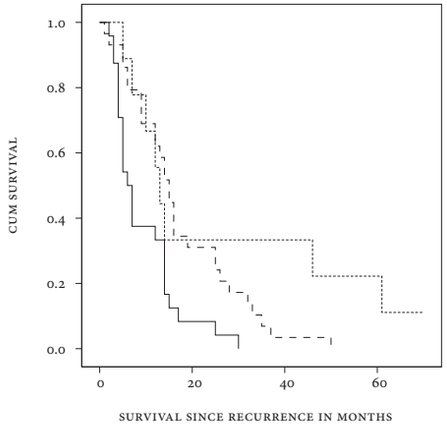

Table 1 Clinicopathological characteristics of advanced cervical cancer patients treated with combined chemo-immunotherapy, isolated immunotherapy or chemotherapy alone.

	Control group	Phase I study with HPV16 E6/E7 SLP vaccination			
	Isolated chemotherapy (n=24)	Isolated immunotherapy (n=33)	Combined chemoimmunotherapy* (n=10)	p-values IC vs CCI	** II vs CCI
FIGO stage				0.03	0.69
IA1-IB2	21 (87.5%)	15 (45.5%)	5 (50%)		
IIA1-IIB	3 (12.5%)	11 (33.3%)	2 (20%)		
IIIA-IIIB	0	4 (12.1%)	2 (20%)		
IV	0	2 (6.1%)	0		
missing (unknown)	0	1 (3%)	1 (10%)		
AGE AT DIAGNOSIS				0.24	0.27
Mean (SD)	40.6 (10.8)	41.3 (10.1)	45.6 (9.7)		
AGE AT RECURRENCE				0.22	0.32
Mean (SD)	41.7 (10.9)	43.2 (9.9)	46.8 (10.3)		
DFI IN MONTHS				0.2	0.88
< 12 months	12 (50%)	16 (48.5%)	5 (50%)		
13-24 months	10 (41.7%)	6 (18.2%)	2 (20%)		
> 24 months	2 (8.3%)	9 (27.3%)	3 (30%)		
unknown	-	2 (6.1%)	-		
SITE OF RECURRENCE				0.267	0.91
locoregional	11 (45.8%)	21 (63.6%)	6 (60%)		
distant metastasis	11 (45.8%)	10 (30.3%)	3 (30%)		
locoregional & distant	2 (8.3%)	-	-		
unknown	-	2 (6.1%)	1 (10%)		
SURVIVAL SINCE RECURRENCE				0.03	0.2
Mean (SD)	9.4 (1.5) months	17.4 (2.2) months	26.4 (7.9) months		

** Maximum interval between chemotherapy and immunotherapy was 3 months*
*** Differences in characteristics were evaluated with Chi-square test. For survival log-lank was used*
Abbreviations: FIGO = International Federation of Gynecology and Obstetrics; DFI = disease free interval (defined as the time from last primary treatment to evidence of recurrent disease); IC = Isolated Chemotherapy; II = Isolated Immunotherapy; CCI = Combined chemo-immunotherapy

PART 2

TREATMENTS TO REINFORCE THE IMMUNE SYSTEM

V

VACCINATION DURING MYELOID CELL DEPLETION BY CANCER CHEMOTHERAPY FOSTERS ROBUST T-CELL RESPONSES

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ABSTRACT

Therapeutic vaccination with human papillomavirus type 16 synthetic long peptides (HPV16-SLPs) results in T-cell-mediated regression of HPV16-induced premalignant lesions but fails to install clinically effective immunity in patients with HPV16-positive cervical cancer. We explored whether HPV16-SLP vaccination can be combined with standard carboplatin and paclitaxel chemotherapy to improve immunity and which time point would be optimal for vaccination. This was studied in the HPV16 E6/E7–positive TC-1 mouse tumor model and in patients with advanced cervical cancer. In mice and patients, the presence of a progressing tumor was associated with abnormal frequencies of circulating myeloid cells. Treatment of TC-1-bearing mice with chemotherapy and therapeutic vaccination resulted in superior survival and was directly related to a chemotherapy mediated altered composition of the myeloid cell population in the blood and tumor. Chemotherapy had no effect on tumor-specific T-cell responses. In advanced cervical cancer patients, carboplatin-paclitaxel also normalized the abnormal numbers of circulating myeloid cells, and this was associated with increased T-cell reactivity to recall antigens. The effect was most pronounced starting 2 weeks after the second cycle of chemotherapy, providing an optimal immunological window for vaccination. This was validated with a single dose of HPV16-SLP vaccine given in this time window. The resulting proliferative HPV16-specific T-cell responses were unusually strong and were retained after all cycles of chemotherapy. In conclusion, carboplatin-paclitaxel therapy fosters vigorous vaccine induced T-cell responses when vaccination is given after chemotherapy and has reset the tumor-induced abnormal myeloid cell composition to normal values.

Introduction

The majority of cervical cancers is induced by human papillomavirus type 16 (HPV16).[1] Up to 70% of the advanced cancers relapse.[2-3] One of the preferred treatments for patients with recurrent, metastatic, or advanced cervical carcinoma is the combination of carboplatin with paclitaxel (CarboTaxol)[4], but this is rarely curative.[5]

The two HPV16-encoded oncoproteins E6 and E7 are required for the transformation of epithelial cells[6] and constitute excellent targets for the immune system. HPV16-specific T-cell reactivity is frequently detected in healthy individuals but usually not in patients with premalignant anogenital lesions or cancer.[7] Installment of robust HPV16-specific immunity by vaccination with therapeutic HPV16 overlapping synthetic long peptides (HPV16-SLPs) admixed with Montanide ISA-51 resulted in regressions of HPV16-induced premalignant lesions of the vulva in two independent studies.[8-10] In contrast, therapeutic vaccination of patients with advanced or recurrent HPV16-positive cervical cancer partly installed HPV16-specific T-cell reactivity, particularly in patients with a less suppressed immune status, but had no clinical effect.[11]

Chemotherapeutic agents act on cancer cells[12], but many of them mediate part of their therapeutic effects through immune mechanisms.[13,14] In murine models, the combination of chemotherapy with activation of T-cells resulted in improved treatment of tumors.[13-16] Therefore, we investigated whether CarboTaxol could be successfully combined with HPV16-SLP vaccination, first in a mouse model[13] and then in an open label observational study with cervical cancer patients.

Materials and Methods

STUDY DESIGN

The aim of the study was to test whether CarboTaxol could be combined with HPV16-SLP vaccination. We first used the HPV16 E6/E7-expressing TC-1 tumor mouse model to define the impact of CarboTaxol on systemic and intratumoral immunological parameters as well as on the clinical efficacy of vaccination. After observing that CarboTaxol did not affect lymphocytes but had a strong effect on myeloid cells and improved tumor control by therapeutic vaccination, we started a multi-center, open label, observational study, entitled 'Immunological aspects of combined chemo-immunotherapy in patients with advanced cervical cancer' (EudraCT 2010-018841-76), consisting of two cohorts of patients. In

the first cohort, 6 patients with advanced, recurrent, or metastatic cancer were treated with 6 cycles of CarboTaxol every 3 weeks, and the composition and function of the myeloid and lymphoid cells in peripheral blood were analyzed. After identifying a specific time window during chemotherapy potentially permitting the best T-cell response, we studied the second cohort of patients. In this cohort, 12 patients were treated with CarboTaxol and one dose of an HPV16 SLP vaccine 2 weeks after the second cycle of CarboTaxol. Blood samples were drawn to validate the observations made in the first cohort as well as to study the vaccine-induced T-cell response. The investigators performing and analyzing immunological assays were blinded to the clinical parameters of the patients. The data from the immunomonitoring studies are reported according to the recommended standard format 'minimal information about T-cell assays.'

MICE AND TUMOR TREATMENT

Female C57BL/6 mice (6 to 8 weeks old; Charles River Laboratories) were housed in individually ventilated cage systems under specific pathogen–free conditions. The experiments were approved by the Animal Experiments Committee of Leiden University Medical Center (LUMC), in line with the guidelines of the European Committee. The tumor cell line TC-1 is of C57BL/6 origin and expresses HPV16 E6 and E7.[17] TC-1 tested negative for rodent viruses by polymerase chain reaction. Mice were subcutaneously inoculated with 1×10^5 TC-1 tumor cells. When a palpable tumor was present on day 8, mice were split into groups with comparable tumor size and treated with carboplatin [40 mg/kg, day 8, intraperitoneally], paclitaxel (20 mg/kg, days 8 and 9, intraperitoneally), and/or subcutaneous synthetic long HPV16 E743–77 peptide (SLP; GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR; 150 μg) dissolved in dimethyl sulfoxide (Sigma), diluted in phosphatebuffered saline (B. Braun), and emulsified in Montanide ISA-51 (Seppic). Chemotherapy was repeated 1 week later (day 15 for carboplatin and days 15 and 16 for paclitaxel), and vaccination was repeated 2 weeks after initial treatment (day 22). Detailed information on the immunomonitoring and statistics is given in the Supplementary Materials.

PATIENTS

Patients with clinical and radiological evidence of advanced-stage, recurrent, or metastatic cervical cancer; with no curative treatment options; and scheduled for CarboTaxol were enrolled between January 2011 and January 2013. Other inclusion criteria were as follows: (I) mentally competent patients 18 years or older, (II) no other active malignancy, (III) no indication of active infectious disease such as HIV, (IV) and no other condition that may jeopardize the health status of the patient. Patients were followed until 2 to 3 weeks after they had received their last chemotherapy cycle and thereafter at standard visits. LUMC, Academic Medical Center (Amsterdam), Free University Medical Center (Amsterdam), and Netherlands Cancer Institute–Antoni van Leeuwenhoek Hospital (Amsterdam) were the participating hospitals.

HPV typing was performed on the tumor and/or smears taken at study entry[8], but it was not part of the inclusion criteria. The study was conducted in accordance with the Declaration of Helsinki (October 2008) and approved by the Central Committee on Research Involving Human Subjects (NL31572.000.10) in agreement with the Dutch law for medical research involving humans.

TREATMENT OF PATIENTS

For the first cohort, six of the nine screened and eligible patients participated and were treated at their hospital with CarboTaxol, consisting of carboplatin (dose based on renal function; area under the curve of six regimen) and paclitaxel (175 mg/kg^2) on day 1 of each cycle, every 3 weeks for a maximum of six cycles. The patients were subjected to serial blood sampling. According to the oncology protocols, routinepremedication consisting of dexamethasone [20 mg, intravenously (iv)], ranitidine (50 mg in 100 ml of NaCl 0.9%, iv), granisetron (1 mg in 100 ml of NaCl 0.9%, iv), and clemastine (2 mg in 100 ml of NaCl 0.9%, iv) was administered immediately before chemotherapeutic treatment. In case of severe hematological toxicity, neurotoxicity, nephrotoxicity, or gastrointestinal toxicity, dose modifications of carboplatin and paclitaxel were made according to the following standard scheme: (i) if the absolute neutrophil count was $< 1.5 \times 10^9$/liter, platelet count was $< 100 \times 10^9$/liter, or other toxicities were higher than grade 2, then CarboTaxol treatment was postponed for at least a week (or longer until the patients had recovered), and the doses of both chemotherapeutic compounds were reduced by 25%; (ii) if the patient experienced neuropathy higher than grade 2, paclitaxel was stopped but carboplatin was continued. After the completion of immunomonitoring of these first 6 patients, a second group of 12 patients (cohort 2) received CarboTaxol at their hospital at the same schedule and dose, as well as a single subcutaneous vaccination of the HPV16-SLP vaccine (300 μg per peptide emulsified in Montanide ISA-51) consisting of two mixes of peptides injected separately in the left and right limb (either arm or leg)[9] at

LUMC, 2 weeks after the second cycle of CarboTaxol. For cohort 2, 18 advanced cervical cancer patients were screened, 5 patients declined participation, and 1 patient (ID6002) died of her disease before receiving the vaccine.

CLINICAL EVALUATION OF SAFETY AND TOLERABILITY

The safety and toxicity of treatment were evaluated according to the National Cancer Institute CTCAE v3.0. Well-known toxicities of CarboTaxol were classified as study-related events. Before the start of CarboTaxol and before vaccination, patients were physically examined and medical history was obtained. Vital signs were measured, and the injection site was inspected 15 min, 1 hour, and 4 hours after vaccination. Patients were followed with routine visits (every 3 months until progression) to monitor for AEs. For each vaccine-related AE, the relationship to HPV16-SLP was defined as definite, probable, or possible. Injection site reactions were classified as definitely vaccine-related. Injection site reaction grade 1 was defined as swelling, erythema, and tenderness (pain/itching). Injection site reaction grade 2 was defined as tenderness or swelling with inflammation or phlebitis. Injection site reaction grade 3 was defined as severe ulceration or necrosis. Venous blood samples were drawn for routine hematological analysis, including leukocyte differential counts and biochemistry assessments. Patients were followed up until date of death or loss to follow-up.

IMMUNOMONITORING OF CLINICAL TRIAL

Blood samples from patients were taken at the time points indicated in figure 4A. In addition, 19 healthy volunteers donated blood. PBMCs were isolated by Ficoll gradient centrifugation, and cells were subjected to LST.[9-11] MRM and influenza peptide pools served as positive controls. The remaining cells were cryopreserved until use. Thawed PBMCs were tested for their response to PHA in a proliferation assay[18,19], for their antigen-presenting capacity in an MLR[19], and for their HPV16-specific T-cell responses by intracellular cytokine staining.[10] The 11-day stimulated nondepleted and CD14-depleted PBMC samples were analyzed by a proliferation assay for antigen recognition.[20] The supernatants of the proliferation assays were used for cytokine analysis by cytometric bead array.[9-11] Immunophenotyping of the PBMC samples was performed by flow cytometry.[19] Detailed information on immunomonitoring and statistics is given in the Supplementary Materials.

Results

COMBINED CHEMOIMMUNOTHERAPY IMPROVES THE ERADICATION OF HPV16-POSITIVE TUMORS IN MICE

To test the effects of CarboTaxol with HPV16-SLP vaccination, HPV16 E6- and E7-positive TC-1 tumor bearing mice were treated when tumors were palpable at day 8 (~4 mm^2, figure 1A). CarboTaxol had little effect on tumor growth, whereas vaccination induced a temporary decrease in tumor size (figures 1B and 1C). The combined treatment had the strongest antitumor effect (figures 1B and 1C). None of the treatments affected the percentages of circulating $CD8^+$ and $CD4^+$ T-cells (figures 1D and 1E; figures S1A and S1B). Vaccination induced HPV16-specific $CD8^+$ T-cells, and this was not influenced by co-treatment with CarboTaxol (figure 1F and figure S1C).

CARBOTAXOL TREATMENT ALTERS CIRCULATING AND INTRA-TUMORAL MYELOID CELL POPULATIONS

To understand the mechanism underlying these improved therapeutic outcomes, immune cells in blood and tumors were analyzed 3 to 4 days after CarboTaxol treatment (and/or 9 to 10 days after peptide vaccination) (figure 2A), which is at the start of the tumor regression phase (figure S2). In untreated tumor-bearing mice, the percentage of circulating myeloid cells increased (figure S3A) because of the increase in circulating $CD11b^{hi}$ cells, in particular, $CD11b^{hi}Gr\text{-}1^{hi}$ cells. However, their numbers decreased markedly in tumor-bearing animals treated with CarboTaxol (figures 2B and 2C, and figure S3). The frequencies of $CD4^+$ and $CD8^+$ T-cells, antigen-specific $CD8^+$ T-cells, monocytes, and dendritic cells in the blood were not affected by CarboTaxol treatment (figure S3). Thus, CarboTaxol treatment normalized the myeloid cell populations in the blood of tumor-bearing mice, making them more similar to those of naïve mice. This effect could not be ascribed to one individual chemotherapeutic compound because the effect on circulating $CD11b^{hi}$ cells was particularly pronounced in animals treated with both compounds (figure S4).

Next, we assessed the effects of CarboTaxol-vaccine combination treatment on the tumor microenvironment.[14] The percentage of intra-tumoral leukocytes increased upon treatment with CarboTaxol and/or vaccine (figure 2D). In vaccinated mice, a markedly high percentage of these leukocytes were $CD8^+$ T-cells (figure 2E), half of which were vaccine-specific (figure 2F) and capable of

producing IFN-γ and TNFα (figure 2G). There was no direct effect of CarboTaxol treatment on the presence and function of these lymphocytes. We then focused on the intra-tumoral CD11b^{hi} myeloid cells because the Gr-1^{hi} subtype of this cell population was increased in the blood of untreated tumor-bearing mice. The Gr-1^{hi} cells in the tumors strongly expressed the granulocytic marker Ly6G and decreased amounts of the macrophage marker F4/80 and the dendritic cell marker CD11c. In contrast, Gr-1^{int}-cells had a higher expression of F4/80, CD11c, CD80, CD86, and major histocompatibility complex class II, but not Ly6G (figure 3A), suggesting a superior immune stimulatory capacity. Treatment with either CarboTaxol or vaccine resulted in a predominance of the CD11b^{hi}Gr-1^{int} population over the Gr-1^{hi} population (figures 3B and 3C). Together, these data demonstrate that treatment of tumor-bearing mice with CarboTaxol results in a relative loss of myeloid cell-associated immunosuppression in both tumor and blood.

A SPECIFIC TIME WINDOW IS ASSOCIATED WITH INCREASED IMMUNITY IN PATIENTS ON CHEMOTHERAPY

On the basis of the above observations, we performed a study in patients with advanced, recurrent, or metastatic cervical carcinoma. The trial was designed to study the impact of chemotherapy on vaccine-induced immunity, and therefore patients were not required to have an HPV16 positive tumor. Patients were screened between January 2011 and January 2013 in 4 Dutch hospitals, and their characteristics are listed in table S1. In the first cohort of 6 patients, the number and function of lymphoid and myeloid cells was studied in blood samples taken at different time points during and after CarboTaxol treatment (figure 4A). CarboTaxol treatment was associated with a decrease in the otherwise high frequency of myeloid cells (median of 32% at baseline), which reached its nadir at 1 to 2 weeks after the second chemotherapy cycle (median of 6% at 1 to 2 weeks after chemo cycle 2; figures 4B and 4C) and coincided with an increase in the percentages of lymphoid cells (figure 4D). Although the relative frequencies of CD4$^+$ and CD8$^+$ T-cells (figure S5) remained unchanged, T-cell function was improved, as evidenced by the increase in their proliferation against a bacterial recall antigen mixture (MRM) in the same time window (figure 4E). T-cell responses to phytohemagglutinin (PHA) stimulation were strong at all time points, indicating that there were no intrinsic problems with the T-cells' response to mitogens (figure S5). The capacity of antigen presenting cells (APC) to stimulate allogeneic T-cell proliferation was slightly improved (figure 4F). Thus, the observations in mice are mirrored by the findings in patients. Furthermore, the results revealed a specific time window throughout CarboTaxol treatment, during which antigen-specific T-cell responses were optimal. This time window, starting at 1 to 2 weeks after the second cycle of CarboTaxol, appeared attractive for the generation of strong T-cell responses by vaccination. We used this observation to select the time window for the application of a single dose of vaccine in the second patient cohort.

CARBOTAXOL MEDIATES NORMALIZATION OF CIRCULATING IMMUNE CELL FREQUENCIES

The second cohort consisted of 13 patients (table S1). One patient (ID6002) died of progressive disease before vaccination and was substituted by ID6102. Compared to 19 healthy donors, the patients from both cohorts displayed an increased frequency of circulating myeloid cells before chemotherapy (figure S6A), confirming that the progressive tumor growth-induced myeloid changes in mice are mirrored in patients with advanced cervical cancer (figure 4). Throughout the CarboTaxol treatment, the absolute numbers of lymphocytes remained similar (figure 5A), but the absolute number of circulating leukocytes was strongly reduced (median -4.7 × 10^9/L) as measured by leukocyte differentiation analyses (figure 5B). This reduction reached its nadir after two cycles of chemotherapy and was retained during the remainder of the chemotherapy cycles. Flow cytometry analysis again revealed a decrease in myeloid (CD45$^+$CD3$^-$CD19$^-$) and a relative increase in lymphoid (CD45$^+$CD3$^+$CD19$^-$) cells (figures S6B and S6C). The frequency of these populations almost normalized to the levels observed in healthy donors (Figures 5C and 5D), and this correlated with an increased T-cell responsiveness to bacterial (MRM; figure 5E) and viral antigens (FLU; figure 5F). Similar to the first cohort, the blood samples of cohort 2 showed no overt changes in APC function or the response to PHA stimulation (figure S5).

Further dissection of the changes within the myeloid (CD45$^+$CD3$^-$CD19$^-$CD1a$^-$) cell population was performed on the basis of HLA-DR expression, to distinguish macrophages and dendritic cells (DCs; HLA-DR$^+$) from myeloid-derived suppressor cells (MDSCs; HLA-DR$^-$/^{low}) and further subdivide them on the expression of CD14 and CD11b within the HLA-DR$^+$ myeloid cell population (figure S7). Of the five identified subpopulations, population 1 (CD14$^+$CD11b$^+$) and population 2 (CD14^{int}CD11b^{int}) were most abundant and increased before CarboTaxol treatment when compared to healthy donors (figures 6A and 6B and figure S8). The other 3 populations each constituted 0.2 to 2.6% of the myeloid cell fraction. During chemotherapy, the frequencies of populations 1, 3, and 5 dropped (figure 6B and figure S8). The treatment-induced decrease in population

1 coincided with improved T-cell reactivity against MRM and FLU (figure 6C). Extended analysis of the various subsets by flow cytometry (figure S7) revealed that population 1 was comprised of M1 monocytes/macrophages ($CD45^+CD3^-CD19^-CD1a^-HLA\text{-}DR^+CD14^+CD11b^+CD206^-CD163^-CD16^-CD11c^+$) and M2c monocytes /macrophages ($CD45^+CD3^-CD19^-CD1a^-HLA\text{-}DR^+CD14^+CD11b^+CD206^-CD163^+CD16^-CD11c^+$). The frequency of both subpopulations was increased in patients but normalized upon treatment (figures 6D and 6E). The frequency of 10 distinct circulating MDSC populations[21] was not different between patients and healthy donors. Only the main MDSC population ($CD45^+CD3^-CD19^-CD1a^-HLA\text{-}DR^{low}$) displayed a slight decrease during chemotherapy (Figure 6F and figure S7).

Analysis of the T-cell populations (figure S7) revealed no changes in $CD4^+$ and $CD8^+$ T-cell frequencies (figures S9A and S9B), confirming our findings in mice. The frequency of TIM3 (T-cell immunoglobulin domain and mucin domain-3) and/or PD-1 (programmed cell death protein 1)-expressing $CD4^+$ or $CD8^+$ T-cells (figures S9C and S9D) and $CD4^+CD25^+CD127^-FOXP3^+$ regulatory T-cells (figure S9E) was higher in patients when compared to healthy controls. The percentage of $CD4^+TIM3^+PD1^-$ and that of regulatory T-cells slightly decreased during chemotherapy (figures S9C and S9E).

Together these results showed that CarboTaxol treatment strongly affected myeloid cells but not lymphocytes. CarboTaxol treatment normalized the amounts of different myeloid cell populations found to be increased in the blood of cervical cancer patients. This normalization of myeloid cell numbers coincided with improved T-cell reactivity to antigens from common pathogens, suggesting a relief from general immune suppression.

TIMED VACCINATION DURING CHEMOTHERAPY RESULTS IN A STRONG AND SUSTAINED HPV16-SPECIFIC T-CELL RESPONSE

Twelve patients received a single vaccination with the HPV16-SLP vaccine[8,10,11] at 2 weeks (13 to 17 days) after the second (n = 11) or third cycle of chemotherapy (n = 1; ID6008). None of the patients had a demonstrable pre-existing response to HPV16 E6/E7. Vaccination with the HPV16-SLP vaccine induced proliferative T-cell responses in 11 patients (figure 7A). The median stimulation index to all 6 peptide pools was 25.0 (range 4.3 to 133.4) at 3 weeks after vaccination in these responders. Vaccine-induced HPV16-specific proliferation was retained after 6 cycles of chemotherapy and in some cases even increased (median 21.0; range 5.0 to 141.5; figure 7A, black versus gray bars). The vaccine-specific proliferative T-cell response in the 7 HPV16 positive patients was not statistically higher than in the other patients (figure 7B). For six patients, enough PBMCs were available

to analyze the vaccine-induced T-cell response by intracellular staining for IFN-γ, IL-2, and TNFA. A poly-functional cytokine response to HPV16 E6 was measured in five and to E7 in four out of the six patients. One patient (ID6004) was anergic (figure S10), confirming the results of the proliferation assay (figure 7A). Previously, patients with recurrent HPV16 positive cervical cancer were vaccinated at least 1 month after chemotherapy.[11] In comparison to the responses seen during the earlier trial, the proliferative responses obtained by vaccination during chemotherapy were of far greater magnitude (figure S11).

MYELOID CELL DEPLETION IMPROVES THE RESPONSE OF PBMC TO STIMULATION IN VITRO

To recapitulate *in vitro* the association between a reduced myeloid cell population and improved T-cell reactivity to recall antigens and HPV16 vaccination, we depleted myeloid cells from the PBMC of two patients displaying relatively high frequencies of myeloid cells before chemotherapy and stimulated these PBMC with autologous monocytes pulsed with a mix of recall antigens, a mix of E6 and E7 peptides, or a mix of p53 peptides as control for 11 days before the antigen-specific T-cell response was tested. As a control, we used non-depleted PBMC. Not only was the T-cell response to recall antigens much higher in the culture started with myeloid cell-depleted PBMC, but the HPV16-specific response was also more efficiently boosted during these 11 days. As expected, no reactivity was detected in the cultures stimulated with the control p53 peptides (figure S12).

COMBINATION OF CHEMOTHERAPY WITH VACCINATION IS SAFE IN ADVANCED CERVICAL CANCER PATIENTS

Safety was assessed according to Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Most of the observed adverse events (AEs) were disease-related or chemotherapy-related. All patients developed chemotherapy-related anemia, thrombocytopenia, leucopenia, neutropenia, and alopecia. There were seven AEs, all in different patients, related to the advanced stage of the disease: shortness of breath, pulmonary embolism, abdominal pain (lymphedema), gastroenteritis, erysipelas, hydronephrosis. One patient (ID6002) died before vaccination could take place, and one patient (ID6004) died 11 weeks after vaccination. The cause of death in both cases was progressive disease. Vaccine-related AEs were largely localized to the vaccination site (table S2). One patient developed an ulcer at the injection site, which persisted for more than 6 weeks and required antibiotic treatment.

Discussion

Here, we observed that tumors expressing the HPV16 oncoproteins E6 and E7 cause the numbers of circulating myeloid cells to be abnormally high in TC-1-challenged mice and in HPV-positive cervical cancer patients. Treatment with CarboTaxol normalizes the numbers of circulating myeloid cells but has no negative effect on the number and function of lymphocytes. In mice, CarboTaxol treatment had a similar effect on the myeloid cell composition within the tumors as in the blood. The effects of CarboTaxol are, therefore, not limited to circulating immune cells, and it is likely that similar effects occur within the tumors of cervical cancer patients. The CarboTaxol-mediated normalization of circulating myeloid cells was associated with increased T-cell-mediated tumor control in mice and with higher T-cell reactivity against common microbial recall antigens and response to HPV16-SLP vaccination in patients. This suggests a causal relationship between the normalization of abnormally high myeloid cell frequencies and improved T-cell responsiveness. Because the combination of HPV16-SLP vaccination plus CarboTaxol improved the cure rate of mice with established TC-1 tumors, we expect that the robust and sustained HPV16-specific T-cell responses seen with this combination improved the efficacy of treatment in patients with advanced cervical cancer. This needs to be studied in a future randomized clinical trial.

