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

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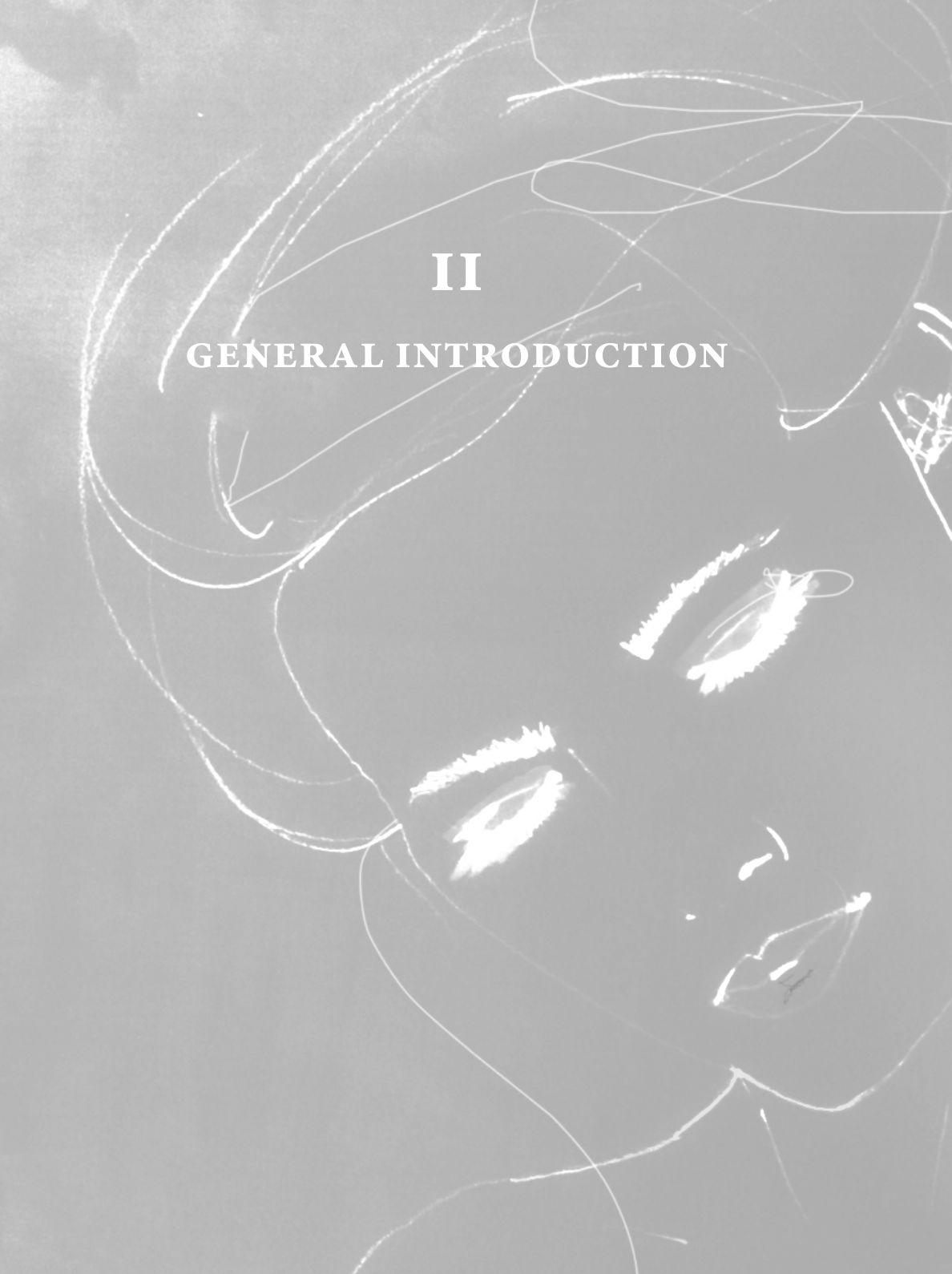

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GENERAL INTRODUCTION

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 subdivided 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] TLRs1-3, 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 immunotherapeutic 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])