CarboTaxol is a standard chemotherapeutic treatment not only in cervical cancer but also in patients with other cancer types, including lung cancer and ovarian cancer. Its effect on the immune system, however, has not been widely studied. Carboplatin and paclitaxel are both known to cause dose-limiting myelotoxicity.[22,23] This is likely a direct effect on precursor cells in the bone marrow, as observed in different animal models.[24] White bone marrow cells display impaired *in vitro* capacity to proliferate when treated with carboplatin.[25] Furthermore, the number of myeloid cells reaching its nadir at 2 weeks and a rebound at 3 weeks after CarboTaxol treatment is in line with the mechanistic models for the development and maturation of leukocytes and drug susceptibility in the bone marrow.[26,27] Lymphopenia has not been reported. We observed an increase in T-cell reactivity 1 to 2 weeks after the second and subsequent cycles of chemotherapy. This was not a result of changes in absolute lymphocyte counts or strong alterations in the number or phenotype of $CD4^+$, $CD8^+$, or regulatory T-cells. Similar observations were made in ovarian cancer patients. Those patients who responded to CarboTaxol displayed a stronger IFN-γ-producing $CD8^+$ T-cell response during treatment 12 to 14 days after chemotherapy.[28,29] Here we show that the positive effect of CarboTaxol on the immune response results from the normalization of abnormal myeloid cell numbers, which are initially high in the presence of larger tumor burden. Leukocytosis has been described in patients and animals with HPV-associated cancers[30,31], but the composition of the increased leukocyte populations was not analyzed in detail. An in-depth analysis of the myeloid cell subsets affected by CarboTaxol revealed that these effects were found across all subsets that are elevated in patients or in tumor-bearing animals. This includes tumor growth-suppressing myeloid cells, but more importantly the tumor-promoting myeloid cell populations which can suppress the function of anti-tumor effector T-cells. Apparently, the balance among these subsets and in particular the decline in immunosuppressive myeloid cells within the tumor microenvironment appears to be important for successful implementation of immunotherapy and improved clinical efficacy. The change in the proportions of myeloid cells and lymphocytes allowed the latter population to respond to antigenic stimulation, most likely through a relief from myeloid cell-mediated immunosuppression. This notion is sustained by the unexpectedly high proliferative responses after timed application of a single vaccination and our *in vitro* experiment showing that removal of excessive $CD14^+$ myeloid cells from pre-chemotherapy PBMC samples of two cancer patients allowed the tumor-specific T-cells to react to antigenic stimulation. This seems to be a general phenomenon, and we observed this also in the context of lung cancer.[20] A recent phase II trial in patients with extensive small-cell lung cancer reported that ipilimumab treatment beginning with the third cycle of CarboTaxol produced better clinical outcomes than giving the drugs during cycles 1 to 4.[32] The effect of CarboTaxol on myeloid cells may have relieved myeloid cell-mediated suppression of T-cells, as in our study, providing ipilimumab the opportunity to release the brakes on activated T-cells in the later phase of treatment. The effects of CarboTaxol on myeloid cells are clear in patients with cancers where myeloid cells have prognostic value[33,34], of which cervical and ovarian carcinomas are prime examples. Other types of cancer in which myeloid cells play an important immunosuppressive and prognostic role are thus also candidates for timed immunotherapy.

Our study has some limitations. First, although abnormal numbers of myeloid cells are found both in the mouse model and in patients, their phenotype differs. In mice, the chemotherapy-related reduction in circulating $CD11b^{hi}Gr\text{-}1^{hi}$ cells reflected their depletion in the tumor. In patients, a number of circulating myeloid cell subsets was reduced but whether this also occurs in the tumor remains to be established. Second, in comparison to the T-cell responses obtained in our earlier studies, the current ones were of far greater magnitude. Although the tests were performed by the same laboratory according to the

same standard operating procedures, we did not perform a formal head-to-head comparison, and future trials should confirm these findings. Finally, both the strength of the vaccine-induced immune response and the reduction in circulating myeloid cells were retained for up to 2 weeks after the sixth cycle of CarboTaxol. It is not clear if stopping chemotherapy will coincide with a quick rebound of the myeloid cells, how this affects the vaccine-induced immune response, and whether the phenotype of myeloid cells will be altered under the influence of vaccine-activated T-cells. These should all be subjects of future investigations.

In conclusion, we have shown that CarboTaxol chemotherapy not only is devoid of immunosuppressive effects on tumor-specific T-cells, but vigorously stimulates tumor-specific immunity by normalizing the abnormal numbers of the immunosuppressive myeloid cell populations. Additional studies will have to demonstrate whether CarboTaxol and adequately timed HPV16-SLP vaccination also produce clinical benefit in patients with advanced cervical cancer.A larger clinical trial is already under way to test this (NCT02128126). If successful, this immunotherapeutic approach should be easy to implement because it combines smoothly with the preferred chemotherapy treatment for advanced cervical cancer.

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Figure 1 CarboTaxol improves the clinical outcome of therapeutic peptide vaccination. **(A)** C57BL/6 mice were injected with 1 x 10^5 TC-1 tumor cells and treated systemically with carboplatin (C) and paclitaxel (P) with or without injection of the HPV16 $E7_{43-77}$ peptide in Montanide vaccine (V) in the flank opposite of the tumor as shown in the schematic diagram. **(B)** Kaplan-Meier survival plots show the combined data from several experiments (number of mice is indicated). Peptide *vs* peptide-CarboTaxol treated group (p = 0.004). **(C)** Tumor growth data from two pooled individual experiments with eight mice per group. Quantification of the percentage of **(D)** $CD4^+$ T-cells, **(E)** $CD8^+$ T-cells, and **(F)** the vaccine-specific $CD8^+$ T-cells as determined by H2-Db $E7_{49-57}$ (RAHYNIVTF) tetramer (TM) staining. Column 3 *vs* 1 (p = 0.03), 2 and 4 (p = 0.005). Column 5 *vs* 2 and 4 (p = 0.03). N = 8 mice in the tumor-bearing groups, N = 4 in the naïve group, data are representative of two individual experiments and expressed as mean plus standard error of the mean (SEM). Data were analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

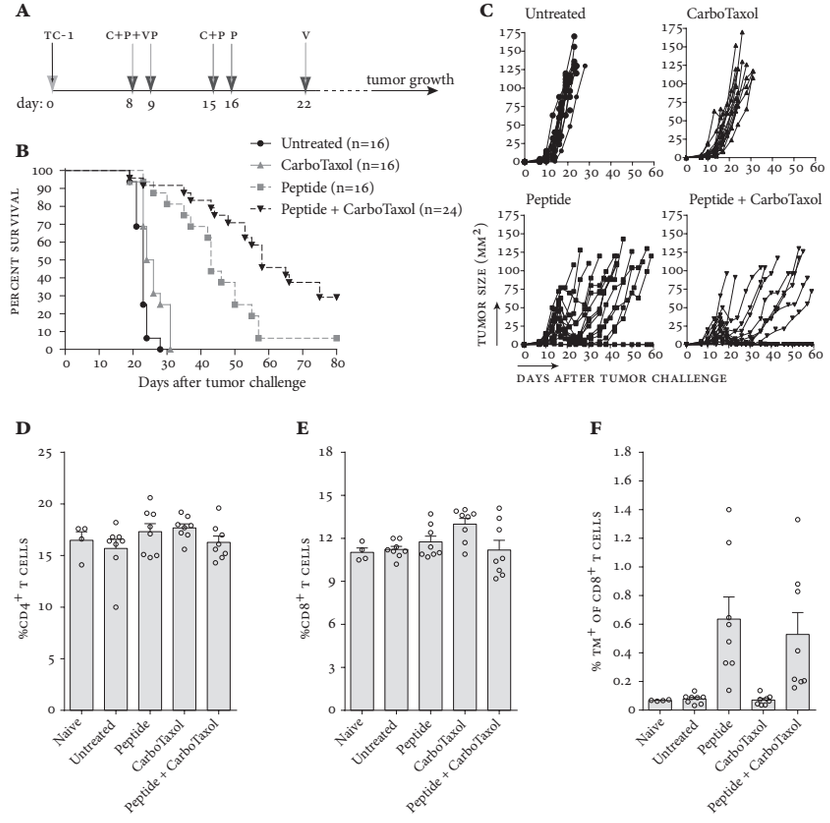
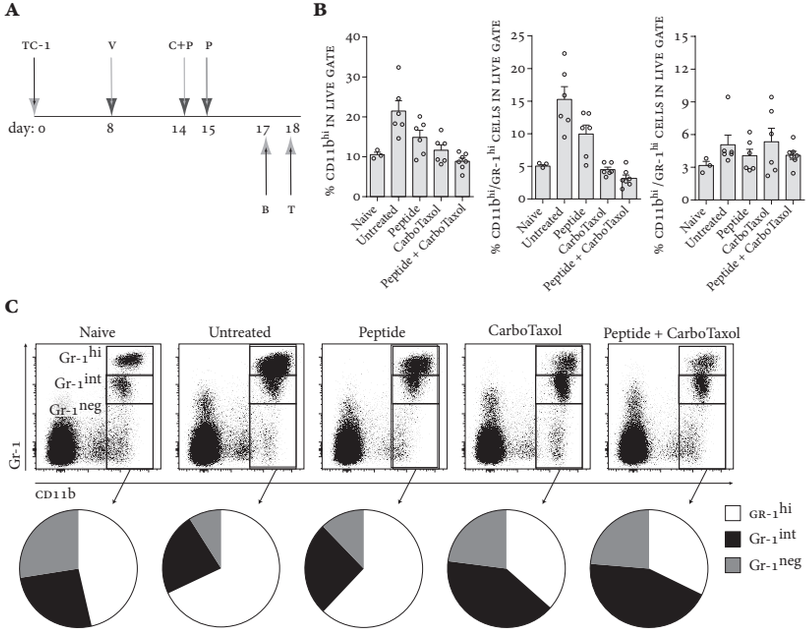


Figure 2 Chemotherapy normalizes systemic tumor-induced myeloid subsets, but intratumoral T-cells are not affected. **(A)** C57BL/6 mice were injected with TC-1 tumor cells (TC-1) and treated with HPV16 $E7_{43-77}$ peptide in Montanide vaccine (V) in the flank opposite of the tumor and with carboplatin (C) and paclitaxel (P) as indicated in the schematic diagram. B and T indicate the time points for blood and tumor analysis, respectively. **(B)** Flow cytometry analysis of the total percentage of myeloid cells [left; column 2 *vs* 1 ($p = 0.007$), 4 ($p = 0.003$) and 5 ($p < 0.0001$)], the $CD11b^{hi}Gr\text{-}1^{hi}$cells [middle; column 2 *vs* 1 ($p = 0.0004$), 3, ($p = 0.03$), 4 and 5 ($p < 0.0001$); column 3 *vs* 4 ($p = 0.02$) and 5 ($p = 0.002$)], and the $CD11b^{hi}Gr\text{-}1^{int}$ (right) cells in the blood. **(C)** Representative flow cytometry plots for each treatment, gated on live ($7AAD^-$) cells (top). Distribution of $Gr\text{-}1^{hi}$, $Gr\text{-}1^{int}$, and $Gr\text{-}1^{neg}$ expressing cells within the total $CD11b^{hi}$ population is indicated in the pie charts (bottom). Tumor samples were collected and analyzed to determine the percentage of



(D) $CD45^+$ cells within the live gate [Column 1 *vs* 2 and 4 ($p < 0.0001$) and 3 ($p = 0.02$); column 3 *vs* 2 and 4 ($p < 0.0001$)], **(E)** $CD8^+$ T-cells in the leukocyte gate [Column 1 *vs* 2 and 4 ($p < 0.0001$); column 3 *vs* 2 and 4 ($p < 0.0001$)], **(F)** vaccine-specific T-cells determined by $H2\text{-}D^b$ $E7_{49-57}$ (RAHYNIVTF) tetramer staining [Column 1 *vs* 2 and 4 ($p < 0.0001$); column 3 *vs* 2 and 4 ($p < 0.0001$)]. **(G)** Single cell suspensions of tumors were co-incubated with HPV16 $E7_{43-77}$ peptide-pulsed D1 cells and stained for intracellular TNFα and IFN-γ. Representative flow cytometry plots (left) and quantification (right) show the frequency of cytokine-producing $CD8^+$ T-cells [IFN-γ graph: column 1 *vs* 2 ($p = 0.009$) and 4 ($p = 0.0001$); column 3 *vs* 2 ($p = 0.004$) and 4 ($p < 0.0001$). TNFα graph: column 1 *vs* 2 ($p=0.006$) and 4 ($p = 0.0009$); column 3 *vs* 2 ($p = 0.008$) and 4 ($p = 0.001$)]. N = 5-7 mice per group, data shown are representative of two individual experiments. Data are expressed as mean plus SEM and analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

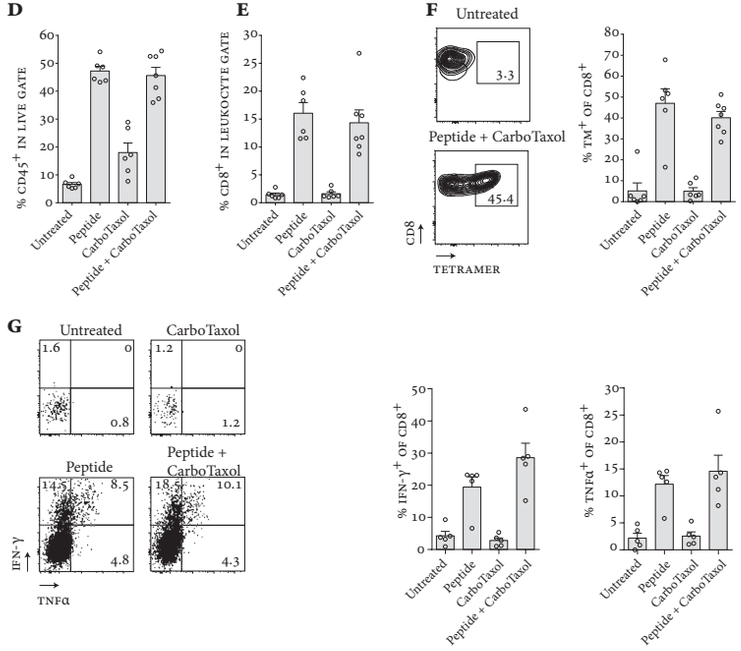


Figure 3 Gr-1^{hi} cells are depleted from the tumor by CarboTaxol treatment. Mice were treated as in figure 2. Tumor samples were isolated and analyzed by flow cytometry. **(A)** Leukocytes from resected untreated tumors were analyzed for the expression of Gr-1 and CD11b. The histograms show the expression of class II, CD80, CD86, F4/80, Ly6G, and CD11c on the Gr-1^{hi} (gray lines) and Gr-1^{int} (black lines) subsets. **(B)** Four days after chemotherapy or ten days after peptide vaccination, leukocytes from resected tumors were analyzed for the expression of Gr-1 and CD11b (top). Distribution of Gr-1^{hi}, Gr-1^{int}, and Gr-1^{neg} expressing cells within the total CD11b^{hi} population is shown in the pie charts (bottom). **(C)** Percentages (mean plus SEM) of Gr-1^{hi} [Column 1 *vs* 2 (p = 0.006), 3 (p = 0.0004) and 4 (p = 0.0008)] and Gr-1^{int} [Column 4 *vs* 1 (p = 0.0006) and 2 (p = 0.02)] subsets were analyzed for untreated and treated tumors. N = 5-7 mice per group, data shown are representative of two individual experiments. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

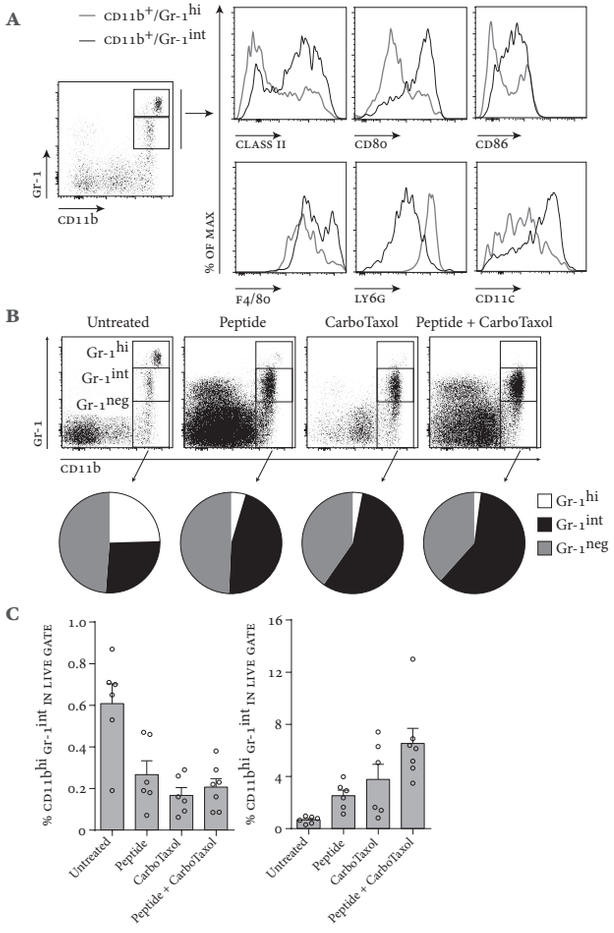


Figure 4 CarboTaxol induces changes in cellular immunity in advanced stage cervical cancer patients. **(A)** Blood draws (B) and CarboTaxol cycles (C) for the 6 patients in cohort 1 are indicated in days (D) and weeks (W) in the schematic outline. **(B)** Representative flow cytometry plots show the myeloid cell gate and lymphocyte gate in the blood of a patient at baseline and after 1 to 2 CarboTaxol cycles in comparison to the blood of a healthy donor. The percentages of myeloid cells and lymphoid cells within the total number of cells are indicated. To determine the relative percentage of each population, the sum of the events in the lymphoid and myeloid cell gates in the forward and side scatter plots was set to 100 and then the frequency of **(C)** myeloid cells [column 1 *vs* 4 (p = 0.0002), 5 (p = 0.02), and 6 (p = 0.005)] and of **(D)** lymphocytes [column 1 *vs* 4 (p = 0.0002), 5 (p = 0.02), and 6 (p = 0.005)] was determined. **(E)** Proliferation of T-cells upon recognition of recall antigens (memory response mix, MRM) shown as stimulation index. Column 1 *vs* 3 and 6 (p = 0.02), 4 (p = 0.001), and 5 (p = 0.005). **(F)** The ability of antigen presenting cells to stimulate T-cells in a mixed lymphocyte reaction shown for the 4 tested patients. Column 1 *vs* 2 (p = 0.005) and 4 (p = 0.049). Data (shown as median plus interquartile range) were analyzed by repeated measures model.

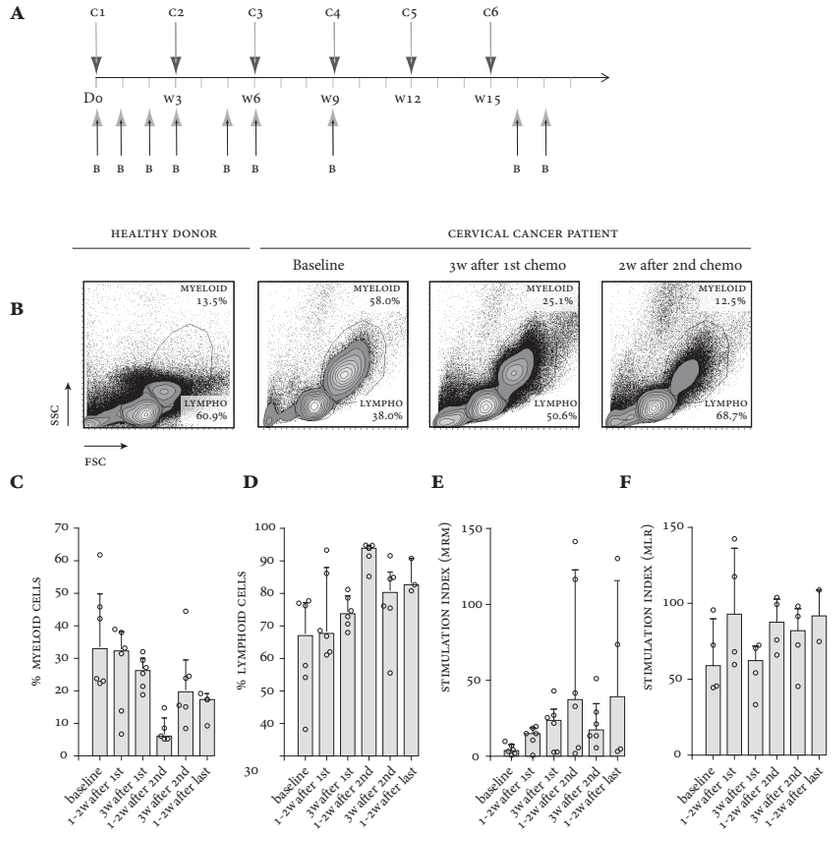


Figure 5 The frequencies of circulating immune cells normalize upon CarboTaxol treatment. Blood samples of the 12 patients in cohort 2 were analyzed for leukocyte differentiation, showing a shift from baseline for the counts of **(A)** lymphocytes and **(B)** leukocytes x 10^9/L. Column 1 *vs* 3 (p = 0.007) and 4 to 6 (p < 0.0001). Column 3 *vs* 4 (p < 0.0001), 5 (p = 0.04) and 6 (p = 0.003). The frequency of **(C)** myeloid cells [Column 1 *vs* 2 (p < 0.0001), 3 (p = 0.002), 4 (p = 0.003), 5 (p = 0.006), 6 (p = 0.008) and 7 (p = 0.009); column 2 *vs* 3 (p = 0.04), 4 (p = 0.02), 5 (p = 0.007), 6 and 7 (p = 0.004)] and **(D)** lymphocytes [Column 1 *vs* 2 (p < 0.0001), 3 (p = 0.0004), 4 (p = 0.0009), 5 (p = 0.002), 6 (p = 0.005) and 7 (p = 0.006); column 2 *vs* 3 (p = 0.02), 4 (p = 0.002), 5 (p = 0.0009), 6 and 7 (p = 0.0002)] were determined in the forward and side scatter plots of these blood samples after acquisition by flow cytometry. Blood samples from healthy donors (HD, n = 19) were included for comparison. Data (shown as median plus interquartile range) were analyzed by repeated measures model. The fold change in stimulation index (SI), which is SI in a sample during/after chemotherapy divided by that of the baseline sample, of the blood samples stimulated with **(E)** recall antigens (MRM) or **(F)** Influenza Matrix 1 peptides (FLU) is shown *vs* the shift in percentage of myeloid or lymphoid cells from baseline. Repeated measures regression analysis was conducted to determine whether there is a slope significantly different from 0, represented with the p-value.

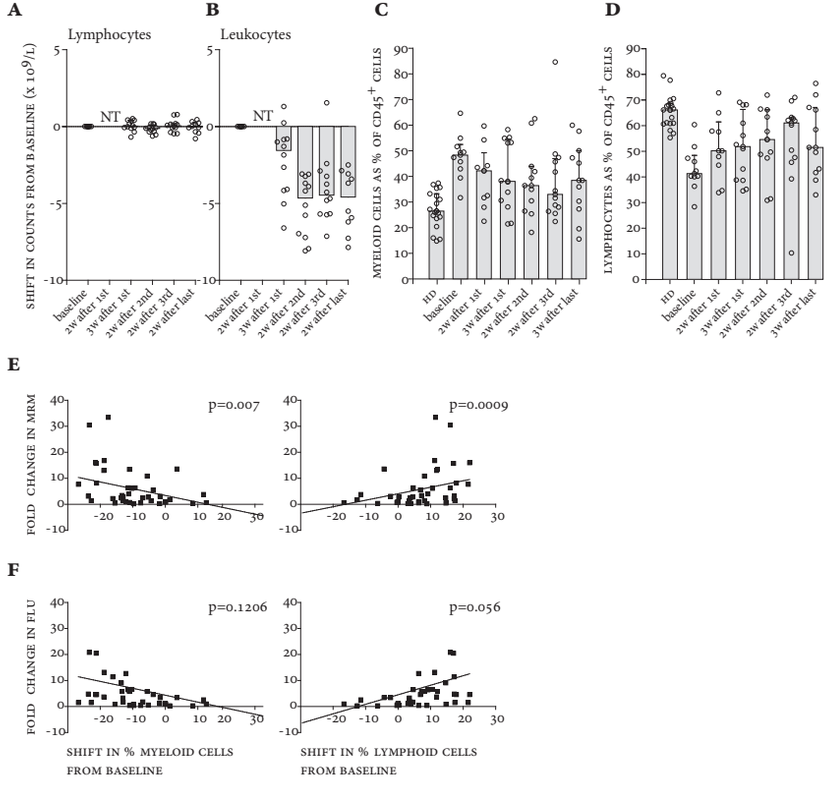


Figure 6 CarboTaxol treatment induces a decline in all subsets of circulating myeloid cells. PBMC from the 12 patients in cohort 2 were subjected to multi-parameter flow cytometry analysis. **(A)** Representative dot plots of the five subpopulations within the $CD45^+CD3^-CD19^-CD1a^-HLA\text{-}DR^+$ population defined by expression of CD11b and CD14 in the baseline blood sample and 2 weeks after the second cycle of chemotherapy. **(B)** The frequency of ($CD45^+CD3^-CD19^-CD1a^-HLA\text{-}DR^+$) $CD11b^+CD14^+$ (population 1) as a percentage of the $CD45^+$ cells in healthy donors (HD, N = 19) and in the patients over time. The time points of blood sampling (X-axis) were 2 weeks after the first (1st), second (2nd), and third (3rd) cycle of chemotherapy and 3 weeks after the sixth (6th) or last chemotherapy cycle. Column 1 *vs* 2 (p < 0.0001), 3 (p = 0.01), 4 (p = 0.02), 5 (p = 0.009) and 6 (p = 0.04). Column 2 *vs* 3 (p = 0.04), 4 (p = 0.01), 5 (p = 0.02) and 6 (p = 0.005). **(C)** The fold change in stimulation index (SI) of the blood samples stimulated with recall antigens (MRM) or Influenza Matrix 1 peptides (FLU) is shown *vs* the absolute shift in percentage of population 1 cells from baseline. Repeated measures regression analysis was conducted to determine whether there is a slope significantly different from 0, represented with the p-value. The frequencies of **(D)** $CD163^-CD206^-CD16^-CD11c^+$ [M1-like cells; column 1 *vs* 2 (p = 0.001) and 3 (p = 0.01); column 2 *vs* 5 (p = 0.01)] and **(E)** $CD163^+CD206^-CD16^-CD11c^+$ [M2c-like cells; column 1 *vs* 2 (p = 0.004) and 5 (p = 0.01)] within population 1 are shown for the healthy donors and patients over time. **(F)** The frequency of myeloid-derived suppressor cells (MDSCs; $CD45^+CD3^-CD19^-CD1a^-HLA\text{-}DR^-/^{low}$) is depicted. Column 1 *vs* 3 (p = 0.03). Column 2 *vs* 4 (p = 0.04). Data (shown as median plus interquartile range) were analyzed by repeated measures model.

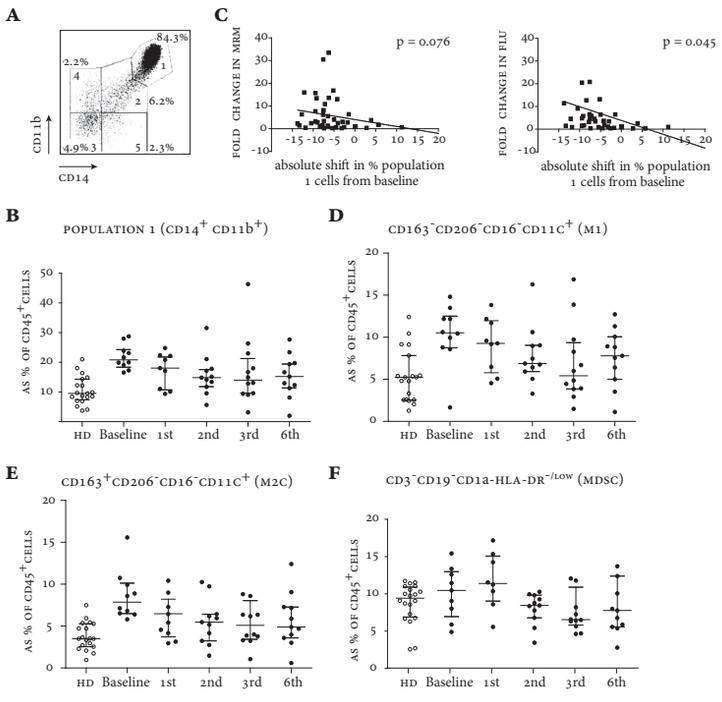
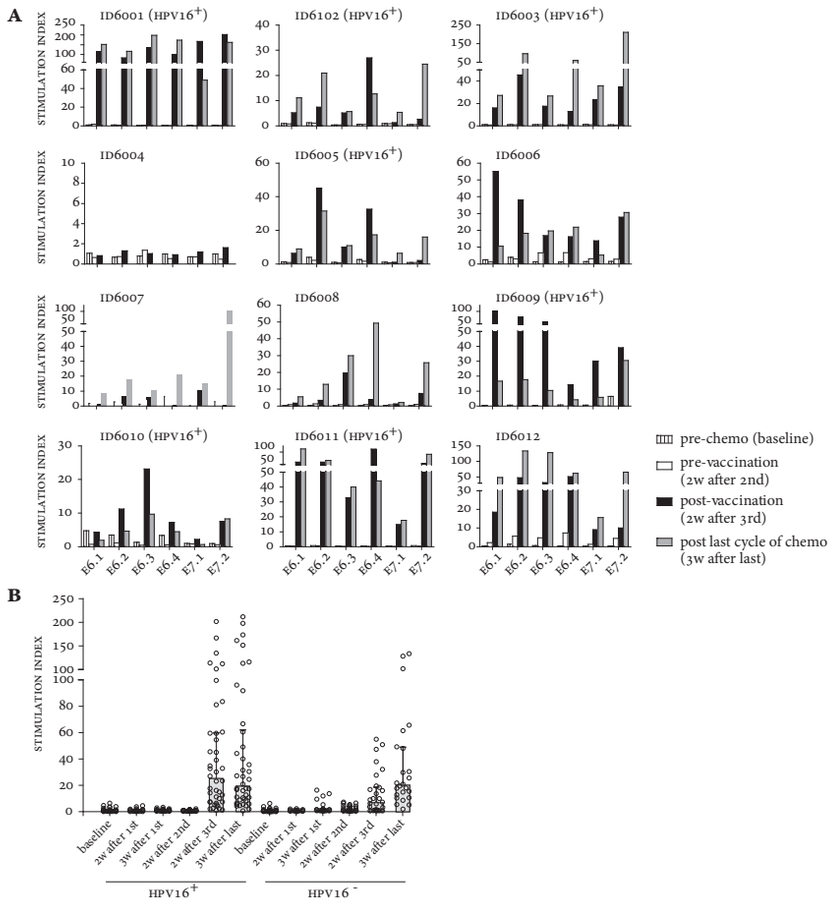


Figure 7 HPV16 SLP vaccination during CarboTaxol treatment results in a strong immune response in patients. The patients in cohort 2 received a single vaccination with HPV16-SLP subcutaneously at 2 weeks after the second cycle of chemotherapy. **(A)** The proliferative responses of T-cells in the lymphocyte stimulation test (LST) are shown as a stimulation index and depicted *vs* the indicated peptide pools used for stimulation of the cells in the blood sample at baseline (hatched bar), 2 weeks after the second cycle of chemotherapy and before vaccination (white bar), 3 weeks after this single vaccination (black bar), and 3 weeks after the sixth or last cycle of chemotherapy (gray bar). **(B)** The patients are grouped by HPV16 status (HPV16$^+$, N = 7; HPV16-, N = 5), and the proliferative response (stimulation index) is plotted *vs* the indicated blood samples. Each dot represents one response against HPV16 E6 and E7. In total 6 peptide pools were tested per blood sample. Data were analyzed by linear mixed model analysis and showed no statistically significant difference.



SUPPLEMENTAL INFORMATION

ANALYSIS OF MURINE TUMOR INFILTRATING IMMUNE POPULATIONS

TC-1 tumor-bearing mice were injected with synthetic long HPV16 $E7_{43-77}$ peptide in the contralateral flank. Chemotherapy was provided intraperitoneally (i.p.) on day 14 (carboplatin) and day 14 and 15 (paclitaxel). On day 17, blood was taken and analyzed by flow cytometry. Tumor-infiltrating immune populations were analyzed as previously described.[13] Briefly, single cell suspensions of tumors from transcardially perfused mice were incubated with 7-Aminoactinomycin D (Life Technologies) to exclude dead cells, with H-$2D^b$ tetramers containing HPV16 $E7_{49-57}$ peptide (RAHYNIVTF) labeled with APC, and with the indicated antibodies from Biolegend: Gr-1-PE Cy7 (clone RB6-8C5), Ly6G-Alexa Fluor 700 (AF700; clone 1A8), CD4-BV605 (clone L3T4); eBioscience: CD11b-Pacific Blue (PC; clone M1/70), F4/80-PE (clone BM8), CD80-FITC (clone 16-10A1), CD86-PE (clone GL1, BD), CD3-PE Cy7 (clone 145-2C11), CD8a-AF700 (clone 53-6.7), CD45.2 eFluor 780 (clone 104) and CD19-APC (clone 1D3); or BD: MHC-class II-Horizon V500 (HV500; clone M5/114.15.2, BD), CD11c Brilliant Violet 605 (BV605; clone HL3, BD).

To determine the capacity of cells to produce pro-inflammatory cytokines, single cell suspensions of tumor infiltrating cells were incubated for 5 hours with 40,000 D1 dendritic cells pre-loaded with HPV16 $E7_{43-77}$ peptide (10 µg/ml) in the presence of Brefeldin A (2 µg/ml, Sigma). After cell surface staining with fluorescently labelled antibodies to mouse CD45, CD8, and CD3, overnight fixation with 0.5% paraformaldehyde solution (Pharmacy LUMC), and permeabilization with Perm/Wash buffer (BD), the cells were stained at 4˚C with antibodies against IFN-γ (APC, clone XMG1.2, eBioscience) and TNFα (FITC, clone MP6-XT22, eBioscience).

IMMUNOMONITORING OF BLOOD SAMPLES FROM CERVICAL CANCER PATIENTS

THE SAMPLES Venous blood samples (45 mL in heparinized tubes and 8.5 mL in clot activator tube) were taken for immunomonitoring before chemotherapy (baseline), 1 or 2 weeks after the first cycle of chemotherapy (1-2 wk after 1st), before (3 wk after 1st) and 1 to 2 weeks after the second cycle (1-2wk after 2^{nd}), 2-3 weeks after the 3rd cycle (2-3wk after 3^{rd}), and 1-2 or 3 weeks after the last cycle indicated in table S1 (maximum 6th cycle; 1-2 or 3 wk after last) of chemotherapy (figure 4A). In addition, blood samples (50 mL) were drawn from 19 healthy blood donor volunteers (≥ 18 years old females) after they had signed an informed consent. The blood (transported at room temperature) was processed within 3 hours and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation, washed, partly used in the lymphocyte stimulation test (LST), and the remaining cells were cryopreserved in 90% fetal calf serum (PAA Laboratories) and 10% DMSO at a concentration of 7-12 million cells per vial in a total volume of 1 mL) using a Mr. Frosty's freezing container (Nalgene). Upon cryopreservation the vials were stored in the vapor phase of the liquid nitrogen until further use.

THE ASSAYS

PROLIFERATION ASSAYS The HPV16-specific proliferative response was determined using freshly isolated PBMCs that were subjected to the LST as described previously.[8-11] In brief, eight replicate wells with 1.5 x 10^5 cells per well were stimulated for 6 days with HPV16 E6 or E7 peptide pools (10 µg/mL per peptide), after which 50 µL supernatant per well was harvested and stored at -20° C for cytokine analysis. The cells were pulsed with [^{3}H]-Thymidine (Perkin Elmer) for 16 hours and harvested, and uptake was determined by Wallac Microbetatrilux (Perkin Elmer). As a positive control, the previously described memory response mix (MRM) and influenza matrix 1 protein-derived overlapping peptides (FLU) were used.[13] The negative control consisted of cells in medium only. A positive response was defined as a stimulation index (SI) of at least 3 under the condition that 6 out of the 8 wells displayed values above the cut-off, which was defined as the mean value of the cells in medium only plus 3 standard deviations (SD).

The capacity to respond to phytohemagglutinin (PHA) was studied using cryo-preserved PBMCs. Cells were thawed and tested in a 3-day proliferation assay as described previously[33], with the minor alteration that now 50,000 cells per well (in quadruplicate) were incubated in medium (IMDM, Lonza) or stimulated with 0.5 µg/mL PHA (Murex Biotech HA16). A positive response was defined as an SI of at least 3.

The antigen-presenting capacity of the PBMC samples from patients was determined in a mixed lymphocyte reaction (MLR).[34] Patients' PBMCs were thawed in Iscove's Modified Dulbecco's Medium (IMDM) plus 10% fetal bovine serum and 30 mg/ml DNase, resuspended in IMDM plus 10% human AB serum, irradiated (3000 rad) to prevent proliferation, washed, and resuspended in IMDM plus 10% human AB serum and then plated at 1 x 10^5 cells per well (in quadruplicate). Third party PBMCs were added (1 x 10^5 million cells/well), making a total volume of 200 µL/well. Irradiated PBMCs alone as well as third party PBMCs alone were used as negative controls. At day 6, 100 µL supernatant per well was harvested for cytokine analysis, and the cells were subjected to [^{3}H]-Thymidine (50 µL/well of 10 µCi/mL) for an additional 16 hours. A positive response was defined as an SI of at least 3.

MYELOID CELL DEPLETION AND STIMULATION OF PBMCS IN VITRO The CD14$^+$ myeloid cells in PBMCs of 2 cervical cancer patients were depleted by magnetic cell sorting (Miltenyi) as described earlier.[28] Depleted and non-depleted PBMC were stimulated for 11 days with autologous monocytes pulsed with either a mix of overlapping FLU peptides and MRM,[9-11] a mix of HPV16 E6/E7 SLP (32-35 amino acid long overlapping peptides[33]), or a mix of p53 SLP (30-mer overlapping peptides) and then tested in a proliferation test (triplicate wells) as described above, with non-pulsed autologous monocytes serving as a negative control.[28]

CYTOKINE ANALYSIS The supernatants of the LST, PHA, and MLR proliferation assays were used for cytokine analysis with a flow cytometer-based cytokine bead array (CBA, human Th1/Th2 kit, BD) according to the manufacturer's instructions as reported earlier.[9-11] The cytokines measured by this kit were IFN-γ, TNFα, IL-10, IL-5, IL-4, and IL-2. A positive response was defined as being above the detection limit, which is 20 pg/mL for each of the cytokines. A three-fold increase above the baseline sample (pre-treatment) was defined as a treatment-related change.

PHENOTYPING OF PMBCS The PBMC samples isolated at different time points during treatment were phenotyped with 4 sets of 6-11 cell surface markers to identify macrophages, myeloid derived suppressor cells (MDSCs), and the expression of co-inhibitory molecules and regulatory t-cells by flow cytometry.[34]

The macrophage set consisted of CD3-PB (Clone UCHT1; Dako), CD1a-FITC (Clone HI149; BD), CD11b-PE (Clone D12; BD), CD11c-AF700 (Clone b-Ly6; BD), CD14-PE CY7 (Clone M5E2; BD), CD16-PE CF594 (Clone 3G8; BD), CD19-BV605 (BV605, Clone SJ25C1; BD), CD45-PerCP Cy5.5 (Clone 2D1; BD), CD163-APC (Clone 215927; R&D), CD206-APC CY7 (Clone 15-2; Biolegend), and HLA-DR HV500 (Clone L243; BD).

The MDSC set contained the same CD3-PB, CD19-BV605, CD45-PerCP Cy5.5, and HLA-DR HV500 antibodies and additionally CD11b-FITC (Clone CBRM1/5; Biolegend), CD14-AF700 (Clone M5E2; BD), CD15-PE CF594 (Clone W6D3; BD), CD33-PE Cy7 (Clone P67.6; BD), CD34-APC (Clone 581; BD), and CD124-PE (Clone HiL4R-M57; BD).

The inhibitory set consisted of the same antibody as above for CD3, and additionally CD4-PE CF594 (Clone RPA-T4; BD), CD8-APC Cy7 (Clone SK1; BD), CD152-PE Cy5 (anti-CTLA-4; Clone BN13, BD), CD279-BV605 (anti-PD-1; Clone EH12.2H7; Biolegend), and TIM3-PE (Clone F38-2E2; Biolegend).

The regulatory T-cell set consisted of CD3-HV500 (Clone UCHT1; BD), CD4-AF700 (Clone RPA-T4; BD), CD8-PerCP Cy5.5 (clone SK1; BD), CD25-PE Cy7 (clone 2A3; BD), CD127-BV650 (clone HIL-7R-M21; BD), Foxp3-PE CF594 (clone 259D/C7; BD), Ki67-FITC (clone 20Raj1; eBioscience), CD45-RA-APC H7 (clone HI100; BD), and live/dead marker (Yellow Amino Reactive Dye (ARD), Life Technologies).

The sets of markers for MDSCs and regulatory t-cells were selected according to the consensus within the CIMT immunoguiding program (CIP).

The cryopreserved PBMCs were thawed and stained. Briefly, for the staining of surface markers the cells were washed in phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA, Sigma), incubated for 10 min at room temperature (RT) in PBS/0.5% BSA/10%FCS (in the dark) to prevent non-specific antibody binding, centrifuged, resuspended in the antibody mixtures described aboveand incubated for 30 min on ice (in the dark). Then, the cells were washed twice with PBS/0.5% BSA and finally resuspended in 1% paraformaldehyde (Pharmacy LUMC). For the regulatory t-cell staining, the cells were first subjected to Yellow ARD (20 minutes at RT in 100 µL/well of 1:800 diluted Yellow ARD), blocked for non-specific staining, and subsequently stained for surface markers as described above, followed by washing in transcription factor fixation and permeabilization buffer (BD) and staining with the intranuclear antibodies for Foxp3 and Ki67 diluted in permeabilization and washing buffer (BD) for 40-50 minutes at 40 C. The cells were then resuspended in 1% paraformaldehyde and analyzed within 24 hours while keeping at 4° C.

INTRACELLULAR CYTOKINE STAINING (ICS)

For the simultaneous detection of surface markers (CD3, CD4, CD8), activation markers (CD154, CD137), and intracellular cytokines (IFN-γ and IL-2), the PBMCs were subjected to the direct ex-vivo multiparameter flow cytometry assay as described previously.[9] Cells in medium only and cells stimulated overnight with Staphylococcal Enterotoxin B (2 µg/mL; Sigma) were taken along as negative and positive control, respectively. A positive response was defined as twice the level of the negative control and at least 10 events in the gate. A vaccine-induced response required at least a 3-fold increase in reactivity compared to the baseline sample.

FLOW CYTOMETRY The acquisition on the Fortessa or LSRII (BD) flow cytometers was performed < 24 hours after the staining was finished. Analysis was performed by using FlowJo (Tree Star; Version 10) or DIVA software (Version 6.2).

LABORATORY ENVIRONMENT Immunomonitoring of patients' PBMCs was performed in the laboratory of the department of Clinical Oncology (LUMC) that operates under research conditions but uses standard operation procedures for all tests, with pre-established definitions of positive responses and trained personnel. This laboratory has been externally and internally audited according to the reflection paper for laboratories that perform immunomonitoring[35] and participated in all proficiency panels of the CIMT Immunoguiding Program (CIP; of which SHVDB and MJPW are steering committee members; http://www.cimt.eu/workgroups/cip/) as well as many of the proficiency panels (including ICS gating and ELISPOT plate reading panels) of the USA-based Cancer Immunotherapy Consortium (CIC of the Cancer Research Institute) to validate its standard operating procedures (SOPS).

STATISTICAL ANALYSIS

Survival of differentially treated tumor-bearing mice was compared by the Kaplan-Meier method and the log-rank (Mantel-Cox) test. Statistical analysis of immune parameters in mice was performed with one-way ANOVA followed by Tukey's post-hoc analysis. Statistical analysis was performed in GraphPad Prism software (version 6).

The immune responses of patients were analyzed with a repeated measures model with fixed factors group, time and group by time, and repeated time within the patient group. The Kenward-Roger approximation was used to estimate denominator degrees of freedom, and model parameters were estimated using the restricted maximum likelihood method. Residual graphs were used to decide which variables would be log transformed before analysis to correct for the expected log-normal distribution of the data. The general group effect was calculated within the model as the average least square means (LSM) over all time points for the patients versus the LSM of the healthy donors. If the general group effect was significant ($p < 0.05$), the various differences (healthy donors vs patients at each time point) were calculated. In a separate analysis with patient data, only the general time effect within patients was estimated. If the time effect was significant ($p < 0.05$), the various differences between time points within the patient group were calculated within the original model. The fold change in MRM and FLU and absolute shift in myeloid, lymphoid, and population 1 cells was analyzed with a repeated measures regression analysis with a compound covariance structure and time as repeated factor within subject. Because the number of myeloid cells reached its nadir after 2 cycles of CarboTaxol and was retained throughout the treatment, the results of patient ID6008, who received the vaccine after the third cycle, were used as if this patient was vaccinated in the same time window as the others. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed using SAS for windows V9.4 (SAS Institute, Inc.). To determine whether a significant difference existed between the patients with a HPV16 positive cervical tumor and those with a HPV16 negative (other HPV type) tumor in the proliferative response to the 6 tested HPV16 peptide pools, we used a mixed linear model with unstructured correlation metric as covariance type using SPSS statistics (version 20). In this model, the possible link between the different peptide pools (although biologically unrelated) was incorporated when the peptide pool was taken as a dependent variable.

Table S1 Patient characteristics. Age at diagnosis (D) of cervical cancer. Age at first recurrence (R), metastatic disease or advanced stage of disease. Age at which the patient was included (I) in the trial, this equals the age at the start of chemotherapy CarboTaxol.

ID	Age			Primary Tumor		Advanced / Recurrenent / metastatic disease		
	D	R	I	FIGO	Treatment	Disease	Interval P-R (months)	Prior treatments
1	51	52	52	IIB	CHRT	recurrent	6	none
2	60	60	60	IV	CHRT	advanced	n.a.	n.a.
3	32	38	39	IB2	SUR + RT	metastatic	73	CH+RT+HT (cisplatin)
4	45	49	50	IB1	SUR + RT	metastatic + recurrent	47	RT
5	55	56	56	IIA	SUR + RT	metastatic	18	none
6	36	36	36	IIIB	CHRT	metastatic	8	none
6001	50	50	50	IV	CH	advanced	N.A.	N.A.
6002	54	56	56	IB1	SUR + RT	metastatic	24	CT + RT (cisplatin)
6102	47	48	48	IB1	SUR + CHRT	recurrent	14	none
6003	42	45	45	IB1	SUR + CHRT	metastatic	35	none
6004	32	33	33	IIB	RT + BT + HT	recurrent	5	none
6005	35	37	37	IB1	SUR	recurrent	22	none
6006	34	35	36	IIB	CHRT + HT	recurrent	22	alternative
6007	49	55	55	IB1	SUR + CHRT	recurrent	64	none
6008	70	71	71	IIIB	RT + BT + HT	metastatic	21	none
6009	29	34	34	IA1	SUR	metastatic	56	none
6010	33	36	37	IIB	SUR + CHRT	metastatic	37	CT + RT (cisplatin)
6011	28	29	31	IB1	SUR	metastatic + recurrent	16	RT
6012	58	58	58	IV	CH	advanced	N.A.	N.A.

Vaccination						
No. of cycles CarboTaxol	Dose Carboplatin	Dose Paclitaxel	Tumor HPV type	Interval CH-vacc (days)	Interval vacc-CH (days)	No. of vacc.
3	normal	normal	16	N.A.	N.A.	0
6	reduced	reduced	16	N.A.	N.A.	0
6	normal	normal	18	N.A.	N.A.	0
6	normal	normal	16	N.A.	N.A.	0
6	normal	normal	16	N.A.	N.A.	0
5	normal	normal	16	N.A.	N.A.	0
6	normal	normal	16	17	6	1
1	normal	normal	16	N.A.	N.A.	0
6	normal	normal	16	18	3	1
6	normal	normal	16	13	8	1
3	normal	normal	-#	15	6	1
6	normal	normal	16	15	6	1
6	reduced	reduced	-	14	15	1
6	normal	normal	-	15	6	1
6	reduced	reduced	-	17*	15$	1
6	normal	normal	16	15	6	1
6	normal	normal	16	13	8	1
6	normal	normal	16	15	6	1
6	normal	normal	-	17	4	1

** For patient ID6008, vaccination took place 17 days after the 3rd cycle of chemotherapy with CarboTaxol (vaccination was postponed due to infection). # HPV16 negative, but not tested for other high-risk HPV types. FIGO: International Federation of Gynecology and Obstetrics representing stage of cancer at diagnosis; CHRT: chemoradiation; SUR: surgery; RT: radiotherapy; BT: brachytherapy; HT: hyperthermia, CH: chemotherapy. Interval P-R: interval between primary tumor and (first) recurrence or metastatic disease (in months). n.a.: not applicable. Prior treatments: other treatment for recurrent, metastatic, or advanced disease, different from the primary treatment and CarboTaxol. Interval CH-vacc: interval between the starting date of the 2nd cycle of CarboTaxol and the date of vaccination with HPV16-SLP. Interval vacc-CH: interval between the date of vaccination and the date of the 3rd cycle (or 4th in case of patient ID6008 $) of chemotherapy with CarboTaxol.*

Table S2 Adverse events systemically and at vaccination site. Systemic adverse events (AE) observed in the 12 patients in cohort 2, which might be vaccine-related and are defined as definite, probable, or possible. Local adverse events (AE at injection site) are shown for all 12 patients in cohort 2 who received the single HPV16-SLP vaccination. Shown are the number (N) and the percentage (%) of a total of 12 patients. Injection site AEs were scored at 15 minutes, 1 and 4 hours, 3 weeks, and at regular visits > 6 weeks after vaccination. * Long-term follow-up (> 6 weeks) could not be established for one patient who was deceased (N = 11 at > 6 weeks). Total reflects the maximal injection site reaction for each patient as determined for the single vaccination consisting of an injection of a mix of E6 peptides and a mix of E6/E7 peptides in two separated sites.

Systemically	< 24H	> 24h; < 3wks	Vaccine related	Remark
Fever	1		definite	
Fever		2	possible	
Myalgia		1	possible	
Nausea		1	possible	
Vomiting		1	possible	
Painfull extremities		1	probable	
Stitch abcess		1	possible	1 wk after vaccination
Nefrodrain infection		1	possible	2 wks after vaccination

	Time after vaccination					
Locally (Injection sites)	**15 min**	**1 hour**	**4 hours**	**3 weeks**	**>6 weeks***	**Total**
SWELLING						
0	2 (17%)	0 (0%)	1 (8%)	3 (25%)	3 (27%)	0 (0%)
< 5	9 (75%)	9 (75%)	9 (75%)	4 (33%)	7 (64%)	5 (42%)
5-10	1 (8%)	3 (25%)	2 (17%)	5 (42%)	1 (9%)	7 (58%)
>10	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0(0%)	0 (0%)
ERYTHEMA						
none	3 (25%)	2 (17%)	4 (33%)	6 (50%)	7 (64%)	1 (8%)
mild	9 (75%)	10 (83%)	7 (58%)	4 (33%)	3 (27%)	8 (67%)
moderate	0 (0%)	0 (0%)	1 (8%)	2 (17%)	0 (0%)	2 (17%)
severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (9%)	1 (8%)
TEMPERATURE						
none	2 (17%)	1 (8%)	4 (33%)	4 (33%)	10 (91%)	0 (0%)
mild	10 (83%)	11 (93%)	8 (67%)	7 (58%)	1 (9%)	11 (92%)
moderate	0 (0%)	0 (0%)	0 (0%)	1 (8%)	0 (0%)	1 (8%)
severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
PAIN						
none	9 (75%)	10 (83%)	9 (75%)	6 (50%)	7 (64%)	6 (50%)
mild	1 (8%)	2 (17%)	3 (25%)	5 (42%)	4 (36%)	3 (25%)
moderate	2 (17%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (17%)
severe	0 (0%)	0 (0%)	0 (0%)	1 (8%)	0 (0%)	1 (8%)
ITCH						
none	12 (100%)	12 (100%)	10 (83%)	7 (58%)	11 (100%)	6 (50%)
mild	0 (0%)	0 (0%)	2 (17%)	5 (42%)	0 (0%)	6 (50%)
moderate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ULCERATION					1 (9%)	1 (8%)
CTCEA	**Administered SLP:**		**E6**	**E6/E7**		
grade 1			5 (42%)	6 (50%)		
grade 2			6 (50%)	6 (50%)		
grade 3			1 (8%)	0 (0%)		

CTCEA: common terminology criteria for adverse events

Figure S1 T-cells are not affected by CarboTaxol treatment. Wild-type C57BL/6 mice were injected on day 0 with 1 x 10^5 TC-1 tumor cells. Eight days later, when tumors were palpable, mice were treated systemically with carboplatin (day 8) and paclitaxel (day 8 and 9) with or without addition of synthetic long HPV16 $E7_{43-77}$ peptide in Montanide in the opposite flank. Chemotherapy treatment was repeated one week after initial treatment, and vaccination was boosted 14 days after initial treatment. Shown is the quantification of the percentage of **(A)** $CD4^+$ T-cells, **(B)** $CD8^+$ T-cells, and **(C)** vaccine-specific cells within the $CD8^+$ population as determined by H2-Db $E7_{49-57}$ (RAHYNIVTF) tetramer staining. Column 1 *vs* 7 (p = 0.008); column 2 *vs* 7 (p = 0.007), column 3 *vs* 7 (p = 0.009), column 4 *vs* 7 (p = 0.007). N = 8 mice in the tumor-bearing groups, N = 4 in the naïve group, data are representative of two individual experiments and expressed as mean plus SEM. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

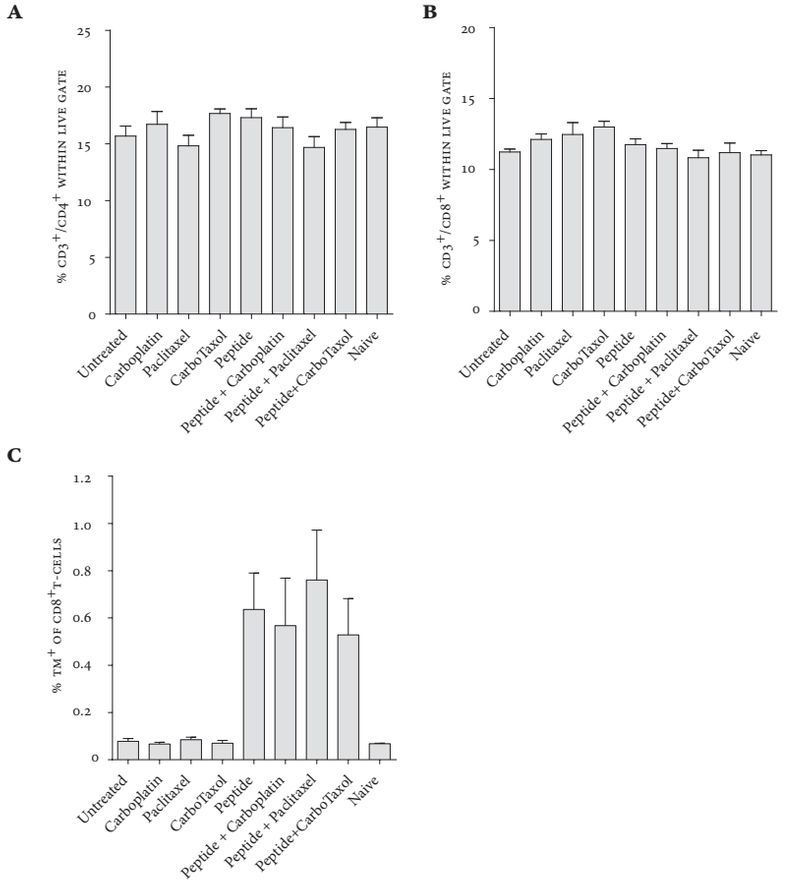


Figure S2 The delay in tumor growth does not differ between the treatment groups. Wild-type C57BL/6 mice were injected with TC-1 tumor cells. Eight days later, when tumors were palpable, mice were treated with synthetic long HPV16 $E7_{43-77}$ peptide in Montanide in the opposite flank. Carboplatin was administered on day 14, paclitaxel on day 14 and 15. Tumor size was measured over time and shown as the mean tumor size measured two-dimensionally (mm^2) plus SEM. The significance of differences in tumor size was calculated for day 18 using one-way ANOVA. The tumor size in the untreated group of mice was significantly different from that of the peptide-treated mice (p = 0.0003), the CarboTaxol-treated mice (p = 0.01), and the peptide plus CarboTaxol treated group (p < 0.0001), whereas there was no significant difference between the different treatment groups. Experiment was performed with 5-7 mice per group; data shown are representative of two individual experiments.

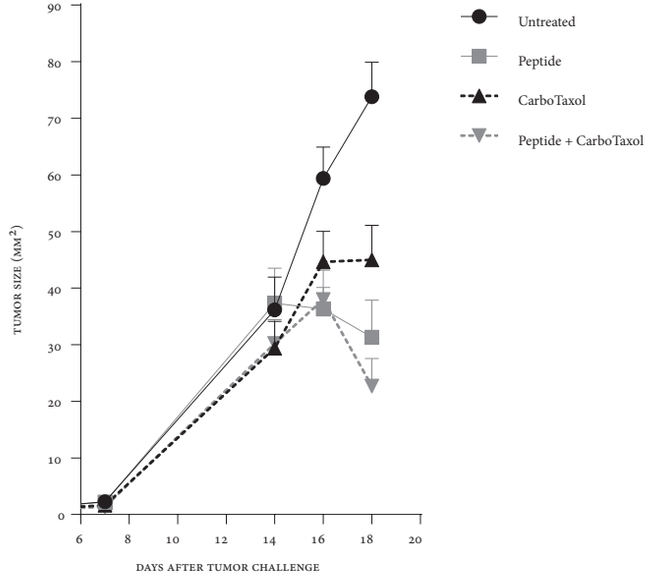


Figure S3 Chemotherapy does not hamper T-cells but decreases myeloid cell frequencies. Wild-type C57BL/6 mice were injected with TC-1 tumor cells. **(A)** As the tumors grew if left untreated, the frequency of myeloid cells increased and the percentage of T-cells decreased as measured in the blood of the tumor-bearing mice. In another experiment, TC-1 tumor cells were injected. Eight days later, when tumors were palpable, mice were treated with synthetic long HPV16 E743-77 peptide in Montanide in the opposite flank. Carboplatin was administered on day 14, paclitaxel on day 14 and 15. Flow cytometry was used to quantify the percentage (mean plus SEM) of **(B)** $CD4^+$ T-cells (Column 1 *vs* 4 (p = 0.001) and 5 (p = 0.008); column 4 *vs* 2 (p = 0.01) and 3 (p = 0.04), **(C)** $CD8^+$ T-cells, **(D)** the vaccine-specific cells within the $CD8^+$ T-cell population [Column 3 *vs* 1 (p = 0.02), 2 (p = 0.003) and 4 (p = 0.004)], **(E)** monocytes, which were identified as F4/80 and CD11b positive [Column 4 *vs* 2 (p = 0.049) and 3 (p = 0.03)], and **(F)** dendritic cells [Column 4 *vs* 1 (p = 0.002), 2 (p = 0.005), 3 (p = 0.0008) and 5 (p = 0.02)]. **(G)** Overlay of $F4/80^+$ and $F4/80^-$ cells in flow cytometry plots that show Gr-1 and CD11b expression of all live cells in the blood of treated mice. **(H)** Flow cytometry plots representing CD11c and CD11b expression of all live cells in the blood of treated mice. The experiment was performed with 5-7 mice per group, and data shown are representative of two individual experiments. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

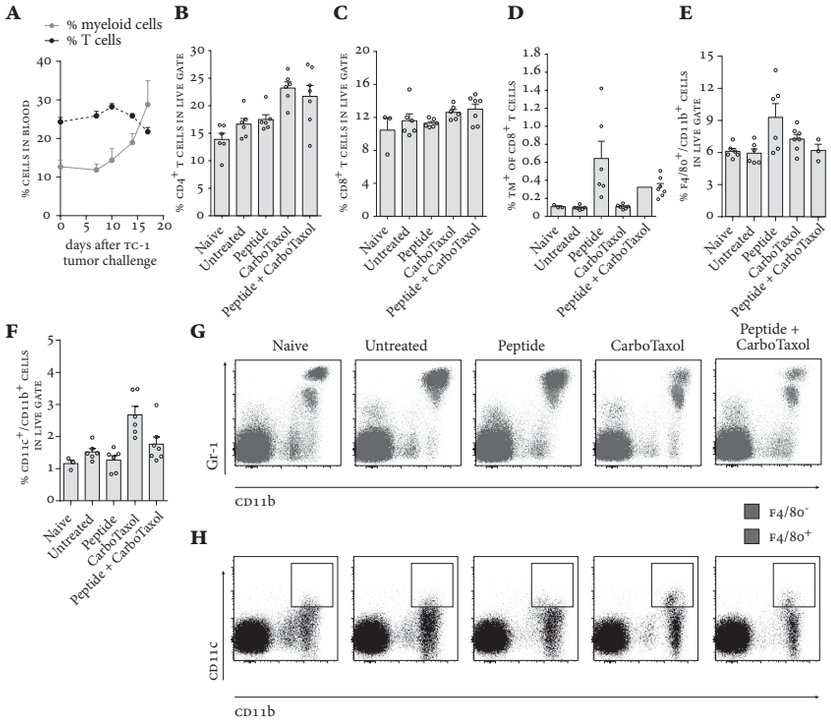


Figure S4 The combination of carboplatin and paclitaxel results in the strongest reduction of circulating myeloid cells. Wild-type C57BL/6 mice were injected with TC-1 tumor cells. Carboplatin was administered on day 14 and paclitaxel on day 14 and 15. Flow cytometry was used to quantify the percentage of $CD11b^{hi}$ cells in the blood. The experiment was performed with 5 mice per group. Data (shown as mean plus SEM) were analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

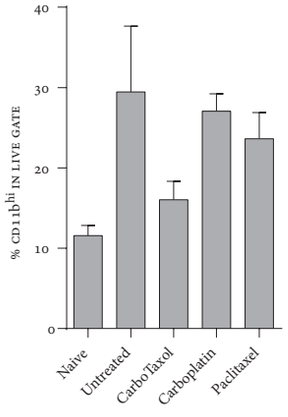


Figure S5 CarboTaxol therapy does not influence general immune parameters. Blood samples obtained at different time points (before, during, and after chemotherapy) from 5 of the 6 cervical cancer patients in cohort 1 were subjected to flow cytometry to determine the frequency of **(A)** $CD4^+$ and **(B)** $CD8^+$ T-cells. Plotted are the percentages of these two types of T-cells within the lymphocyte gate. PBMC samples from **(C)** 5 of the 6 patients in cohort 1 and **(D)** the 12 patients in cohort 2 were stimulated with PHA to test their capacity to proliferate. The results are depicted as stimulation index. **(E)** The quality of the antigen presenting cells in the patients' PBMCs from cohort 2 was analyzed by mixed lymphocyte reaction. The proliferative capacity of third party PBMC is depicted as stimulation index. Data (shown as median plus interquartile range) were analyzed by repeated measures model.

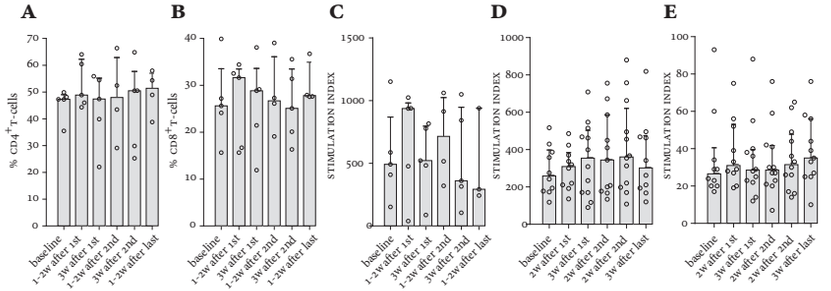


Figure S6 CarboTaxol therapy alters the relative frequencies of myeloid cells and lymphocytes. **(A)** The myeloid cells and lymphocytes as percentage of CD45$^+$ cells are depicted for healthy donors (HD, N = 19) and cervical cancer patients (P, N = 18) at baseline. Column 1 *vs* 2 ($p < 0.0001$); column 3 *vs* 4 ($p < 0.0001$). The shifts in the frequency of **(B)** myeloid cells [Column 1 *vs* 2 (p=0.04), 3 (p = 0.007), 4 and 5 (p = 0.004)] and **(C)** lymphocytes [Column 1 *vs* 2 (p = 0.02), 3 (p = 0.0009), 4 and 5 (p = 0.0002)] compared to baseline values are depicted for the blood samples obtained from the cervical cancer patients of cohort 2 before, during, and after the last cycle of chemotherapy with CarboTaxol. Data (shown as median plus interquartile range) were analyzed by repeated measures model.

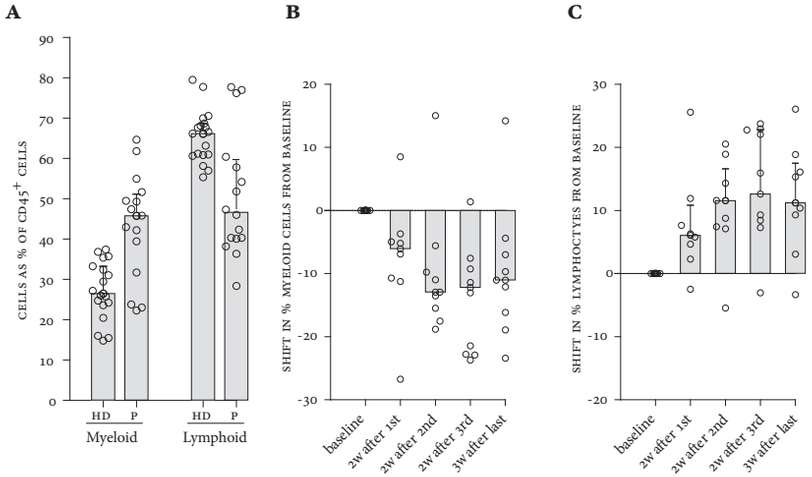


Figure S7 Flow cytometric analysis of myeloid cells and T-cells in blood samples of cervical cancer patients. Blood samples of the cervical cancer patients are stained with three different sets of antibodies and acquired by flow cytometry to determine the composition of immune cells within the sample and over time within an individual patient. An example of the gating strategy is shown for these three sets of antibodies. **(A)** In the macrophage-like set, first the single cells were gated, then the CD45$^+$ cells followed by the selection of non T and non B cells (CD3$^-$CD19$^-$). Then, the HLA-DR$^+$CD1a$^-$ cells were selected. Next, this myeloid cell population was plotted for the expression of CD14 and CD11b, revealing 5 subpopulations. Each of these subpopulations was then analyzed for the expression of CD11c, CD16, CD206, and CD163.

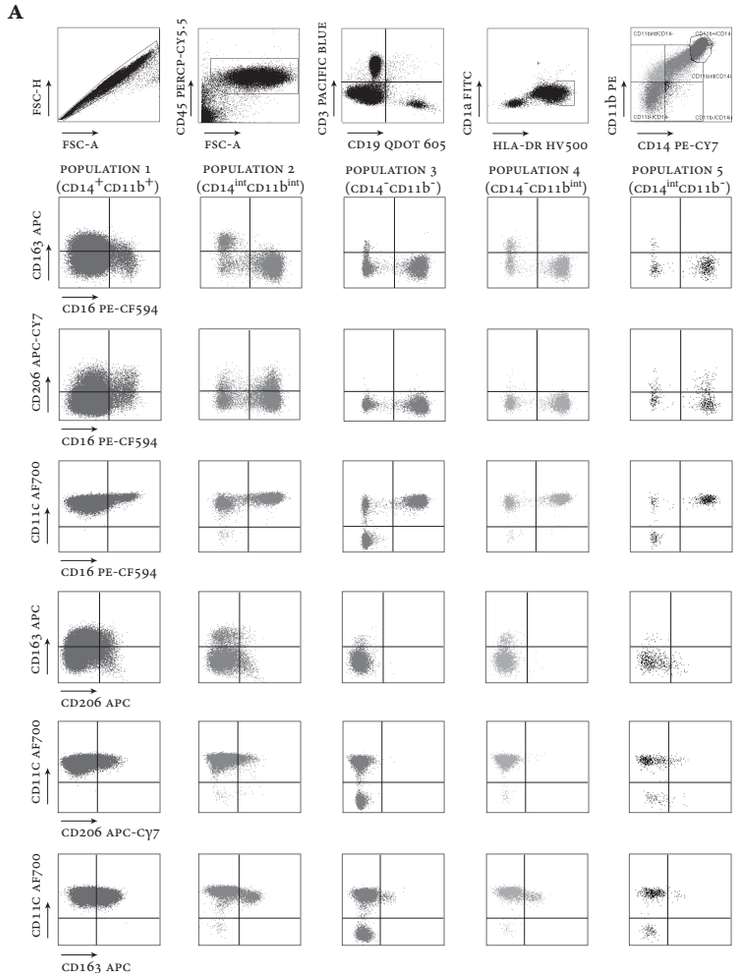


Figure S7(B) In the myeloid-derived suppressor cell (MDSC) set, we used the same strategy as in the macrophage panel for the first three plots. Then, the HLA-DR$^{-/low}$ subpopulation within the myeloid cells (CD3$^-$CD19$^-$) was selected and the expression of CD14 (for myeloid MDSC; mMDSC) and CD15 (for granulocytic MDSC; gMDSC) plotted. Both subsets of MDSC as well as the double negative cells (CD14$^-$CD15$^-$) were analyzed for the expression of the other markers: CD11b, CD33, CD34, and CD124.

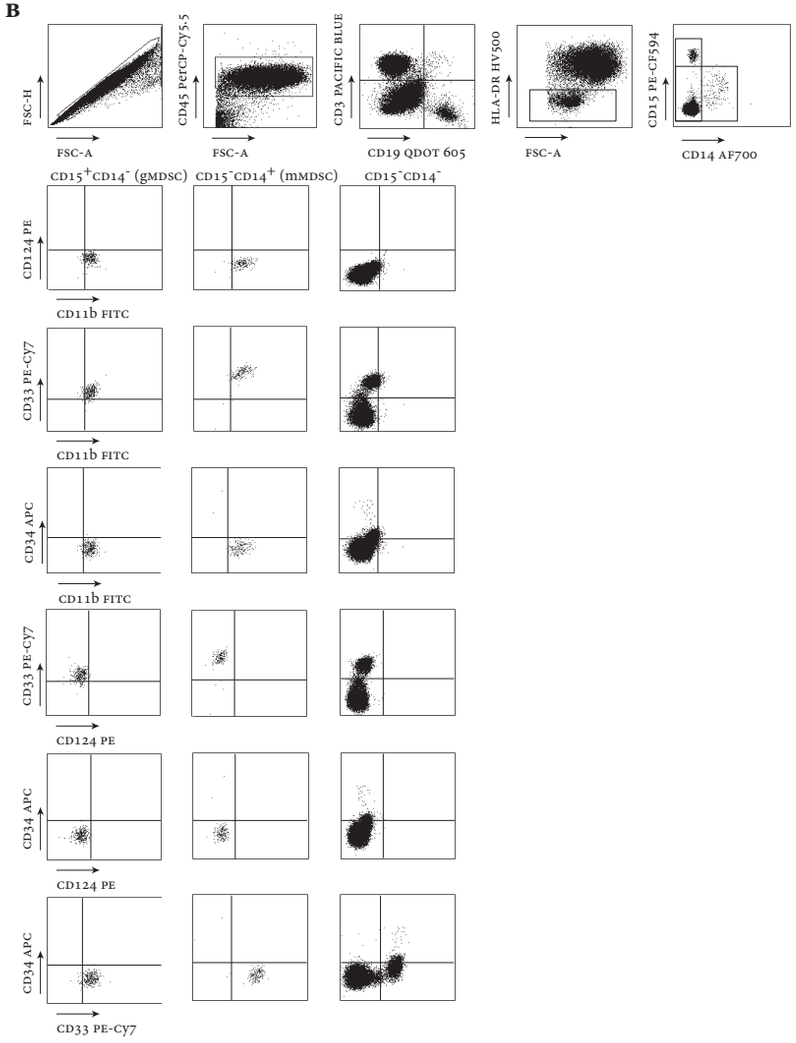


Figure S7(C) In the inhibitory receptors set, the CD3$^+$ cells were selected from the single cells, followed by the live gate. Then, CD4 was plotted versus CD8 and within each of these two T-cell populations, the expression of TIM-3, PD-1, and CTLA-4 was determined.

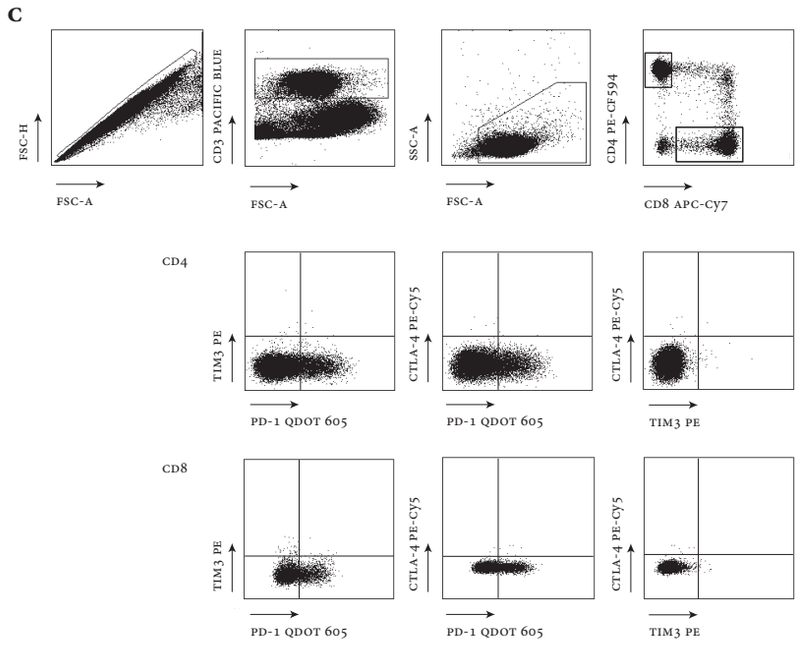


Figure S8 CarboTaxol treatment affects different subpopulations of CD11b and/or CD14 positive myeloid cells. The phenotypic analysis of the blood samples of cervical cancer patients was performed with the macrophage antibody set and using the gating strategy shown in fig. S7A, for the 5 different populations which could be defined within the $CD45^+CD3^-CD19^-CD1a^-HLA\text{-}DR^+$ subpopulation of cells on the basis of differential expression of the markers CD14 and CD11b. These subsets of cells were followed over time in all 12 vaccinated patients of cohort 2 and are plotted as median plus interquartile range. **(A)** Population 2, with intermediate expression of both CD14 and CD11b. Column 1 *vs* 2 (p = 0.005), 3 (p = 0.03), 4 (p = 0.01) and 6 (p = 0.004). **(B)** Population 3, lacking the expression of both markers. Column 1 *vs* 3 to 6 (p < 0.0001). Column 2 *vs* 3 and 6 (p = 0.04), 4 (p = 0.004). **(C)** Population 4, with intermediate CD11b expression and no CD14. Column 1 *vs* 6 (p = 0.03). **(D)** Population 5, with intermediate CD14 expression and no CD11b. Column 1 *vs* 4 (p = 0.008). Column 2 *vs* 3 and 6 (p = 0.02), and 4 (p = 0.002). Data (shown as median plus interquartile range) were analyzed by repeated measures model.

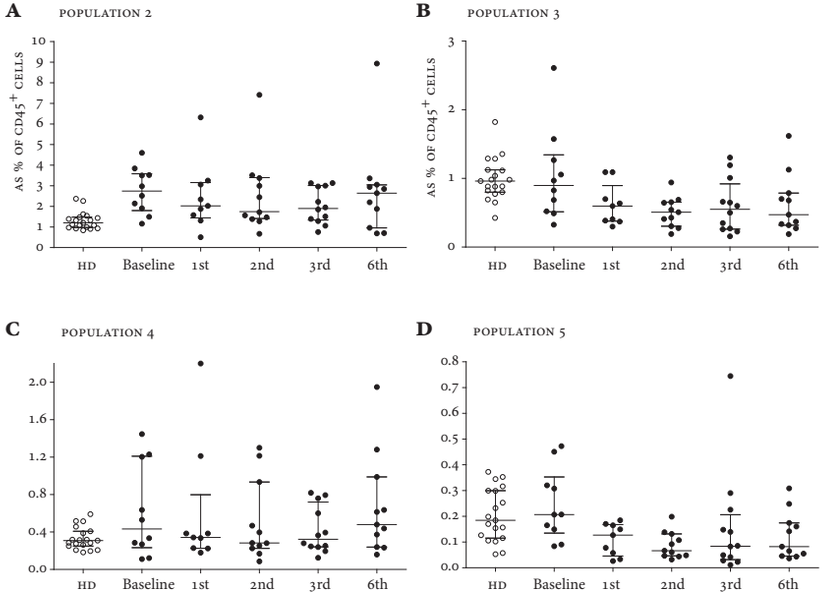


Figure S9 CarboTaxol therapy reduces the number of regulatory T-cells in cervical cancer patients. The T-cells in the blood samples of cervical cancer patients of cohort 2 as well as from healthy donors (HD) were stained for multiple markers indicative of inhibitory receptors. The frequency is depicted as percentage of the $CD45^+$ cells. **(A)** $CD4^+$ T-cells [Column 1 *vs* 2 (p = 0.009)]. **(B)** $CD8^+$ T-cells. **(C)** The frequency within the $CD4^+$ T-cells was calculated as a percentage of $CD45^+$ cells that express TIM3 alone [left; column 1 *vs* 2 (p = 0.01); column 2 *vs* 4 (p = 0.002), 5, and 6 (p = 0.01)], both TIM3 and PD-1 [middle; column 1 *vs* 2 (p = 0.007), 3 (p = 0.01), 4 (p = 0.04), and 5 (p = 0.004)], or PD-1 alone [right; column 1 *vs* 2 (p = 0.0008), 3 (p = 0.001), 4 (p = 0.0001), 5 (p < 0.0001), and 6 (p = 0.002)]. **(D)** The frequency within the $CD8^+$ T-cells calculated as percentage of $CD45^+$ cells that express TIM3 alone [left; column 1 *vs* 2 (p = 0.01), 3 (p = 0.004), 4 (p = 0.02), 5 (p = 0.004), and 6 (p = 0.009)], both TIM3 and PD-1 [middle; column 1 *vs* 2 (p = 0.008), 3 (p = 0.01), 5 (p = 0.03)], or PD-1 alone [right; column 1 *vs* 2 (p = 0.04)]. **(E)** Measurement of regulatory T-cells within the $CD3^+CD8^-CD4^+$ population by expression of $CD25^+$, $CD127^-$, and $Foxp3^+$ (Column 1 *vs* 2 and 3 (p < 0.0001), 4 (p = 0.001), and 5 (p = 0.002); column 2 *vs* 4 (p = 0.007) and 5 (p = 0.005). Data (shown as median plus interquartile range) were analyzed by repeated measures model.

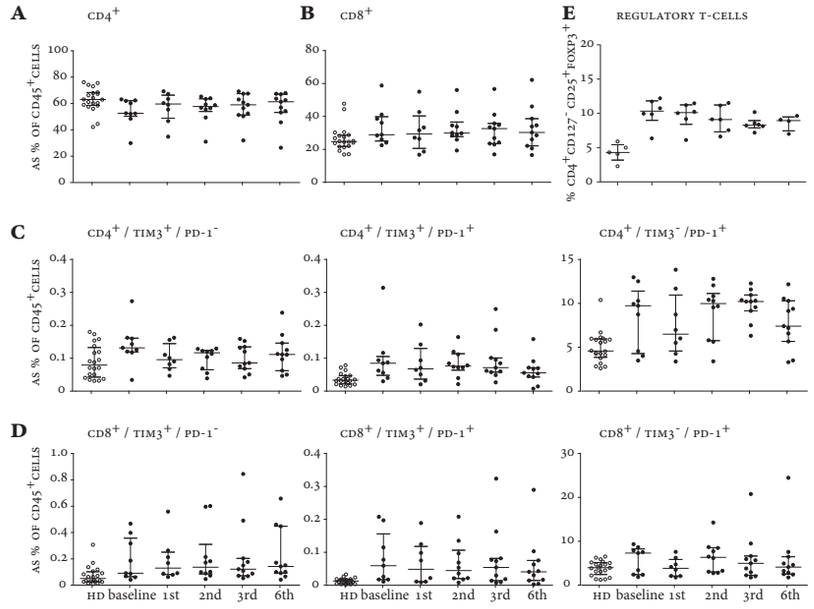


Figure S10 HPV16-SLP vaccination induces poly-functional T-cells. An in depth analysis by intracellular cytokine staining could be performed to determine the cytokine production specifically upon recognition of HPV16 E6 and/or E7 peptides for 6 patients in the second cohort. The stacked bars show the frequency of $CD4^+$ T-cells producing only TNFα ($TNF\alpha^+$ $IL\text{-}2^-$ $IFN\gamma^-$; white bars), both TNFα and IL-2 ($TNF\alpha^+$ $IL\text{-}2^+$ $IFN\gamma^-$; gray bars), and all three cytokines ($TNF\alpha^+$ $IL\text{-}2^+$ $IFN\gamma^+$; black bars) for the two viral oncoproteins in the indicated blood samples.

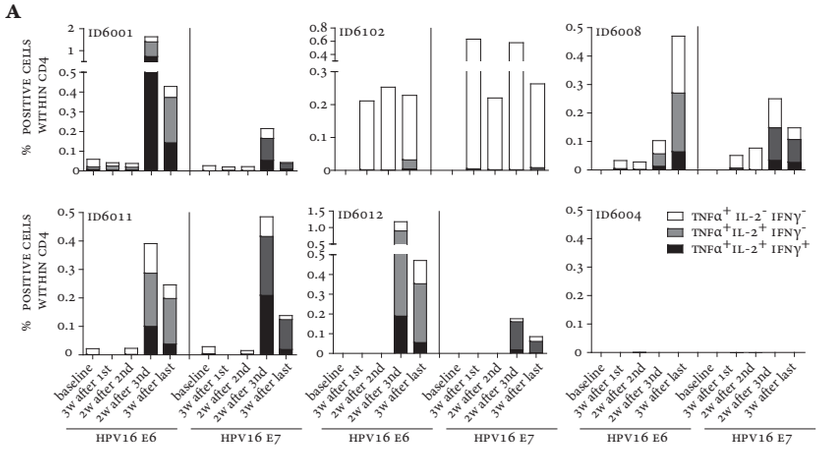


Figure S11 The vaccine-induced HPV16-specific T-cell response is stronger in patients vaccinated during chemotherapy. The median stimulation index (plus interquartile range) of the 6 tested peptide pools per patient was calculated for all patients at each indicated time point and depicted for **(A)** recurrent cervical cancer patients in the previously conducted clinical trial [15], in which the patients received HPV16-SLP vaccination at least one month after they had undergone chemotherapy and **(B)** advanced cervical cancer patients who were vaccinated during chemotherapy as described in the current trial. The blood samples were taken before vaccination (pre-vac), or after 1 (1-vac), 2 (2-vac), or 4 (4-vac) vaccinations as indicated. FU, follow-up blood sample taken after the last cycle of chemotherapy. In both graphs, the pre-vaccinated median stimulation index is significantly different from the two post-vaccinated responses ($p < 0.0001$). Data were analyzed by paired T-test.

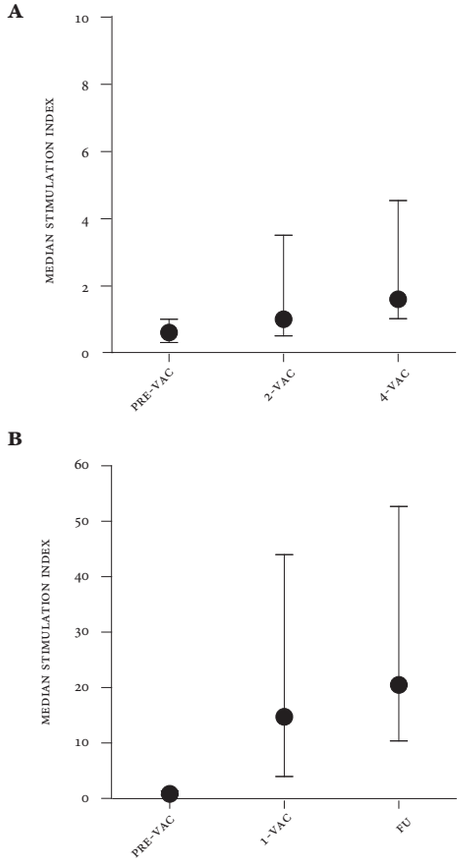
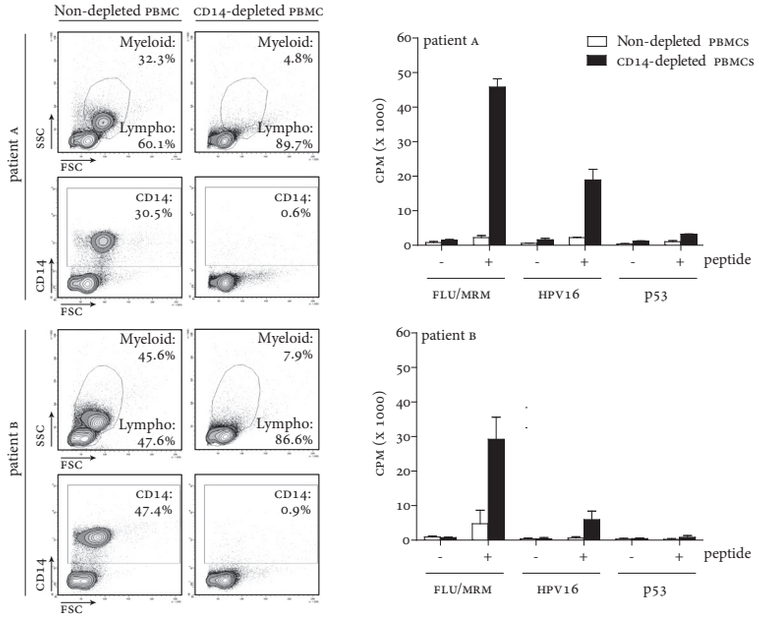


Figure S12 Myeloid cell depletion improves the response of PBMC to stimulation in vitro. The $CD14^+$ myeloid cells were depleted in the pre-chemotherapy PBMC samples of two advanced cervical cancer patients via magnetic cell sorting. The depleted and non-depleted PBMCs were stimulated with autologous monocytes, which were pulsed with a mix of recall antigens (FLU and MRM), a pool of HPV16 E6 and E7 SLP, or a pool of p53 SLP, for 11 days after which the bulk culture was tested in a 3-day proliferation assay. At the top, the forward (FSC) and side (SSC) scatter plots are shown for both the non-depleted and depleted PBMC. The percentages indicate the frequencies of lymphoid and myeloid cells. In the FSC and CD14 plots (bottom), the $CD14^+$ cell frequencies in PBMC before and after the CD14 depletion are shown. On the right, the graphs display the antigen-specific proliferation (in counts per minute, cpm, shown as mean of triplicate wells plus standard deviation) for the three different bulk cultures after stimulation with non-pulsed (peptide -) or antigen-pulsed (peptide +) autologous monocytes



VI

IMPACT OF (CHEMO) RADIOTHERAPY ON IMMUNE CELL COMPOSITION AND FUNCTION IN CERVICAL CANCER PATIENTS

OncoImmunology 2017; 1;e1267095

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ABSTRACT

New treatments based on combinations of standard therapeutic modalities and immunotherapy are of potential use, but require a profound understanding of immune modulatory properties of standard therapies. Here, the impact of standard (chemo)radiotherapy on the immune system of cervical cancer patients was evaluated. Thirty patients with cervical cancer were treated with external beam radiation therapy (EBRT), using conventional three-dimensional or intensity modulated radiation therapy without constraints for bone marrow sparing. Serial blood sampling for immunomonitoring was performed before, midway, and at 3, 6 and 9 weeks after EBRT to analyze the composition of lymphocyte and myeloid-cell populations, the expression of co-stimulatory molecules, T-cell reactivity and antigen presenting cell (APC) function. Therapy significantly decreased the absolute numbers of circulating leukocytes and lymphocytes. Furthermore, the capacity of the remaining T-cells to respond to antigenic or mitogenic stimulation was impaired. During treatment the frequency of both $CD4^+$ and $CD8^+$ T-cells dropped and $CD4^+$ T-cells displayed an increased expression of programmed cell death-1 (PD-1). In vitro blocking of PD-1 successfully increased T-cell reactivity in all 5 samples isolated before radiotherapy but was less successful in restoring reactivity in samples isolated at later time points. Moreover, (chemo)radiotherapy was associated with an increase in both circulating monocytes and myeloid-derived suppressor cells (MDSCs) and an impaired capacity of APCs to stimulate allogeneic T-cells. T-cell reactivity was slowly restored at 6-9 weeks after cessation of therapy. We conclude that conventional (chemo)radiotherapy profoundly suppresses the immune system in cervical cancer patients, and may restrict its combination with immunotherapy.

Introduction

Radiotherapy is used as primary or adjuvant therapy in the curative treatment of patients with cervical cancer. Primary (chemo)radiotherapy is an effective treatment for locally advanced cervical cancer with a 5-year pelvic control rate of 87% and a cancer-specific survival of 79%.[1] However, especially in cases with tumor cell positive lymph nodes and patients with higher stages, systemic failure of current therapies represents a major challenge. Radiotherapy was thought to mediate its effect through direct cytotoxic or cytostatic effects on malignant cells, but (pre)clinical findings suggest that its therapeutic effect also contains vascular and immunogenic components. Normalization of the vasculature by radiotherapy facilitates the delivery of chemotherapeutic compounds and promotes the infiltration by effector immune cells into the tumor bed.[2-4] In addition, radiation therapy may alleviate immune suppression in the tumor microenvironment[5-7] as well as (re)activate a tumor specific cellular immune response.[6] Changes in the tumor microenvironment contribute substantially to treatment success or failure, particularly in so-called immunogenic tumors.[8] Interestingly, it was shown that a pre-treatment peripheral blood lymphocyte count at or above the median value was associated with higher clinical responses and survival rates. Apparently, peripheral blood lymphocyte count and lymphocyte subsets are independent predictors of survival and tumor regression in cervical cancer patients treated with concurrent chemoradiation.[9,10]

Cervical cancer is regarded as an immunogenic tumor since it is induced by a persistent infection with human papilloma virus (HPV), most often HPV16 or HPV18.[11] The number and functional orientation of tumor-infiltrating $CD4^+$ and $CD8^+$ T-cells, and the presence of M1 type macrophages is strongly associated with survival in patients with cervical cancer after primary treatment.[12-15] The up-regulation of signaling through negative co-stimulatory molecules on T-cells, such as Cytotoxic T-lymphocyte Antigen 4 (CTLA-4) and programmed cell death-1 (PD-1), is another mechanism through which T-cell infiltration and function can be impaired in cervical cancer.[16,17] Whereas studies in mouse tumor models suggest that certain radiotherapy schedules can be combined with immunotherapy[18-20], the effect of standard radiation therapy in patients with cervical cancer has not been extensively studied. Most clinical studies investigated the baseline lymphocyte count as a prognostic predictor of treatment response[9,10,21,22], rather than the effects of therapy on the composition and function of these cells during treatment. Therefore, this study focused on the influence of pelvic radiation on immune responses in patients with cervical cancer during and after treatment. We prospectively

analyzed changes in the immune cell composition and function during radiation therapy, with or without concomitant platinum-based chemotherapy, in serial blood samples from 30 patients with cervical cancer. We examined alterations in different lymphocytes subtypes, myeloid cell populations, the expression of co-stimulatory molecules, T-cell reactivity to antigens and the capacity of antigen presenting cells (APCs) to stimulate T-cells. Our study showed that (chemo)radiotherapy for cervical cancer induced unfavorable immune changes reflected by a decreased number of circulating lymphocytes and an increased percentage in myeloid-cell populations, including myeloid-derived suppressor cells (MDSCs) and monocytes. Moreover, radiotherapy subverted the reactivity of T-cells to antigenic stimulation and the capacity of APCs to enhance allogeneic T-cell proliferation. We further demonstrated that radiotherapy upregulates PD-1 expression on the circulating $CD4^+$ T-cells, which can partially explain their lower reactivity to antigenic stimulation.

Materials and methods

ETHICS

The Medical Ethics Committees of the Leiden University Medical Center (LUMC) and the Netherlands Cancer Institute - Antoni van Leeuwenhoek hospital (NKI-AVL) formally approved the protocol of this study. The study was conducted according to the Dutch Act on Medical Research involving Human Subjects (WMO) and was registered under number NL36829.058.11. Written informed consent was obtained from all patients before study inclusion and participation. All participating patients were acknowledged that they were fully anonymized, and cannot be identified via the paper.

PATIENTS AND TREATMENTS

This observational study was performed at the gynecology clinics of the LUMC and the NKI-AVL from October 2011 to December 2014. Thirty patients with invasive cervical cancer (FIGO stage IB1 to IV[23]) with an indication for external beam radiation therapy (EBRT) were recruited for participation. Additionally, the eligibility of patients required all of the following criteria: mentally competent patients of 18 year and older, no other active malignancy than cervical cancer, no indication of active infectious disease such as HIV and hepatitis B, and no medical condition that may interfere with the study objectives.

Radiotherapy was used as primary treatment for locally advanced disease (FIGO stages IB2-IIIB, or lymph node positive), in combination with concurrent cisplatin chemotherapy and brachytherapy (BT). As adjuvant therapy after radical hysterectomy and pelvic lymphadenectomy for early stage disease, radiotherapy was applied in case of high-risk early stage disease with two or three unfavorable tumor characteristics.[24-27] Unfavorable tumor characteristics included tumor diameter exceeding 40 mm, tumor depth more or equal to 15 mm, and lymphovascular space involvement (LVSI). Patients with 2 or more tumor-positive lymph nodes, parametrial infiltration, or tumor-positive surgical margins were treated with radiotherapy and concurrent cisplatin chemotherapy. Patients suffering from recurrent cervical cancer with only surgical treatment in history were treated with EBRT in combination with chemotherapy, with or without BT. Patients with a contra-indication for cisplatin treatment were treated with 5 weekly courses of deep tissue hyperthermia.

All treatments were carried out in accordance with the guidelines of the radiotherapy departments of the participating hospitals. EBRT was delivered in 23 fractions of 2 Gy (total 46 Gy) or an equivalent dose given in fractions of 1.8 Gy; 5 times a week. An EBRT boost was given in patients with PET-CT suspected lymph node metastases, aiming for a total dose of 60 Gy, taking the BT dose contribution into account. Standard EBRT was delivered using either conventional three-dimensional (3D-CRT) or intensity modulated radiation therapy (IMRT) without constraints for bone marrow sparing. Concurrent chemotherapy consisted of 5 or 6 weekly cycles of intravenous cisplatin (40mg/m^2 per cycle). Dose adjustments, omissions and delays were implemented as the standard intravenous cisplatin administration protocol of the institutes. Upon completion of EBRT, MRI-guided intracavitary alone or combined interstitial-intracavitary high dose rate BT was administered in three or four fractions of 7 Gy.[28] The dose rate and fractionation was performed according to the department policy, aiming at an equivalent dose in 2 Gy fractions (EQD2 dose) of at least 80-85 Gy in high risk – clinical target volume (HR-CTV) according to the Groupe Européen de Curiethérapie (GEC) and the European Society for Radiotherapy & Oncology (ESTRO) guidelines.[29] The aim was to maintain the overall treatment time within 7 weeks.

BLOOD SAMPLING AND FOLLOW-UP

Venous blood sampling for routine leukocyte differential count analysis took place daily during the first week of EBRT. These samples were analyzed for leukocyte differentiation at the routine laboratories of LUMC or NKI-AVL.

Additionally, a full blood count and blood sampling for immunomonitoring were performed before start of radiotherapy (baseline), after 15 fractions of EBRT (midway), and at 3 weeks after completion of EBRT. Analysis of the data obtained in the first 15 patients at 3 weeks after therapy showed substantial decreases in lymphocyte counts and an impaired capacity of peripheral blood mononuclear cells (PBMCs) to respond to antigenic stimulation. Therefore, the protocol was amended to allow extra blood samples for immunological analysis at 6 and 9 weeks after completion of radiation therapy for the subsequent 15 patients. Twelve patients of the second group consented for these additional blood draws at 6 and 9 weeks. Figure 1 shows treatment schedules in detail, with exact intervals between treatments and blood samples.

IMMUNOMONITORING

Venous blood samples for immunomonitoring were taken in 6 heparinized tubes of 9 mL to isolate PBMCs and in one 9 mL clot activator tube to obtain serum. Blood samples were transported at room temperature and PBMCs were isolated by Ficoll gradient centrifugation within 6 hours. Part of these freshly obtained PBMCs was used for the lymphocyte stimulation test (LST). The remaining cells were cryopreserved in 90% fetal calf serum (PAA Laboratories, Pashing, Austria) and 10% DSMO at a concentration of 7 to 12 million cells per vial in a total volume of 1 mL using a mister Frosty's freezing container (Nalgene). Upon cryopreservation the vials were stored in the vapor phase of the liquid nitrogen until further use. Immunological assays were performed and analyzed under blinding for clinical parameters of the participating patients.

T-CELL PROLIFERATION ASSAYS The proliferative response to the memory response mix (MRM) and influenza matrix 1 protein-derived peptides (FLU)[30] was determined using freshly isolated PBMCs that were subjected to the Lymphocyte Stimulation Test (LST) as described previously.[30,31] In short, eight replicate wells with 1.5 x 10^5 cells per well were stimulated for 6 days with the indicated antigens (10 µg/mL), after which 50 uL supernatant per well was harvested, pooled for the 8 similar wells and stored at -20°C for cytokine analysis. The cells were pulsed with 10 µCi/mL [^{3}H]-Thymidine (Perkin Elmer, the Netherlands). The negative control consisted of cells in medium (Iscove's Modified Dulbecco's Medium, IMDM (Lonza) plus 10% human AB serum (Life Technologies)) only. A positive response was defined as a stimulation index (S.I.) of at least 3 under the condition that 6 out of 8 wells displayed values above the cut off value, which was defined as the mean value of the cells in medium only plus 3 x standard deviation (SD).

The capacity to respond to Phytohemagglutinin (PHA) was studied using cryopreserved PBMCs.[31] Cells were thawed and tested in a 3-days proliferation assay with the minor alteration that 50.000 cells per well (in quadruplicate) were incubated in medium (IMDM) or stimulated with 0.25 µg/mL PHA (Murex Biotech HA16). At day 2, 100 µL supernatant per well was harvested for cytokine analysis, and cells were subjected to [^{3}H]-thymidine (50 µL/well of 10 µCi/mL) for an additional 16-20 hours. A positive response was defined as an S.I. of 3 or higher.

ANTIGEN PRESENTING CAPACITY ASSAY The antigen presenting capacity of the patient's PBMCs was determined in a mixed lymphocyte reaction (MLR).[31] PBMCs were thawed in IMDM plus 10% fetal calf serum and 30 µg/mL DNase (Sigma, St Louis, USA), pelleted and suspended in IMDM plus 10% human AB serum. Then, irradiated (3000 rad) to prevent proliferation, washed, suspended in IMDM plus 10% human AB serum and plated at 1x10^5 cells per well (in quadruplicate). Third party PBMCs of 2 donors were added to determine their proliferative capacity upon encountering the irradiated patients' APCs. Third party PBMCs only as well as irradiated patients PBMCs only were used as negative controls. At day 6, 100 µL supernatant per well was harvested for cytokine analysis, and the cells were subjected to [^{3}H]-thymidine (50 µL/well of 10 µCi/mL) for an additional 16-20 hours. A positive response was defined as an S.I. of at least 3.

CYTOKINE ANALYSIS The supernatants harvested in the proliferation and MLR assays were subjected to a flow cytometer based cytokine bead array (CBA, human Th1/Th2 kit, BD), which was conducted according to the manufacturer's instructions and as reported earlier.[30] The cytokine panel consisted of IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2. A positive response was defined as a cytokine concentration above the detection limit as indicated by the manufacturer, which was 20 pg/mL for each cytokine. Treatment-related change in cytokine production was defined as a cytokine concentration above the cut-off value and a three-fold increase or decrease above the baseline sample (pre-radiotherapeutic treatment).

PHENOTYPING OF PBMCS The PBMC samples isolated at different time points were phenotyped as described earlier by using 3 sets of 10-13 cell surface markers to identify immune cell subsets and the expression of co-inhibitory molecules by flow cytometry.[31]

The myeloid set consists of CD3-HV450 (Clone UCHT1; BD), CD1a-FITC (Clone HI149; BD), CD11b-PE (Clone D12; BD), CD11c-BV650 (Clone B-ly6; BD), CD14-AF700 (Clone M5E2; BD), CD15-PE-CF594 (Clone W6D3; BD), CD19-BV605

(Clone SJ25C1; BD), CD33-PE-Cy7 (Clone P67.6; BD), CD56-PerCP-Cy5.5 (Clone HCD56; Biolegend), CD163-APC (Clone 215927; R&D), CD206-APC-Cy7 (Clone 15-2; Biolegend) and HLA-DR-V500 (Clone L243; BD).

The inhibitory set consists of CD3-HV450 (Clone UCHT1; BD), and CD4-PE-CF594 (Clone RPA-T4; BD), CD8-APC-Cy7 (Clone SK1; BD), CD56-AF700 (Clone B159; BD), CD94-FITC (Clone 131412; R&D), CD152-PE-Cy5 (anti-CTLA-4, Clone BN13; BD), CD279-PE-Cy7 (anti-PD-1, Clone EH12.2H7; Biolegend), TIM-3-BV605 (Clone F38.2E2; Biolegend) and CD159a-PE (NKG2a, Clone Z199; Beckman Coulter).

The regulatory T-cell set consists of CD3-HV500 (Clone UCHT1; BD), and CD4-AF700 (Clone RPA-T4; BD), CD8-PerCP-Cy5.5 (Clone SK1; BD), CD25-PE-Cy7 (Clone 2A3; BD), CD127-BV650 (Clone HIL-7R-M21; BD), CD45RA-APC-H7 (Clone HI100; BD), CD152-BV421 (CTLA-4, Clone BNI3, BD); FOXP3-PE-CF594 (Clone 259D/C7; BD), Helios-APC (Clone 22F6; Biolegend) and Ki67-FITC (Clone 20Raj1; eBioscience).[32]

The cryopreserved PBMCs were thawed and first subjected to live-dead marker (Yellow amino reactive dye (ARD); dilution 1:800) incubation for 20 minutes at room temperature in 100 µL/well. Then, the cells were pelleted and suspended in phosphate buffered saline (PBS), washed and supplemented with 0.5% bovine serum albumin (BSA, Sigma) and 10% FCS for an incubation of 10 minutes on ice (4°C and in the dark) to prevent non-specific antibody binding to free Fc-receptors on the cells. Subsequently, the cells were centrifuged, washed and suspended in the antibody mixtures described above and incubated in the dark for 30 minutes on ice. Finally, the cells were washed twice with PBS/0.5% BSA and suspended in 1% paraformaldehyde (LUMC Pharmacy). For the regulatory T-cell staining, following the Yellow ARD incubation and blocking step, cells were stained for surface markers as described above, washed twice with PBS/0.5% BSA and subsequently fixated in transcription factor fixation and permeabilized by buffer (BD) and intranuclear stained with the antibodies CD152, Foxp3, Helios and Ki67 (diluted in permeabilization and washing buffer (BD)) for 40-50 minutes on ice. Cells were finally suspended in 1% paraformaldehyde and assessed within 24 hours while keeping them in the dark at 4°C.

FLOW CYTOMETRY Acquisition on the BD Fortessa flow cytometers was performed within 24 hours after staining of the cells was finished. Analysis was performed using DIVA software (BD Biosciences, version 6.2).

PD-1 BLOCKING AND STIMULATION OF PBMC IN VITRO Thawed autologous monocytes (1-4 x 10^6 cells/mL) were adhered to the bottom of the wells in a 96-wells plate in X-vivo 15 medium (Lonza) during 2 hours incubation at 37°C, 5% CO2 in a humidified incubator. The wells were subsequently gently washed to remove non-adherent responder cells (which were centrifuged and stored in a 15 mL tube in IMDM with 10% human AB serum and rested overnight in the incubator) and the adhered monocytes were replenished in 75 µL X-vivo 15 medium with 800 U/ml granulocyte macrophage colony-stimulating factor (GM-CSF) and incubated for 5 hours in the incubator. Then, the monocytes were overnight loaded in triplicate wells with 75 µL of the FLU peptide pool (at a concentration of 5 µL/mL) diluted in X-vivo 15 medium. Medium only served as a negative control. The next day, responder cells (50.000-100.000 cells/well) were added as well as the anti-PD-1 antibody Nivolumab (final concentration of 1 µg/mL). After 5 days of incubation, 50 µL supernatant per well was harvested and stored at -20°C for cytokine analysis. The cells were pulsed with [^{3}H]-Thymidine (Perkin Elmer, the Netherlands), and filters were counted using the Betaplate counter.

LABORATORY ENVIRONMENT Immunomonitoring of patient's PBMCs was performed in the laboratory of the department of Medical Oncology at LUMC that operates under research conditions, following standard operating procedures (SOPs) and using trained personnel. The authors acknowledge the reporting of results from T-cell assays according to the minimal information about T-cell assays (MIATA). Definitions of positive responses were pre-established. This laboratory has been audited both internally and externally, according to the reflection paper for laboratories that perform immunomonitoring.[33] The laboratory has participated in all proficiency panels of the CIMT Immunoguiding Program (CIP; http://www.cimt.eu/workgroups/cip/) as well as many of the proficiency panels of the USA-based Cancer Immunotherapy Consortium (CIC of the Cancer Research Institute), whose aim is to harmonize reporting and assays used for immunomonitoring and to validate SOPs.

STATISTICAL INTERPRETATION

The repeated measured immune responses of patients were analyzed with a mixed model analysis of variance with fixed factors time, if feasible chemotherapy and chemotherapy by time, random factor subject. Contrasts calculated within the model included: different time points, with or without chemotherapeutic treatment. The fold change in MRM and FLU, absolute shift in lymphoid and myeloid cells and PD-1 expressing $CD4^+$ T-cells, were analyzed with a repeated measures regression analysis with a compound structure covariance structure and time as repeated factor within each subject. Analysis results per variable are generated with estimates of the difference of the different contrasts and a

back transformed estimate of the difference in percentage for log transformed parameters, 95% confidence intervals (in percentage for log-transformed parameters) and least square means (LSM, geometric means for log transformed parameters), and a p-value of the contrasts. A p-value < 0.05 was considered statistically significant. All calculations and statistical analysis were performed using SAS for Windows V9.4 (SAS Institute, Inc., Cary, NC, USA)

Results

CHARACTERISTICS OF THE PATIENTS

Thirty patients with histologically proven invasive cervical cancer FIGO stage IB1 to IV, who were to receive EBRT with a curative intention, were enrolled between October 2011 and December 2014. Patient characteristics are summarized in table 1. Twenty-three patients (76.6%) received combined radiotherapy and chemotherapy, of which 18 with brachytherapy and 5 without. Four patients (13.3%) received radiotherapy alone, two patients (6.7%) received radiotherapy with brachytherapy and 1 patient (3.3%) received radiotherapy with hyperthermia. The patient receiving hyperthermia was initially scheduled for radiotherapy in combination with chemotherapy, but chemotherapy was canceled due to pre-existent renal impairment. Supplemental table 1 shows detailed information about all participating patients including treatment indication, dosages of the different treatment regimens and existence of high or low tumor load (high in case of primary or recurrence therapy; low in case of adjuvant, post-operative therapy). Three patients refused further participation after the first blood sampling and were considered as lost to immunological follow-up.

RADIATION THERAPY HAS A PROFOUND EFFECT ON CIRCULATING IMMUNE CELLS

The routine leukocyte differential count obtained daily during the first week of EBRT, after 15 fractions of EBRT, and 3 weeks after completion of EBRT in the first 18 patients showed a decreased number of leucocytes in blood, which included a decrease in the absolute number of lymphocytes. This decrease in lymphocytes occurred early during treatment; already within 48 hours after the first fraction a decrease of 27% was observed (from 1.916 x 10^9/L (mean, range 0.96 - 3.37) at baseline to 1.397 x 10^9/L (mean, range 0.5 - 1.99) after the 2nd fraction; p = 0.0004) (figure 2A), and lasted at least until 3 weeks after completion of (chemo) radiation therapy (figure 2B). It was therefore decided to extend the observation period. An extended follow up of 6 weeks after completion of EBRT was done in 12 patients, and follow-up of 9 weeks after completion of EBRT was possible in 10 patients. All 30 patients showed the most distinct decrease of peripheral lymphocytes halfway the radiation treatment (after 15 fractions) with a mean absolute lymphocyte count of 0.39 x 10^9/L, and showed a slight increase of lymphocyte count when radiation treatment was finished (figure 2B). Although at 6 weeks after completion of radiotherapy, the lymphocyte count was slightly increased compared to mid-treatment, it was still significantly lower when compared to baseline (p < 0.0001). This decrease in lymphocytes occurred regardless of tumor-load and of concurrent cisplatin treatment (supplementary figures 1A and 1B). These results suggest that (chemo)radiotherapy as used in cervical cancer patients is immunosuppressive.

RADIOTHERAPY REDUCES T-CELL REACTIVITY AGAINST COMMON RECALL ANTIGENS AND MITOGENS

PBMCs were stimulated with a pool of Influenza M1 peptides (FLU) and with a mix of bacterial recall antigens (MRM) to test if radiation therapy suppresses the capacity of T-cells to respond to antigenic stimulation. In 29/30 patients (96.7%) the T-cell reactivity to FLU and MRM was strongly decreased during and shortly after completion of EBRT when compared to baseline (figure 3). Strikingly, the T-cell reactivity to MRM and FLU remained suppressed at 6 weeks and 9 weeks after completion of EBRT. The combined data show a severe decrease in the capacity of T-cells to respond to stimulation with MRM and FLU after 15 fractions of EBRT (p = 0.0027 for MRM and p < 0.0001 for FLU). This situation continued until 6 weeks after termination of treatment (p = 0.0001 for MRM and p < 0.0001 for FLU) and was still retained at 9 weeks post-treatment, with mean stimulation index of 3.71 (p = 0.0091; Figure 3). A sub-analysis comparing patients receiving either EBRT only or the combination of EBRT with cisplatin, showed a decrease in T-cell reactivity against both MRM and FLU regardless of the type of treatment (supplementary figure 1C and 1D). Interestingly, patients receiving EBRT only displayed higher baseline reactivity than those receiving chemoradiotherapy which nonetheless collapsed demonstrating the profound impact of EBRT on T-cell responsiveness.

Upon analysis of individual patient data it was noticed that four patients (ID3, ID5, ID8 and ID13) showed a high baseline T-cell response, a less severe decrease in T-cell reactivity and a persistent positive response during radiation therapy (ID3, ID8) or chemoradiotherapy (ID5, ID13) (supplementary figure 2A). Furthermore, in six (ID16, ID17, ID20, ID21, ID27, ID29, all treated with chemoradiation) of the

12 patients (50%) with the extended follow-up, a restoration of the proliferative T-cell response against FLU was noted at 6 and/or 9 weeks post-chemoradiation (supplementary figure 2B). These data showed that EBRT is associated with a loss in T-cell reactivity against common recall antigens, slowly recovering after cessation of therapy.

To gain more insight in the dynamics of T-cell reactivity during and after (chemo)radiotherapy, we decided to study the immune-cell composition and function of these 10 patients in more detail. First the T-cell responses to PHA stimulation was studied and found to be strong for all 10 patients at baseline, with a mean stimulation index of 200.6 (range 54.0 - 586.0). The 4 patients who retained T-cell reactivity against the recall antigen FLU also showed a strong T-cell response to PHA stimulation throughout radiotherapy (mean stimulation index after 15 fractions was 325.75, range 121.0 - 446.0). For the 6 patients showing a chemoradiotherapy-induced reduction in recall antigen responsiveness, T-cell reactivity to PHA also significantly ($p = 0.03$) decreased to a mean stimulation index of 67.6 (range 15.0 - 123.0), albeit that reactivity was never completely lost (data not shown).

RADIOTHERAPY IMPAIRS THE T-CELL STIMULATORY CAPACITY OF APC

The antigen presenting capacity of the patient's PBMCs was determined in a MLR.[23] At baseline, the capacity of APCs to stimulate allogeneic T-cells to proliferate was strong for all patients but a significant decrease in APC capacity was observed upon treatment (figure 4), with a mean fold change in stimulation index of 0.62 after 15 fractions, 0.56 at 3 weeks, 0.66 at 6 weeks, and 0.62 at 9 weeks after treatment compared to baseline. Two of the patients were treated with EBRT only, and the eight other patients concurrently received cisplatin. Nonetheless, the suppressive treatment effect on APC capacity occurred regardless the therapy. Note, however, that the group of EBRT only is very small. Altogether, (chemo)radiation not only altered the number of circulating lymphocytes but also impaired the capacity of circulating APC to stimulate allogeneic T-cell proliferation in mixed lymphocyte reactions, shown as the decreased T-cell responsiveness within patient's PBMC to recall antigens and mitogens.

SUPPRESSIVE MYELOID CELL POPULATIONS ARE MORE RADIORESISTANT THAN LYMPHOCYTES

The loss of T-cell reactivity and stimulatory capacity of APC in these 10 patients was associated with changes in the immune cell composition. After 15 fractionated doses of radiation therapy a significant decrease in the percentage of circulating lymphoid ($CD3^+CD19^-$) cells, and a concomitant increase in myeloid ($CD3^-CD19^-$) cell populations occurred ($p = 0.0002$ and $p = 0.0006$, respectively). This effect on circulating lymphoid cells ($p < 0.0001$) and myeloid cells ($p < 0.0001$) was still present after completion of the therapy, and was most prominent at 3 weeks after last EBRT (Figure 5). Six to nine weeks after completion of radiotherapy, a slight increase of lymphoid cells and decrease of myeloid cells was observed, but the percentages remained significantly changed when compared to baseline. The effect on circulating lymphoid and myeloid cells occurred regardless the administration of cisplatin since similar kinetics in the percentages of lymphoid cells and myeloid cells are seen in patients treated with EBRT only (2 patients) and the patients with cisplatin + EBRT (8 patients; supplementary figures 3A and 3B).

A more in-depth analysis of the myeloid cell populations was based on of the expression of HLA-DR, to distinguish between macrophages or dendritic cells (DCs) (both $HLA\text{-}DR^+$) and MDSC ($HLA\text{-}DR^{-/low}$). In addition, the differential expression of CD14 and CD11b within the $HLA\text{-}DR^+$ myeloid cell population was used to identify 5 previously reported subpopulations.[31] For all 10 patients a significant ($p = 0.009$) increase in the percentage of $CD3^-CD19^-CD1a^-HLA\text{-}DR^+$ myeloid cells was observed after 15 fractions of radiotherapy. This effect was stronger at 3 weeks ($p < 0.0001$), and the percentage of circulating myeloid cells remained significantly elevated at 9 weeks after completion of the radiotherapy ($p=0.0005$), when compared to baseline (supplementary figure 4A). This effect was most pronounced for the population of $CD14^+CD11b^+$ expressing monocytes (supplementary figure 4B; $p = 0.0038$) and was retained after completion of the radiotherapy. Although the percentage of circulating $CD3^-CD19^-CD1a^-HLA\text{-}DR^-CD14^+CD15^-$ monocytic MDSC (mMDSC) were very low at baseline (average 0.11%, range 0.02 - 0.31%), they increased in 9 out of the 10 patients upon radiotherapy, with a mean percentage in mMDSCs of 0.28% (range 0.04 - 1.32%) (data not shown). Interestingly, the loss of T-cell reactivity against the recall antigen FLU was paralleled by the decrease in lymphoid cells and an increase in subpopulations of less stimulatory or even suppressive types of myeloid cells (supplementary figure 5). These data show that some myeloid cell populations are more radio-resistant than lymphocytes and that their presence is associated an impaired T-cell response to recall antigens as well as an impaired antigen presenting cell capacity to stimulate allogeneic T-cells. This suggests an overall immunosuppressive effect of (chemo)radiotherapy on systemic immunity in patients with cervical cancer.

A SPECIFIC INCREASE IN PD-1 EXPRESSION BY T-CELLS UPON RADIOTHERAPY

Analysis of the T-cell populations in PBMCs showed a strong reduction in the percentage of both $CD4^+$ and $CD8^+$ T-cells in 9 of the 10 analyzed patients. However, the percentage of $CD4^+CD25^+CD127^-Foxp3^+$ regulatory T-cells (Treg) and $CD4^+CD25^+CD127^-Foxp3^+CD45RA^-$ activated regulatory T-cells (aTreg) were not significantly altered during treatment (supplementary figure 6). We previously demonstrated that the frequency of $CD4^+$ and $CD8^+$ T-cells expressing the co-inhibitory marker program death-1 (PD-1) was increased in cervical cancer patients when compared to healthy controls.[23] Therefore, the expression of PD-1, cytotoxic T-lymphocyte Antigen 4 (CTLA-4), and T-cell immunoglobulin mucin-3 (TIM-3) was analyzed to determine whether radiotherapy influences the expression of these inhibitory markers. While the percentage of CTLA-4 and/or TIM-3 expressing $CD4^+$ or $CD8^+$ T-cells remained similar upon treatment, we observed a high expression of PD-1 on a minority of circulating $CD4^+$ T-cells at baseline (range 5.8 - 40.6 %), which even increased up to 2.7 fold upon radiotherapy (range 10.8 – 71.4 %). These higher percentages of PD-1 expressing $CD4^+$ T-cells occurred in all patients (regardless whether EBRT was combined with concurrent cisplatin (8 patients) or not (2 patients; supplementary figure 7) and remained elevated for up to 9 weeks after radiotherapy (figures 6A and 6B). A similar effect was seen on circulating $CD8^+$ T-cells, with a significant increase of PD-1 expression 3 weeks after (chemo) radiotherapy when compared to baseline ($p = 0.002$, data not shown). The increase of PD-1 expression of $CD4^+$ T-cells upon treatment was accompanied with the decline in T-cell response against viral (FLU) antigens (supplementary figure 7). Notably, compared to the other 9 patients, patient ID29 displayed a remarkably high level of $CD4^+$-$PD1^+$ T-cells at baseline (39%) and concurrently the lowest T-cell reactivity in the LST, suggesting that PD-1 expression may have contributed to the insufficient immune response upon treatment with chemoradiotherapy. We subsequently explored whether blocking of the PD-1 signaling could improve T-cell responses. We used the PD-1 blocking antibody Nivolumab and stimulated 5 baseline and 3 post-chemoradiotherapy PBMC samples, that were still available to us, with autologous monocytes pulsed with a pool of FLU peptides. As a control, non-blocked PBMCs were used. We observed higher antigen-specific T-cell proliferation in 4 and increased IFNγ production in 5 out of the 5 tested baseline PBMC samples when Nivolumab was present (figures 6C and 6D), indicating that PD-1 expression contributed to the immune suppression in patients with cervical cancer. Unfortunately, this

effect of anti-PD-1 was seen in only 1 out of the 3 post-treatment samples; the antigen-specific IFNγ production was partly restored for patient ID5 (figures 6E and 6F). Apparently, radiotherapy disturbs immunity via multiple other pathways, rendering concurrent PD-1 blocking less adequate for restoration of T-cell reactivity against recall antigens in this setting.

Discussion

In this study, the immunological effects of standard radiotherapy in patients with cervical cancer were studied. Radiotherapy without bone marrow sparing induced a substantial and long-lasting immune suppression. From the first fractions onwards, a decrease in the number of circulating lymphocytes, a decrease in T-cell reactivity to common recall antigens and a decrease in the capacity of APC to stimulate T-cell responses were found. Immune reactivity slowly recovered after cessation of therapy with only half of the patients responding 9 weeks after therapy. These effects on peripheral immune cells, T-cell function and APC capacity were similar for patients treated with radiation therapy alone or with concomitant chemotherapy, confirming an earlier report showing that radiotherapy alone and radiotherapy with concomitant cisplatin decreased the absolute number of all lymphocyte subsets and decreased PHA-induced T-cell proliferation.[34]

Chemo-radiotherapy was also reported to be immune suppressive in patients with HPV-related oropharyngeal cancer.[35] A decrease in $CD4^+$ and $CD8^+$ T-cells, an increase of MDSCs and an unfavorable $CD8^+$/Treg ratio upon radiation treatment was seen. Furthermore, an up-regulation of PD-1 expression on $CD4^+$ T-cells was noted, which occurred 3 weeks after completion of therapy and remained elevated for up to 1 year following therapy.[35] Although the radiation field in this patient group contains less active bone marrow, the immunosuppressive effects were long lasting (up to 1 year after treatment), indicating the direct effect of radiotherapy on the peripheral blood cells. PD-1 is a key immune checkpoint protein expressed on activated and exhausted T-cells, which leads to the suppression of T-cell activity through interaction with its ligand PD-L1. We also observed elevated PD-1 expression on $CD4^+$ T-cells during and following radiotherapy and showed that this was associated with the impaired T-cell reactivity against FLU and impaired ability of APCs to stimulate allogeneic T-cells. Together, this is strongly suggestive for radiotherapy induced immune suppression. There are a number of clinically available antibodies to block PD-1 signaling (e.g. nivolumab, pembrolizumab, lambrolizumab). We

hypothesized that PD-1 blockade could reverse radiotherapy-induced immune suppression. While our in vitro experiments in blocking PD-1 in PBMC samples isolated before radiotherapy provide further support for targeting PD-1 and its ligand in cervical cancer, this was hardly the case after the initiation of radiotherapy. PD-1 blocking in patients treated with (chemo)radiotherapy partly restored cytokine production but not proliferation of antigen-stimulated T-cells. These data do not support a combination of the (chemo)radiotherapy together with PD-1/PD-L1 blockade to obtain more clinical benefit.

An increase in circulating MDSCs and macrophages upon (chemo) radiotherapy and a change in capacity of circulating APCs, reflected in a subverted reactivity of T-cells to recall antigen stimulation as well as a lower response of allogeneic T-cells to become activated was observed after completion of radiotherapy. This suggests that the loss of T-cell reactivity and stimulatory capacity of APC may also be caused by changes in the immune cell composition, in favor of (suppressive) myeloid cell populations.

Our results indicate that it will be a considerable challenge to establish the optimal delivery and dosing strategies when combining radiotherapy and immunotherapy before combinations hereof can successfully be applied to cervical cancer patients. In pre-clinical models, it was demonstrated that alpha radiation-based therapy (an in situ ablation treatment based on intra-tumoral ^{224}Ra-loaded wires that release its daughter atoms) inhibited breast, colon and lung tumor growth by stimulating anti-tumor immunity. It was suggested that combinations of local ablation treatments and immunotherapy could further augment powerful anti-tumor immunity.[36] However, translation of radio-immunotherapy to the clinic requires careful consideration of the radiation dose and fractionation for both the tumor and organs at risk, particularly the bone marrow.

Hematologic toxicity, including lymphopenia, is frequently noted in women undergoing pelvic radiotherapy for cervical cancer, because approximately 40% of the active bone marrow is located in the pelvic region, and T-cells constantly circulate though this irradiation field.[37,38] The extreme sensitivity of active pelvic bone marrow was recently demonstrated by McGuire et al. using [^{18}F] Fluorothymidine (FLT) imaging using positron emission tomography (FLT-PET) to identify active bone marrow before, during and after radiotherapy.[39] As little as a radiation dose of 4-5 Gy resulted in an approximately 50% decrease in FLT uptake and the suppression of bone marrow activity was measurable up to 1 year after radiotherapy, especially in pelvic cancer patients receiving radiation doses of more than 35 Gy.[39] Based on empirical experience, the use of daily fractions of 2 Gy to a total dose of approximately 46-50 Gy, has evolved as a

standard radiotherapy approach to control microscopic disease for most tumor types including cervical cancer. It is possible that different treatment regimens, depending on fractioning, dosing and delivery may have different effects on antitumor immunity. In the setting of cervical cancer, an immune enhancing effect was seen in the tumor-draining lymph nodes of patients undergoing low-dose radiation (total dose 39.6 Gy), while an immunosuppressive effect was observed in patients treated with high-dose radiation (total dose 50 Gy). Although dose differences were only minor, lower-dose radiation was associated with an increase in the anti-tumor Th1 and cytotoxic T-cell subsets, while a lower frequency of Tregs was noticed when compared to higher-dose radiation therapy.[40] Within another study, it was demonstrated that local low-dose gamma irradiation (2 Gy) caused normalization of aberrant vasculature and increased recruitment of tumor-specific T-cells into human pancreatic tumors, by the polarization of M2-like toward M1-like macrophages.[41]

This underlines again the importance of further characterization of the effect of radiotherapy on the immune system. To reduce the incidence and severity of hematologic toxicity, the use of techniques that limit pelvic bone marrow irradiation is of interest.[42,43] Especially bone marrow sparing (BMS) intensity modulated radiotherapy (IMRT) is a technique that could reduce the volume of bone marrow receiving high dosages, while maintaining target coverage, resulting in less hematologic toxicity[39], and potentially limit the suppressive effect on lymphoid populations and immune responses. Until now, there is no clear evidence for an optimal radiation dose fractionation schedule based on clinical data to elicit anti-tumor immune responses, and there is a lack of (randomized) studies comparing radiation regimens (with or without bone marrow sparing) for their ability to synergize with immunotherapy. The optimal dose and fractionation schedule should cause sufficient cytotoxic effects for tumor eradication, while reducing myeloid cell associated suppressive effects and foster lymphoid cell populations and effective immune responses. As peripheral blood lymphocyte count and lymphocyte subsets have shown to be independent predictors of survival and tumor regression in cervical cancer patients treated with concurrent chemoradiation[9,10], such immunological markers could be used to select the optimal (combination) treatment schedule for the patient that benefits most.

There were some limitations of this study. Our analyses were limited to the systemic immunity, rather than direct examination of the intra-tumoral cell composition itself. It remains to be established whether (chemo)radiotherapy-induced alterations in circulating immune cells also occurs at the tumor site. It has been shown in head and neck cancer patients that tumor infiltrating T-cells

have a higher expression of PD-1 compared to circulating T-cells[44]. In addition, cryopreservation may cause down-regulation of PD-1 and PD-L1 expression on PBMCs. This implies that our findings on radiotherapy-induced increased PD-1 expression on $CD4^+$ T-cells may underestimate the effect of radiotherapy on the tumor and its microenvironment. Another limitation is the relatively small number of patients participating in this trial (N=30) and the variation in the amount of blood samples provided by patients. Due to clinical conditions and disease burden, motivating patients to donate blood was difficult. A further limitation is the heterogeneity in the participating patients. Differences in FIGO stage, treatment (adjuvant versus primary) and clinical performance status, made it difficult to study potential interesting differences between patient groups. In addition, there was a high variation in treatment modalities. Although every patient was treated with high dose EBRT without bone marrow sparing, there were different additional treatment regimens including concurrent chemotherapy, hyperthermia and/or brachytherapy.

In conclusion, our data show that standard non-BMS radiotherapy affects circulating immune cells and immune responses, causing immune suppression in patients with cervical cancer. Relevant mechanisms underlying this (chemo) radiotherapy-induced immunosuppression include decrease in lymphoid cells, decrease of APC function, increase of different subsets of myeloid cells and PD-1 up-regulation on T-cells. Studies on the immunological effects of BMS radiotherapy should be made, in order to determine whether alternative treatments as immunotherapy could synergistically improve immune responses and outcomes.

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Table 1 Clinical and tumor characteristics of patients. For patients suffering from locally advanced cervical cancer, tumor size was measured before primary radiation therapy by magnetic resonance imaging (MRI). Age of the patients is given in years.

Characteristics	
Age (average)	48.9 (range 19-82)
FIGO stage	
IB1	11/30 (36.7%)
IB2	3/30 (10%)
IIA	1/30 (3.3%)
IIB	12/30 (40%)
IIIB	2/30 (6.7%)
IV	1/30 (3.3%)
Pre-treatment tumor size (average)	32.8 mm (range 10-75 mm)
Treatment with	
EBRT + ChTh + BT	18/30 (60%)
EBRT + ChTh	5/30 (16.7%)
EBRT alone	4/30 (13.3%)
EBRT + BT	2/30 (6.7%)
EBRT + HT	1/30 (3.3%)
Treatment for	
Primary disease	18/30 (60%)
Adjuvant	8/30 (26.7%)
Recurrent disease	4/30 (13.3%)

EBRT = external beam radiation therapy; ChTh = chemotherapy; BT = brachytherapy; HT = hyperthermia.

Figure 1 Treatment and blood sampling schedule. Blood samples 1-5: blood samples for immunomonitoring. Numbers above the blood samples indicate the amount of patients who provided blood for immunomonitoring.

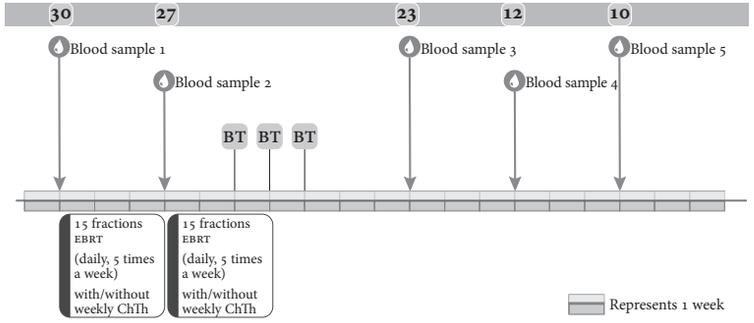


Figure 2 (Chemo)radiotherapy induced reduction in the absolute numbers of leukocytes and lymphocytes. **(A)** Time course of changes in the absolute number of leukocytes and lymphocytes before (baseline) treatment, and during the first 5 days of fractionized radiotherapy. **(B)** Time course of changes in absolute number of leukocytes and lymphocytes before (baseline), during (15 fractions) and after (chemo)radiotherapy (3, 6 and 9 weeks after completion). Data are expressed as mean ± SD. * $p < 0.05$.

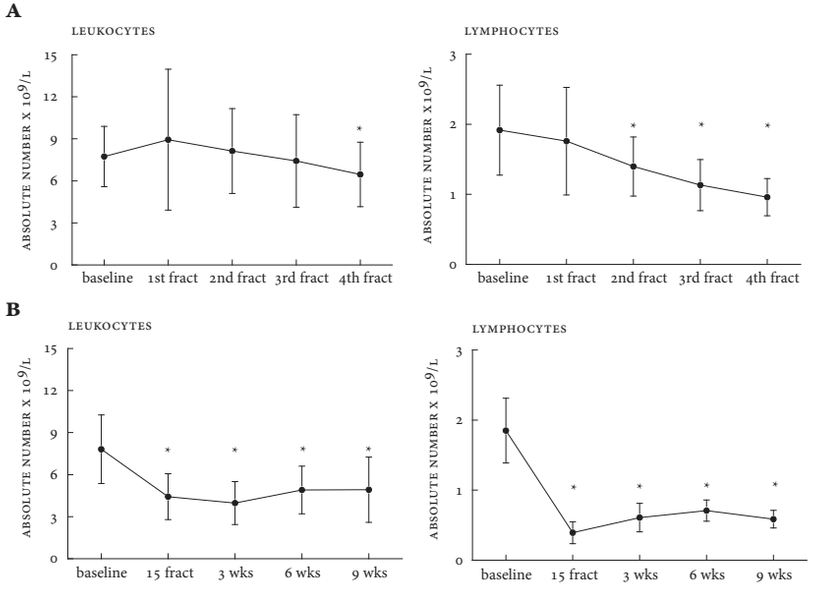


Figure 3 The effect of (chemo)radiotherapy on T-cell reactivity. The response of circulating T-cells against memory response mix (MRM; black bars) and influenza M1 protein-derived peptides (FLU; white bars) was measured in the lymphocyte stimulation test (LST). T-cell proliferation is expressed as Stimulation Index + standard error of the mean (SEM) and shown at different time points, including baseline, after 15 fractions of EBRT, and 3, 6 and 9 weeks after completion of EBRT. Data were analyzed by repeated measures model. *$p < 0.05$ with respect to baseline.

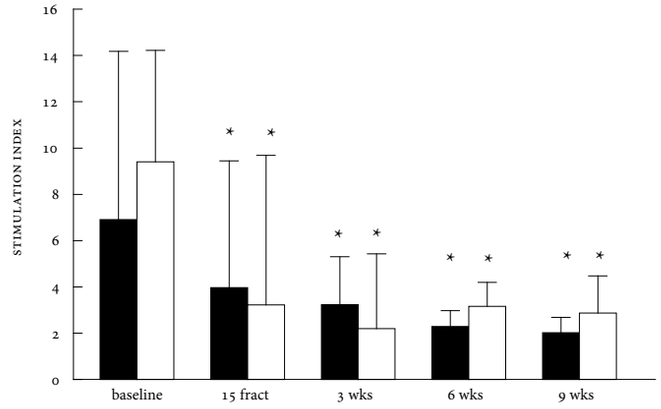


Figure 4 (Chemo)radiotherapy impairs the ability of antigen presenting cells to stimulate allogeneic T-cells. Antigen presenting capacity of PBMCs as determined in a mixed lymphocyte reaction (MLR) in the blood samples from 10 patients are plotted over time. Treatment-induced changes in lymphocyte reactions as observed in the MLR based on **(A)** Stimulation Index (S.I., expressed as mean ± SEM) and **(B)** expressed as the fold changes (mean + SEM) of these S.I. over baseline. Time point include baseline, after 15 fractions of EBRT, and at 3, 6 and 9 weeks after completion of EBRT. *p < 0.05 with respect to baseline.

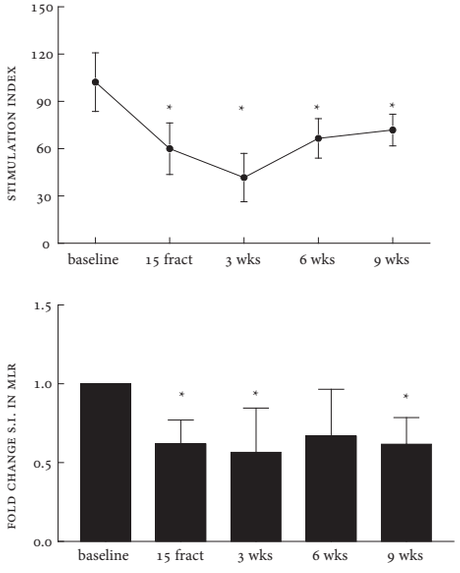


Figure 5 (Chemo)radiotherapy alters the relative frequencies of circulating myeloid and lymphoid cells. (A) Percentages of lymphoid cells ($CD3^+CD19^-$) and myeloid cells ($CD3^-CD19^-$) of viable cells as measured by flow cytometry. Mean percentages are shown for 10 patients with a complete follow-up at different time points, including baseline, after 15 fractions of EBRT, and at 3, 6 or 9 weeks after completion of EBRT. Percentages are expressed as mean ± SD. **(B)** Fold changes in lymphoid and myeloid cells over baseline. Fold changes are expressed as mean + SEM. *p < 0.05 with respect to baseline.

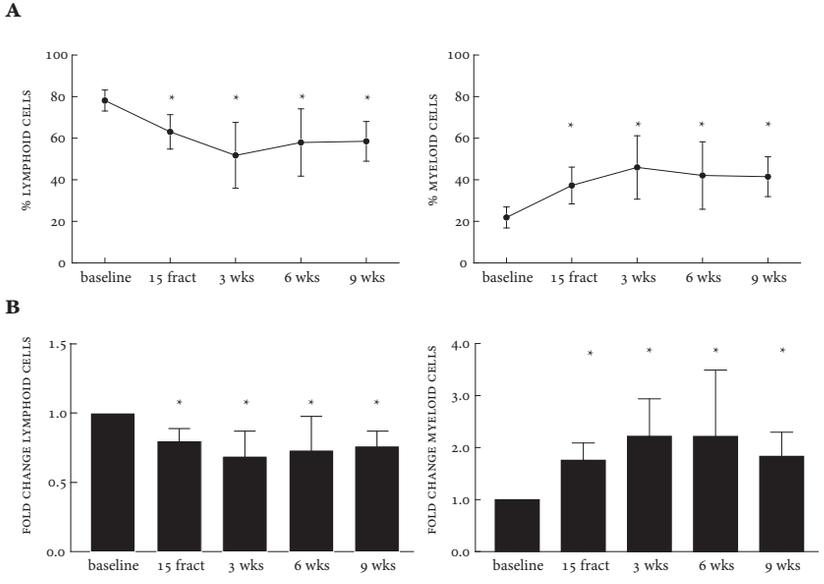
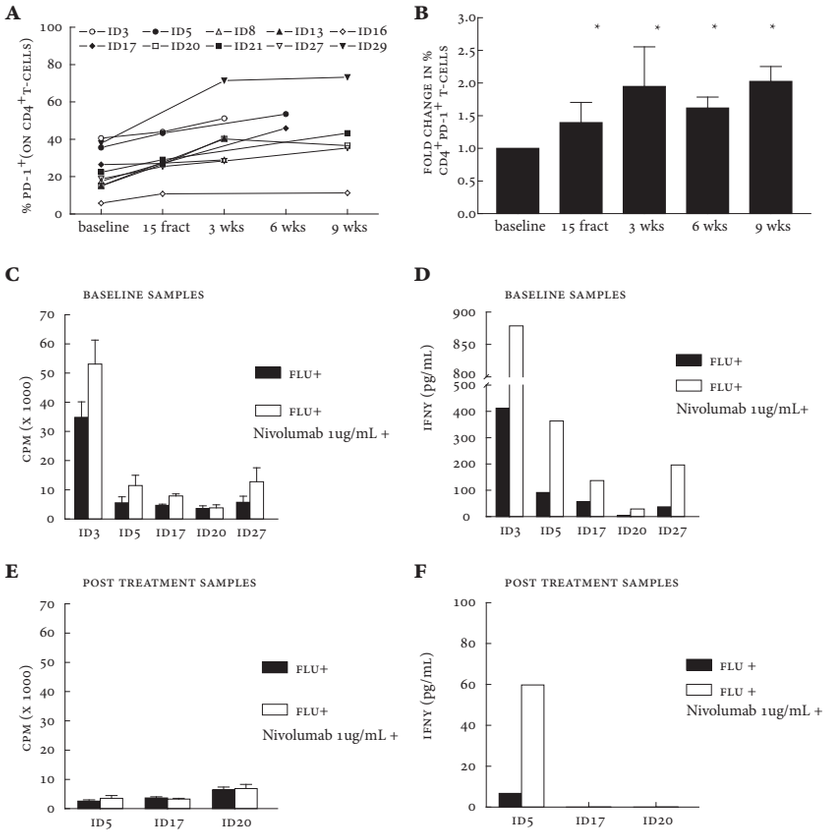


Figure 6 (Chemo)radiotherapy induces CD4$^+$ T-cell suppression via PD-1. **(A)** Percentage of PD-1$^+$ expressing CD4$^+$ T-cells for individual patients. **(B)** Aggregated fold changes of percentages with respect to baseline. *p < 0.05 with respect to baseline. **(C)** and **(D)** Stimulation of 5 baseline PBMC samples with influenza Matrix 1 protein-derived peptides (FLU) *in vitro* in the presence (white bars) or absence (black bars) of PD-1 blocking using 1 µg/mL Nivolumab. **(E)** and **(F)** Stimulation of 3 post-treatment samples with FLU *in vitro* in the presence (white bars) or absence (black bars) of PD-1 blocking with 1 µg/mL Nivolumab. Displayed in C and E is the proliferation, expressed as counts per minute (cpm), shown as mean of triplicate wells plus standard deviation after stimulation. D and F display cytokine IFNγ production as measured within the supernatant of the proliferation assay, with and without PD-1 blocking.



SUPPLEMENTAL INFORMATION

Table S1 Detailed patient treatment characteristics. Age is expressed in years. Tumor burden was identified as high or low, with high tumor burden in case of primary or recurrence therapy; and low tumor burden in case of adjuvant, post-operative therapy. All treatments were highly dosed: chemotherapy with cisplatin at a dose of 40 mg/square meter, in 5-6 cycles ; radiotherapy in 23 to 30 fractions of 1.8 or 2 Gray (Gy).

Patient ID	Age	Treatment	Dosages of ChTh, EBRT and BT	Treatment for	Tumor burden
1	31	EBRT + CHTH + BT	23X2GY EBRT; 6XCIS; 4XBT	primary disease	high
2	54	EBRT + BT	23X2GY EBRT; 3XBT	recurrent disease	high
3	41	EBRT	23X2GY EBRT	adjuvant	low
4	40	EBRT	20X2GY EBRT	adjuvant	low
5	56	EBRT + CHTH + BT	27X1.8GY EBRT; 6XCIS; 3XBT	recurrent disease	high
6	67	EBRT + CHTH	30X2GY EBRT; 6XCIS	adjuvant	low
7	28	EBRT + CHTH + BT	23X2GY EBRT; 5XCIS; 3XBT	primary disease	high
8	19	EBRT	30X2GY EBRT	adjuvant	low
9	82	EBRT + BT	23X2GY EBRT; 2XBT	adjuvant	low
10	26	EBRT + CHTH + BT	28X2GY EBRT; 3XCIS; 3XBT	primary disease	high
11	49	EBRT + CHTH + BT	28X2GY EBRT; 6XCIS; 3XBT	primary disease	high
12	36	EBRT + CHTH	30X2GY EBRT; 6XCIS	recurrent disease	high
13	53	EBRT + CHTH + BT	27X1.8GY EBRT; 6XCIS; 3XBT	primary disease	high
14	62	EBRT + CHTH + BT	25X1.8GY EBRT; 3XCIS; 3XBT	primary disease	high
15	35	EBRT + CHTH + BT	23X2GY EBRT; 6XCIS; 3XBT	primary disease	high
16	68	EBRT + CHTH + BT	23X2GY EBRT; 6XCIS; 3XBT	primary disease	high
17	50	EBRT + CHTH	33X2GY EBRT; 6XCIS	recurrent disease	high
18	55	EBRT + CHTH + BT	25X1.8GY EBRT; 5XCIS; 3XBT	primary disease	high
19	33	EBRT	23X2GY EBRT	adjuvant	low
20	45	EBRT + CHTH	27X1.8GY EBRT; 6XCIS	adjuvant	low
21	35	EBRT + CHTH	23X2GY EBRT; 5XCIS	adjuvant	low
22	69	EBRT + CHTH + BT	25X1.8GY EBRT; 5XCIS; 3XBT	primary disease	high
23	69	EBRT + CHTH + BT	27X1.8GY EBRT; 5-FU; 3XBT	primary disease	high
24	51	EBRT + CHTH + BT	23X2GY EBRT; 6XCIS; 3XBT	primary disease	high
25	53	EBRT + CHTH + BT	23X2GY EBRT; 6XCIS; 3XBT	primary disease	high
26	79	EBRT + HT	23X2GY EBRT; 4XHT	primary disease	high
27	51	EBRT + CHTH + BT	23X2GY EBRT; 6XCIS; 3XBT	primary disease	high
28	54	EBRT + CHTH + BT	27X1.8GY EBRT; 5XCIS; 3XBT	primary disease	high
29	49	EBRT + CHTH + BT	27X1.8GY EBRT; 6XCIS; 3XBT	primary disease	high
30	28	EBRT + CHTH + BT	30X1.8GY EBRT; 5XCIS; 3XBT	primary disease	high

CIS = cisplatin ; 5-FU = 5 Fluorouracil

Figure S1 The effect of therapy on circulating lymphocytes as measured in whole blood, comparing patients receiving EBRT only or EBRT in combination with chemotherapy (A), and comparing patients receiving (chemo)radiation with a high or low tumor load (B). The effect on T-cell responsiveness against MRM and FLU with a lymphocyte stimulation test (LST), is also compared between patients receiving EBRT alone or in combination with concurrent chemotherapy **(C, D)**.Time course of changes before, during and 3, 6 and 9 weeks after radiotherapy. The suppressing effect of radiotherapy on peripheral lymphocytes and T-cell responses was similar for patients treated with radiotherapy alone or with concomitant chemotherapy (A, C, D) and regardless of whether tumor-load was high or low (B). Data are expressed as means ± SD ; * $p < 0.05$ compared to baseline.

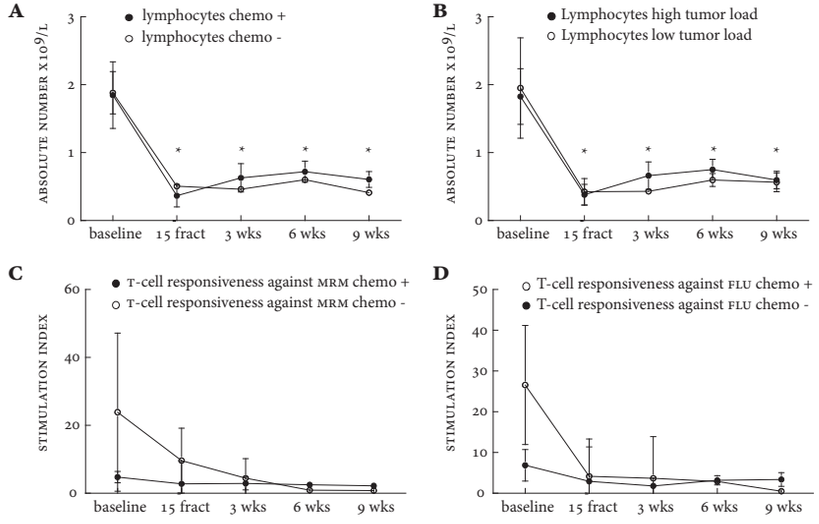


Figure S2 The effect of EBRT on circulating T-cell responses in individual patients as measured in the LST against FLU. (A) Dynamics for 4 patients with a strong T-cell response as indicated by the stimulation index (S.I.), at baseline (black bars), during (white bars) or after (gray bars) EBRT treatment. **(B)** T-cell response dynamics for 6 patients showing a rebound of T-cell responses after completion of EBRT. Baseline is indicated by black bars. Nadir (white bars) indicates midway or close after last EBRT treatment. Rebound (gray bars) is at 6 to 9 weeks after last EBRT. – - line represents S.I. value of 3, a positive T-cell response is defined as a S.I. of 3 or higher.

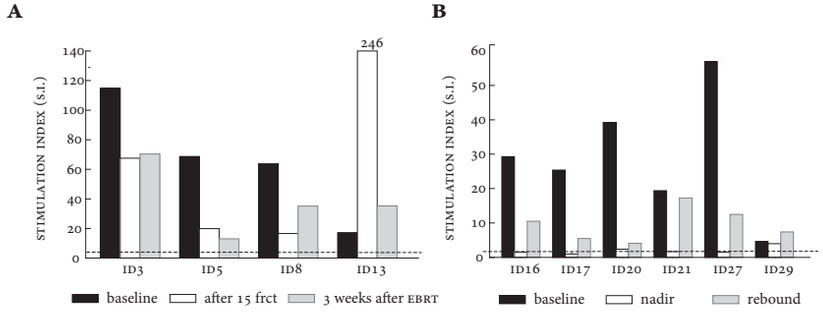


Figure S3 The treatment effect on (A) circulating lymphoid cells ($CD3^+CD19^-$) and (B) myeloid cells ($CD3^-CD19^-$) of viable cells as measured by flow cytometry, compared between patients receiving EBRT alone or in combination with concurrent chemotherapy. Both radiotherapy alone (2 patients) and radiotherapy with concurrent cisplatin chemotherapy (8 patients) altered the relative frequencies of circulating myeloid and lymphoid cells. Longer follow-up (6 and 9 weeks after completion of EBRT) is missing for the 2 patients treated with EBRT alone. Percentage are expressed as mean ± SD. *$p < 0.05$ with respect to baseline.

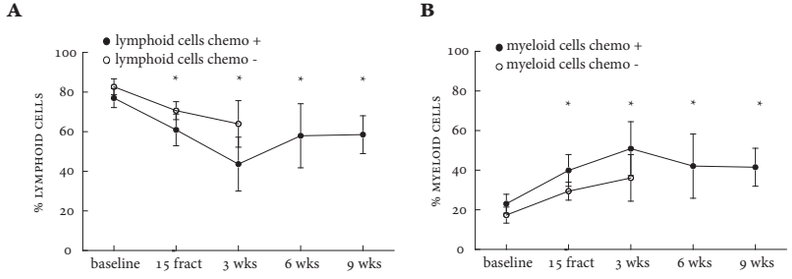


Figure S4 The effect of (chemo)radiotherapy on the percentage of circulating $CD3^-CD19^-CD1a^-HLA\text{-}DR^+$ population (A) and on the subpopulation of monocytes expressing both CD14 and CD11b (B). Data is expressed as percentage of viable cells. *p < 0.05 with respect to baseline.

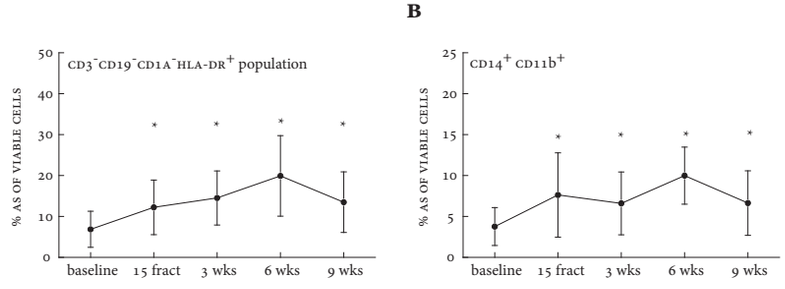


Figure S5 T-cell responsiveness against FLU within LST, presented for 10 individual patients showing high responsiveness at baseline, severe drop upon (chemo)radiotherapy (except for subject 13) and slight rebound 6-9 weeks after completion of EBRT in 6 cases. The strong decrease in T-cell responsiveness occurred concurrently with a decrease in percentage of circulating $CD3^+CD19^-$ lymphoid cells **(A)**, and an increase in percentage of circulating $CD3^-CD19^-$ myeloid cells **(B)** in 9 of the 10 patients. Visit 1: baseline. Visit 2: after 15 fractions of EBRT. Visit 3: 3 weeks after EBRT. Visit 4: 6 weeks after EBRT. Visit 5: 9 weeks after EBRT.

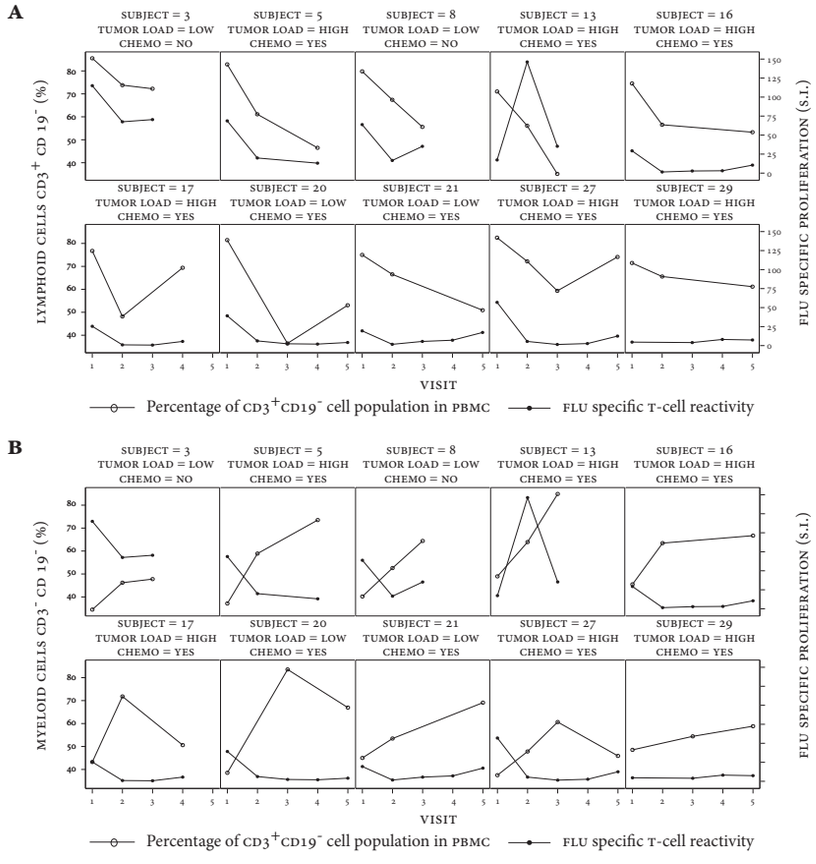


Figure S6 The effect of radiotherapy on the percentage of circulating CD4$^+$ T-cells, CD8$^+$ T-cells, CD4$^+$CD25$^+$CD127$^-$FOXP3$^+$ regulatory T-cells (Treg), and CD4$^+$CD25$^+$CD127$^-$FOXP3$^+$CD45RA$^-$ activated Tregs (aTreg). Data is expressed as percentage of viable cells and presented using mean values ± SEM. *p < 0.05 with respect to baseline.

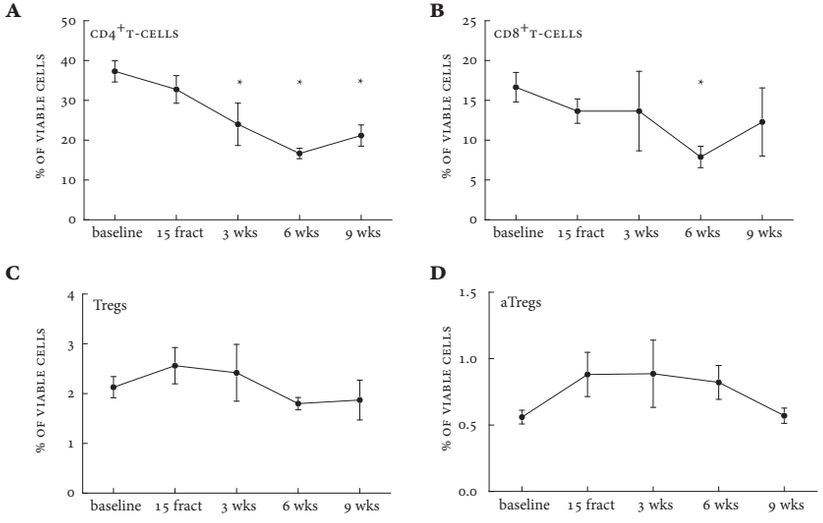
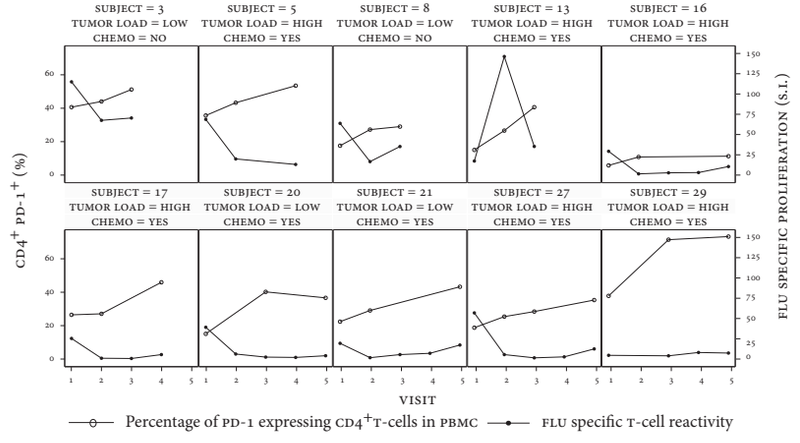
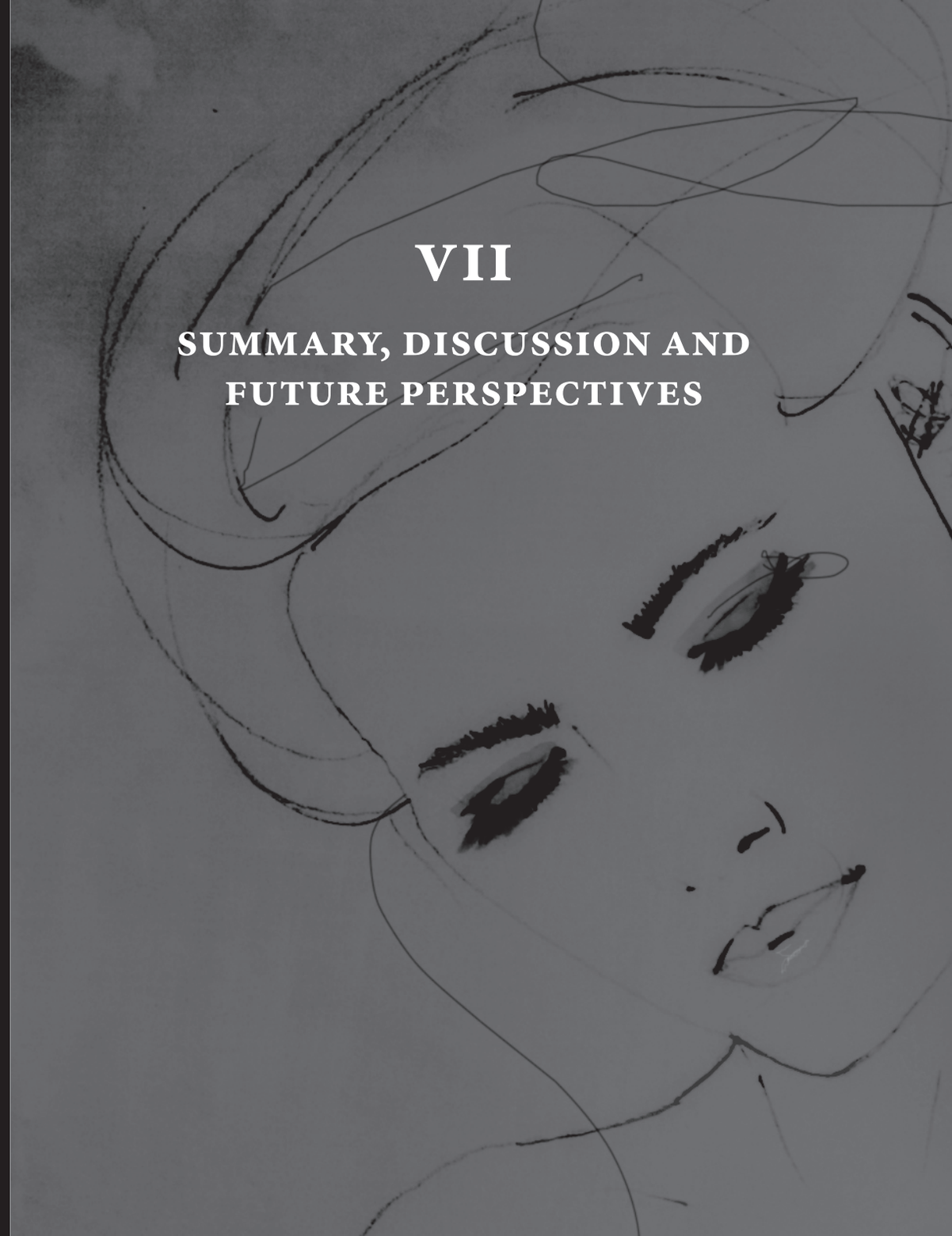


Figure S7 Individual plots, showing T-cell responsiveness against FLU within LST. At baseline, positive responsiveness (S.I. ≥ 3) is seen in all patients. A severe drop in T-cell responsiveness occurs upon EBRT (except for subject 13) and slight rebound 6-9 weeks after completion of EBRT in 6 cases. Except for ID13, the strong decrease in T-cell responsiveness occurred concurrently with an increase in PD-1 expression on CD4$^+$ T-cells.Visit 1: baseline. Visit 2: after 15 fractions of EBRT. Visit 3: 3 weeks after EBRT. Visit 4: 6 weeks after EBRT. Visit 5: 9 weeks after EBRT



CONCLUSIONS AND DISCUSSION



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 $Gr\text{-}1^{int}CD11b^{hi}$ cells in the tumor, together with markedly reduced circulating $Gr\text{-}1^{hi}CD11b^{hi}$ cells *(chapter 5)*. The remaining intra-tumoral $Gr\text{-}1^{int}CD11b^{hi}$ 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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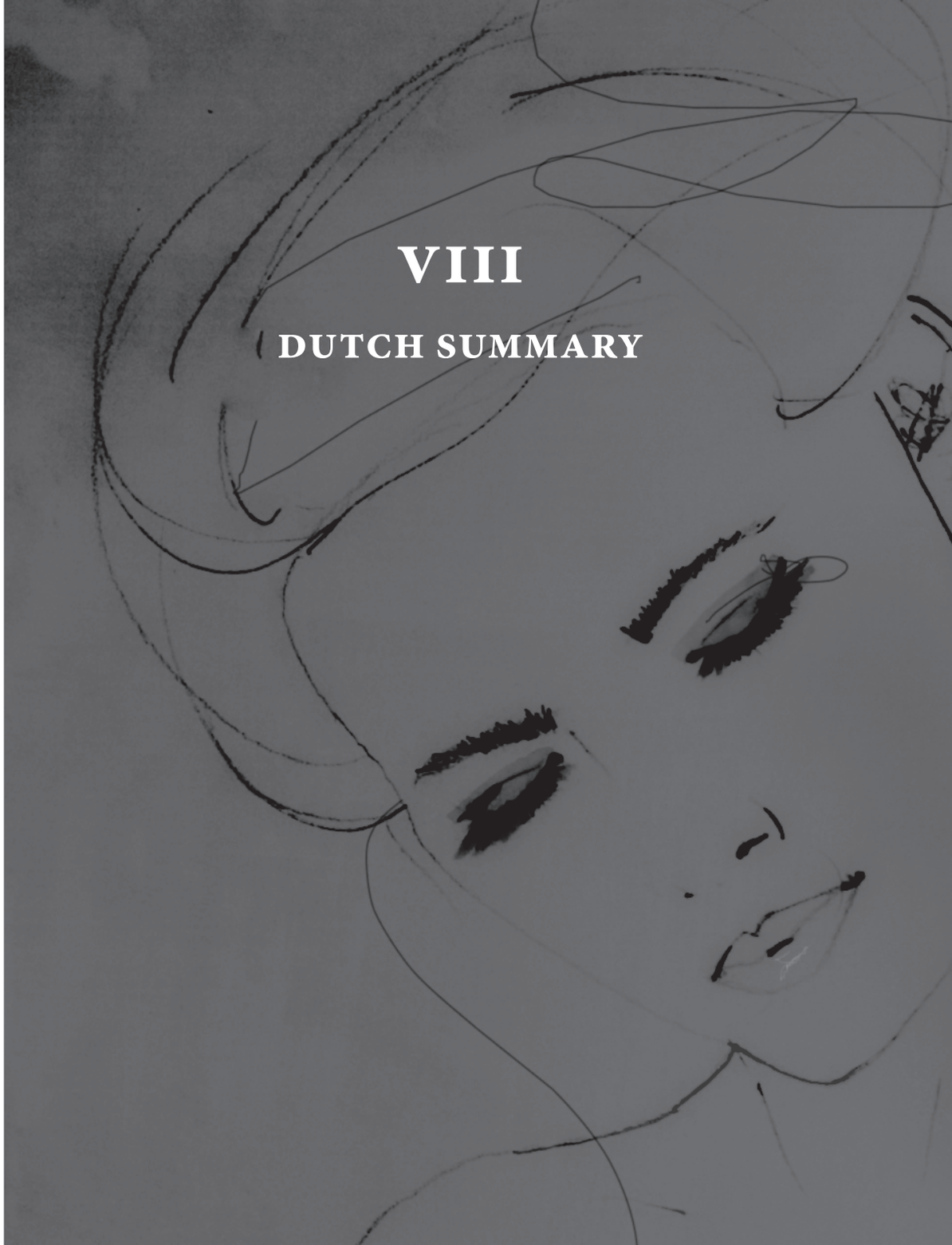

VIII

DUTCH SUMMARY

Nederlandse samenvatting

Jaarlijks wordt bij ongeveer 700 vrouwen in Nederland de diagnose baarmoederhalskanker gesteld. Klinische stadiëring vindt plaats volgens de Internationale Federatie van Gynaecologie en Obstetrie (FIGO) richtlijnen. In geval van een vroeg stadium baarmoederhalskanker kan succesvolle behandeling plaatsvinden met een operatie of bestraling (radiotherapie) van het kleine bekken al dan niet gecombineerd met chemotherapie. De prognose voor deze patiënten is over het algemeen goed, met een 5-jaars overleving tussen de 80-90% bij een FIGO stadium I tot IIA. Bij een operatieve behandeling, waarbij een verwijdering van de baarmoeder en de lymfeklieren uit het bekken plaatsvindt, wordt extra informatie verkregen over de lokale uitbreiding en specifieke tumor. De aanwezigheid van kwaadaardige cellen in de lymfeklieren, evenals de grootte van de tumor *(hoofdstuk 3)* zijn de belangrijkste risicofactoren voor terugkeren van ziekte en of er nog aanvullende behandeling nodig is zoals bestraling al dan niet in combinatie met chemotherapie. Als de baarmoederhalskanker – ondanks uitgebreide behandeling(en) – weer terug komt, gebeurt dat in praktisch 80% van de gevallen in de eerste twee jaar na de primaire behandeling.

In geval van een vergevorderd stadium baarmoederhalskanker, is de prognose ongunstig en is het risico groot dat de ziekte terugkomt of uitzaaiingen geeft. Bij uitgezaaide of teruggekeerde baarmoederhalskanker, is genezing niet meer mogelijk en is chemotherapie met carboplatin en paclitaxel de standaard behandeling. Aangezien slechts bij 15-25% van de patiënten de tumor hierop reageert, is deze behandeling er vooral op gericht om snelle uitbreiding te voorkomen en de kwaliteit van leven te verbeteren ("palliatieve behandeling"). De laatste jaren zijn er verschillende strategieën bedacht om patiënten waarbij de baarmoederhalskanker uitgebreid, uitgezaaid of teruggekeerd is effectiever te kunnen behandelen. Tot op heden heeft dit nog weinig klinisch succes opgeleverd en gesteld kan worden dat voorlopig het uitgebreide stadium van baarmoederhalskanker niet optimaal te behandelen is, en patiënten er uiteindelijk aan zullen overlijden. Daarom is het belangrijk om voor deze groep patiënten nieuwe behandelingen te onderzoeken.

HET AFWEERSYSTEEM

Het afweersysteem beschermt ons lichaam tegen indringers van buitenaf zoals bacteriën en virussen en is daarnaast in staat om afvalstoffen en zieke lichaamseigen cellen op te ruimen. Het afweersysteem bestaat uit verschillende soorten witte bloedcellen (leukocyten), waarbij de subgroep van T-cellen een essentiële

rol hebben bij de bestrijding van de zieke lichaamseigen cellen zoals virus-geïnfecteerde cellen en tumorcellen. Binnen de T-cellen kunnen er verschillende subtypes onderscheiden worden, met elk een specifieke functie. De helper $CD4^+$ T-cellen kenmerken zich door de productie van signaalstoffen zoals cytokines (interferon γ) en interleukines (o.a. IL-2), deze hebben een regulerende functie waarbij ze een afweerreactie activeren en onderhouden. De cytotoxische $CD8^+$ T-cellen zijn in staat om geïnfecteerde cellen en kankercellen aan te vallen en te doden. Er zijn daarnaast ook regulatoire T-cellen (Treg) die de ontwikkeling en functie van de andere afweercellen kunnen onderdrukken door de productie van afweer onderdrukkende stoffen.

Een andere groep van witte bloedcellen wordt gevormd door de myeloide cellen. Dit zijn immuun cellen die enerzijds een rol kunnen spelen bij de stimulatie van T-cellen tijdens het opruimen van tumor cellen, maar anderzijds in staat zijn T-cellen te onderdrukken en de groei van tumorcellen te bevorderen.

HET AFWEERSYSTEEM EN BAARMOEDERHALSKANKER

Bij baarmoederhalskanker speelt de afweer tegen het humaan papillomavirus (HPV) een belangrijke rol. Baarmoederhalskanker wordt veroorzaakt door een langdurige en persisterende infectie met HPV. Er zijn meer dan 100 verschillen typen HPV, waarvan er 15 zogenoemd een hoog risico type zijn omdat deze kanker kunnen veroorzaken. HPV is de meest voorkomende seksueel overdraagbare infectie en ongeveer 80% van de bevolking raakt ooit geïnfecteerd met een hoog-risico type HPV. De meerderheid van de mensen die hiermee geïnfecteerd raken, zijn goed in staat om het virus binnen 2 jaar uit het lichaam op te ruimen door middel van een virus-specifieke afweerreactie. Hiervoor lijkt een krachtige HPV-specifieke reactie van het grootste belang. In iets minder dan 10% van de gevallen blijft de infectie met HPV echter langdurig bestaan en kunnen (voorstadia van) kwaadaardige afwijkingen van de baarmoederhals, schaamlippen of vagina ontstaan. Blijkbaar weet in die gevallen het HPV virus via verschillende mechanismen te ontsnappen aan het afweersysteem.

Dat het afweersysteem ook een belangrijke rol speelt bij baarmoederhalskanker blijkt uit het feit dat baarmoederhalskanker vaker voorkomt bij vrouwen met HIV en AIDS en bij vrouwen die medicijnen gebruiken die het afweersysteem onderdrukken (bv. na een orgaantransplantatie). Gebleken is dat deze vrouwen een verzwakt afweersysteem hebben en niet gemakkelijk een HPV-infectie kunnen opruimen. In het geval van baarmoederhalskanker, is de aanwezigheid van veel T-cellen en het juiste type myeloide cellen rondom de tumor sterk geassocieerd met een goede levensverwachting bij kankerpatiënten, terwijl bijvoorbeeld

de aanwezigheid van regulatoire T-cellen samenhangt met een lagere levensverwachting. Kwaadaardige cellen proberen echter een afweerreactie van T-cellen te vermijden door middel van verschillende ontsnappingsmechanismen. Zo passen de tumorcellen hun celmembraan aan, waardoor de T-cellen de tumorcel niet meer herkennen en dus niet aanvallen of waardoor deze T-cellen in hun functie geremd worden. Tevens kan de functie van T-cellen onderdrukt worden door de aanwezigheid van zogenoemde afweer-onderdrukkende myeloide cellen en de productie van afweersysteem remmende stofjes. Deze vormen een beschermende laag rondom de tumor, en maken de tumor onbereikbaar voor aanvallende T-cellen, waardoor deze ongestoord en uitgebreid kan groeien.

IMMUNOTHERAPIE VOOR BAARMOEDERHALSKANKER

Omdat bepaalde elementen binnen het afweersysteem een belangrijke rol spelen bij enerzijds het ontstaan en de groei van kanker en anderzijds de bestrijding hiervan, zijn er in de afgelopen decennia verschillende behandelingen ontwikkeld die een effect hebben op het afweersysteem ("immunotherapie"). Het doel van immunotherapie in het geval van kanker, is het herstellen van een goede afweer tegen de kankercellen met als gevolg het doden ervan. Er zijn grofweg drie immunotherapeutische strategieën: antilichaam- of cytokinetherapie, adoptieve T-cel therapie en therapeutische vaccinatie. Helaas hebben tot op heden slechts enkele immunotherapieën (kortdurend) klinisch effect laten zien bij veelal een kleine groep patiënten. Vermoedelijk heeft dit te maken met het feit dat deze middelen getest worden in patiënten met uitgebreide of uitgezaaide tumoren. Bij deze patiënten is de ziekte zo uitgebreid dat kwaadaardige cellen een afweerreactie gemakkelijk kunnen ontwijken. Het afweersysteem wordt bij deze patiënten veelal op verschillende niveaus onderdrukt en is daardoor te zwak om goed te reageren als maar één van de problemen wordt aangepakt. Men kan dan van een bepaald type immunotherapie niet verwachten dat deze in staat is om een goede afweerreactie uit te lokken, om de tumor kleiner te maken.

Onderzoek in cellijnen, dieren en mensen heeft laten zien dat de klassieke behandelingen zoals chemotherapie en radiotherapie ook een effect hebben op het afweersysteem. Oorspronkelijk werd er gedacht dat dit een afweer-onderdrukkend effect was, echter gebleken is dat chemotherapie en radiotherapie het afweersysteem ook gunstig, en dus afweer-stimulerend, kunnen beïnvloeden. Wanneer deze klassieke behandelingen het afweersysteem gunstig beïnvloeden, zouden ze ook een betere conditie kunnen creëren voor immunotherapie en dus versterkend of synergistisch kunnen werken. Enerzijds zou dan tumorvernietiging door chemo- en/of radiotherapie een afweerreactie uit moeten lokken, terwijl de afweer onderdrukkende condities uitgeschakeld worden en het afweersysteem versterkt wordt.

In de studies die worden beschreven in dit proefschrift, proberen we meer informatie te verkrijgen over het precieze effect dat chemotherapie en radiotherapie, zoals ze gebruikt worden voor de behandeling van baarmoederhalskanker, hebben op het afweersysteem. We hebben in kaart gebracht hoe deze therapieën het afweersysteem beïnvloeden en wanneer eventueel aanvullende immunotherapie mogelijk zou zijn. Dit alles is van belang om uiteindelijk een zo sterk mogelijke tumor-specifieke afweerreactie te verkrijgen en de kanker via het afweersysteem op te ruimen.

DIT PROEFSCHRIFT

In *hoofdstuk 1* worden 2 klinische casus van patiënten met baarmoederhalskanker beschreven. Deze casus laten zien dat geen enkele baarmoederhalskanker patiënte hetzelfde is en dat we het beloop van de ziekte vaak lastig kunnen voorspellen. De vele verschillen in klachten patroon, behandeling strategieën, tumor kenmerken, afweerreacties en klinische uitkomst, motiveren artsen en wetenschappers om hun kennis op het gebied van gynaecologie, oncologie, radiologie, pathologie, farmacologie en immunologie te combineren. Deze combinatie van kennis is van essentieel belang, aangezien er ook een enorm aantal verschillende mechanismen verantwoordelijk zijn voor de effectiviteit van bepaalde behandelingen. *Hoofdstuk 2* geeft een overzicht van de betrokkenheid van het afweersysteem bij het ontstaan en de uitgroei van kanker, in het bijzonder baarmoederhalskanker. Daarnaast worden de huidige behandelingen met chemotherapie en/of bestraling bij uitgebreide of uitgezaaide baarmoederhalskanker besproken. De huidige kennis van het effect van deze klassieke behandelingen op het afweersysteem worden samengevat en de ervaringen met immunotherapie bij patiënten worden aangehaald. Geconcludeerd wordt dat de combinatie van klassieke therapieën met immunotherapie mogelijk het effect op het afweersysteem zou kunnen versterken en idealiter ook een goed effect zou hebben op de klinische uitkomst van patiënten met baarmoederhalskanker. Hiervoor is het echter noodzakelijk dat eerst de effecten van de klassieke behandelingen op het afweersysteem gedetailleerd in kaart worden gebracht om hiermee te bepalen of combinatie therapie mogelijk is en wat de ideale tijdspanne is waarbinnen dit plaats zou moeten vinden.

In *hoofdstuk 3* van dit proefschrift wordt beschreven dat het belangrijk is om een groep patiënten te identificeren die een hoog risico heeft op het terugkeren

van baarmoederhalskanker na primaire (operatieve) behandeling. Aangezien deze groep een slechte prognose heeft ten aanzien van overleving, zou dit een geschikte populatie kunnen zijn om aanvullende behandeling te geven in de vorm van bijvoorbeeld immunotherapie. In de studie beschreven in *hoofdstuk 3*, werd gezien dat bij patiënten met een grote tumor (> 4cm) en/of lymfeklier uitzaaiingen de kans groter is dat de ziekte terugkeert dan bij patiënten die dit niet hebben. Deze hoog-risico patiënten worden veelal reeds bestraald of krijgen chemotherapie na de operatieve behandeling. Desondanks blijken uitzaaiingen zich toch te manifesteren en voor deze groep patiënten is mogelijk nog aanvullende behandeling, in de vorm van immunotherapie, noodzakelijk. De mogelijkheid om immunotherapie met standaard therapieën zoals bestraling en chemotherapie te combineren, is ontstaan nadat uit meerdere onderzoeken is gebleken dan zowel chemotherapie als radiotherapie het afweersysteem beïnvloeden. De verschillende effecten van chemotherapie op het afweersysteem worden in *hoofdstuk 4* besproken. Beschreven wordt hoe het ingewikkelde netwerk van tumorcellen, afweercellen en signaalstoffen op diverse manieren door de verschillende soorten chemotherapieën wordt gemoduleerd. Met name de gunstige effecten van chemotherapie op het afweersysteem bieden de kans om in de toekomst chemotherapie met immunotherapie te combineren. In de aanloop naar het mogelijke succes van gecombineerde behandelingen met immunotherapie voor baarmoederhalskanker, is het essentieel om het verloop (kinetica) van de afweerreacties in kaart te brengen. Gebleken is dat het combineren van immunotherapie met de huidige standaard therapieën (zoals chemotherapie en bestraling) gedetailleerd onderzoek vereist, waarbij intensieve monitoring van het afweersysteem ('immunomonitoring') noodzakelijk is om uiteindelijk de ideale tijdsspanne waarin combinatie plaats kan vinden te identificeren.

In de studie beschreven in *hoofdstuk 5*, hebben we getracht om het afweersysteem te monitoren tijdens de chemotherapeutische behandeling van baarmoederhalskanker. We hebben zowel bij muizen als bij patiënten op verschillende tijdspunten voor, tijdens en na de chemotherapie het bloed onderzocht en verschillende afweercellen getypeerd en afweerreacties gemeten. Bij patiënten met baarmoederhalskanker was er vóór aanvang met de chemotherapie, een abnormaal hoog aantal afweer-onderdrukkende ("myeloide") cellen aanwezig, vergeleken met het bloed van gezonden mensen. Ongeveer 1 à 2 weken na de tweede chemokuur met carboplatin-paclitaxel zagen we een sterke daling / normalisatie van deze myeloide cellen, waarbij de T-cel aantallen niet daalden. Gebaseerd op deze resultaten, hebben we patiënten met lokaal uitgebreid, op afstand uitgezaaid of teruggekomen baarmoederhalskanker behandeld met gecombineerde chemo-vaccinatie therapie. Chemo-immunotherapie in deze patiënten resulteerde in een zeer sterke vaccin-geïnduceerde afweerreactie gemeten in het bloed. Deze reacties waren opvallend sterk, sterker dan in eerdere klinische studies, en bleven tevens langdurig bestaan. Momenteel wordt er een klinische studie uitgevoerd waarbij deze multimodaliteit in de behandeling van baarmoederhalskanker wordt uitgebreid. Patiënten krijgen chemotherapie met carboplatin-paclitaxel, 4 vaccinaties met HPV16 SLP vaccin, en cytokine therapie met IFNα. Het doel is om bij deze patiënten effectieve tumor-specifieke afweerreacties te krijgen, welke uiteindelijk kunnen leiden tot klinische reactie en een betere overleving van deze patiëntengroep.

In *hoofdstuk 6* beschrijven we een soortgelijke studie, waarbij opnieuw in het bloed van patiënten met baarmoederhalskanker afweercellen en –reacties zijn gemeten. Aan deze studie namen patiënten deel die radiotherapie, al dan niet in combinatie met chemotherapie, ondergingen vanwege baarmoederhalskanker. Hierbij zagen we dat het afweersysteem door de bestraling ernstig onderdrukt wordt. Dit effect was onafhankelijk van het feit of patiënten wel of geen aanvullende chemotherapie kregen en onafhankelijk van het stadium van de ziekte. Dit afweer onderdrukkende effect vond al heel snel na het starten met de bestraling plaats en herstelde zeer traag. Daarnaast werd er gezien dat de functie van de antigen presenteerde cellen verstoord werd en de helper $CD4^+$ T-cellen meer "programmed cell death-1" (PD-1) tot expressie brachten. PD-1 is een eiwit dat zich aan de buitenkant van een T-cel bevindt en kan binden aan PD-ligand-1 (PD-L1) wat aan de buitenkant van tumorcellen zit. Als het eiwit PD-L1 aan PD-1 bindt, wordt een T-cel inactief. De verhoogde expressie van PD-1 bij $CD4^+$ T-cellen tijdens bestraling, zou kunnen verklaren waarom de functie van T-cellen tijdens bestraling zo fors achteruit gaat. Het in het bekken gelegen beenmerg blijkt dus extreem gevoelig voor radiotherapie en dit resulteert bij vrouwen met baarmoederhals kanker tot een sterke onderdrukking van het afweersysteem. Aangezien bestraling bij patiënten met baarmoederhalskanker een afweer onderdrukkend effect blijkt te hebben, lijkt het niet handig om immunotherapie met de huidige vorm van bestraling te combineren. Er ligt een grote uitdaging in de optimalisatie van dosis en frequentie van de bestraling, zodanig dat het voldoende anti-tumor effect heeft maar het afweersysteem niet meer onderdrukt. Daarnaast zou beenmerg-sparende radiotherapie het volume van blootgesteld beenmerg kunnen beperken, en potentieel het afweer onderdrukkende effect kunnen beperken.

CONCLUSIE

Omdat de levensverwachting van patiënten met uitgezaaide of teruggekeerde baarmoederhalskanker ondanks veranderingen in de behandeling in de laatste decennia nauwelijks is verbeterd, wordt er gezocht naar nieuwe behandeltechnieken voor deze vreselijke ziekte. Immunotherapie is een nieuwe behandelstrategie, maar is als mono-therapie nog niet effectief gebleken bij patiënten met een vergevorderd stadium baarmoederhalskanker. Standaard therapieën zoals chemotherapie en bestraling kunnen het afweersysteem op verschillende manieren beïnvloeden, wat een klinisch effectieve combinatie met immunotherapie mogelijk zou kunnen maken. Ons onderzoek geeft nieuwe inzichten in het effect van chemotherapie en radiotherapie op het afweersysteem. Immunotherapie met HPV16 SLP vaccinatie kan mogelijk succesvol gecombineerd worden met chemotherapie, wanneer het binnen een optimale tijdspanne wordt toegediend. Zo weet chemotherapie met carboplatin-paclitaxel de afweer-onderdrukkende mechanismen te verdringen, en lokt immunotherapie een sterke, HPV-specifieke afweerreactie uit. Om ook de klinisch effectiviteit te testen zijn intensieve vervolg studies nodig waarbij verschillende behandelstrategieën optimaal gecombineerd dienen te worden. Daarbij is van groot belang om dosering, tijdspanne en toediening van zowel immunotherapie als de klassieke behandelingen nauwkeurig te testen. Daarnaast is er behoefte aan afweer-specifieke biomarkers die de selectie van de geschikte behandeling voor de juiste patiënt gemakkelijker maken en daarbij de (immunologische én klinische) effectiviteit van de behandelingen kunnen voorspellen.

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LIST OF ABBREVIATIONS

ACT	Adoptive cell transfer
ATREG	Activated regulatory T-cell
APC	Antigen presenting cell
BT	Brachytherapy
CBA	Cytometric bead array
CGOA	Center for Gynecologic Oncology Amsterdam
CD	Cluster of differentiation
CHDR	Centre for Human Drug Research
CIN	Cervical intraepithelial neoplasia
CTL	$CD8^+$ Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte Antigen 4
DC	Dendritic cell
EBRT	External beam radiation therapy
EGFR	Epidermal growth factor receptor
FOXP3	Forkhead box P3
GMDSC	Granulocytic myeloid derived suppressor cell
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HRHVP	High-risk human Papilloma Virus
ICS	Intracellular cytokine staining
IFN	Interferon
IL	Interleukin
LC	Langerhans cells
LST	Lymphocyte stimulation test
LUMC	Leiden University Medical Center
M1	Macrophage type 1
M2	Macrophage type 2
MOABS	Monoclonal antibodies
MMDSC	monocytic myeloid derived suppressor cell
MDSC	Myeloid derived suppressor cell
MRM	Memory Response Mix
MLR	Mixed Lymphocyte Reaction
NTREG	Naïve regulatory T-cell
NK	Natural Killer
PBMC	Peripheral blood mononuclear cells
PD-1	Program death 1
PD-2	Program death 2
PD-L	Program death ligand
PHA	Phytohemagglutinin
PRR	Pattern recognition receptor
SI	Stimulation index
SLP	Synthetic Long Peptide
TAM	Tumor associated macrophages
TGF	Tumor growth factor
TH CELL	$CD4^+$ T-helper
TIL	Tumor infiltrating T-cells
TIM	Tumor infiltrating myeloid cells
TIM-3	T cell immunoglobulin mucin 3
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREG	Regulatory T-cell
VEGF	Vascular endothelial growth factor

CURRICULUM VITAE

Hélène van Meir was born on the 20th of September 1981 in Goes. From the age of 10, she went to the girls-only boarding school Sint-Bavo humaniora in Ghent, Belgium, from which she graduated in 1999. In the same year she started with Pharmacy at the Catholic University of Leuven, Belgium, and switched to medical school at the Leiden University the year after. During her study, she worked as an allocation officer at the Eurotransplant International Foundation. She performed her graduation project at the department of Cardiac Surgery and Pediatric Cardiology at the Leiden University Medical Center, under supervision of Prof. dr. Hazekamp. She studied the use of bovine jugular vein graft for the reconstruction of the right ventricular outflow tract in a pediatric population at the Leiden University Medical Center and Centre Hospitalier Universitaire Vaudoise, Lausanne, Switzerland. In 2008 she obtained her medical degree (cum laude) and started working as a physician at the Department of Obstetrics and Gynecology at the Bronovo Hospital, The Hague. In 2009 she was appointed as a research physician in the Vascular Medicine research group at the Centre for Human Drug Research (CHDR). She focused on the integration of immunology in clinical pharmacology, and participated in several research projects, supervised by prof. dr. A.F. Cohen and prof. dr. J. Burggraaf. The studies in this thesis were performed in close collaboration with the departments of Clinical Oncology (prof. dr. S.H. van der Burg) and Gynecology (dr. M.I.E. van Poelgeest) of the Leiden University Medical Center and the Center for Gynecological Oncology Amsterdam (prof. dr. G.G. Kenter). Whilst working as a research physician at CHDR, she was trained as a clinical pharmacologist and obtained her degree in clinical pharmacology in 2016.

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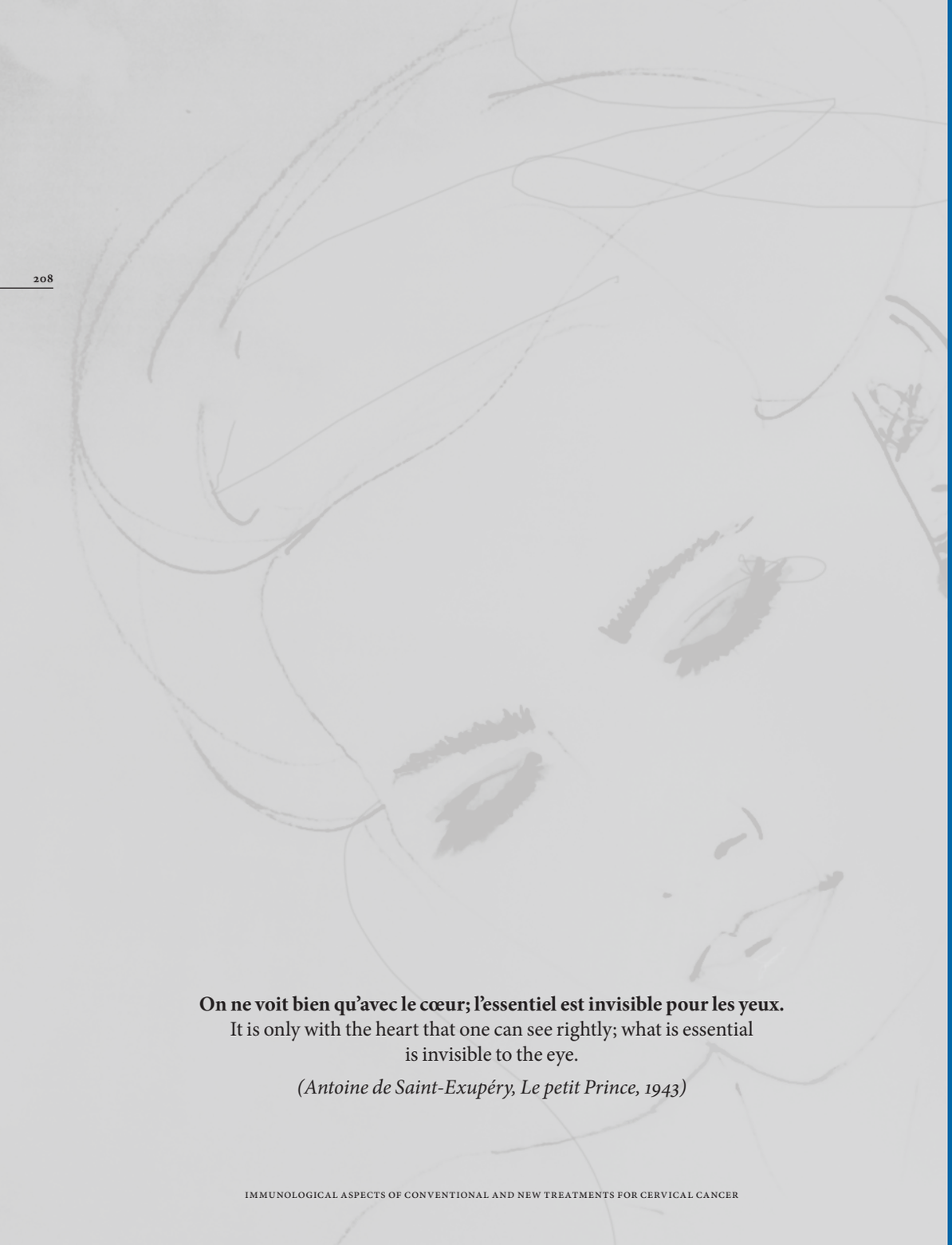

On ne voit bien qu'avec le cœur; l'essentiel est invisible pour les yeux.
It is only with the heart that one can see rightly; what is essential
is invisible to the eye.

(Antoine de Saint-Exupéry, Le petit Prince, 1943)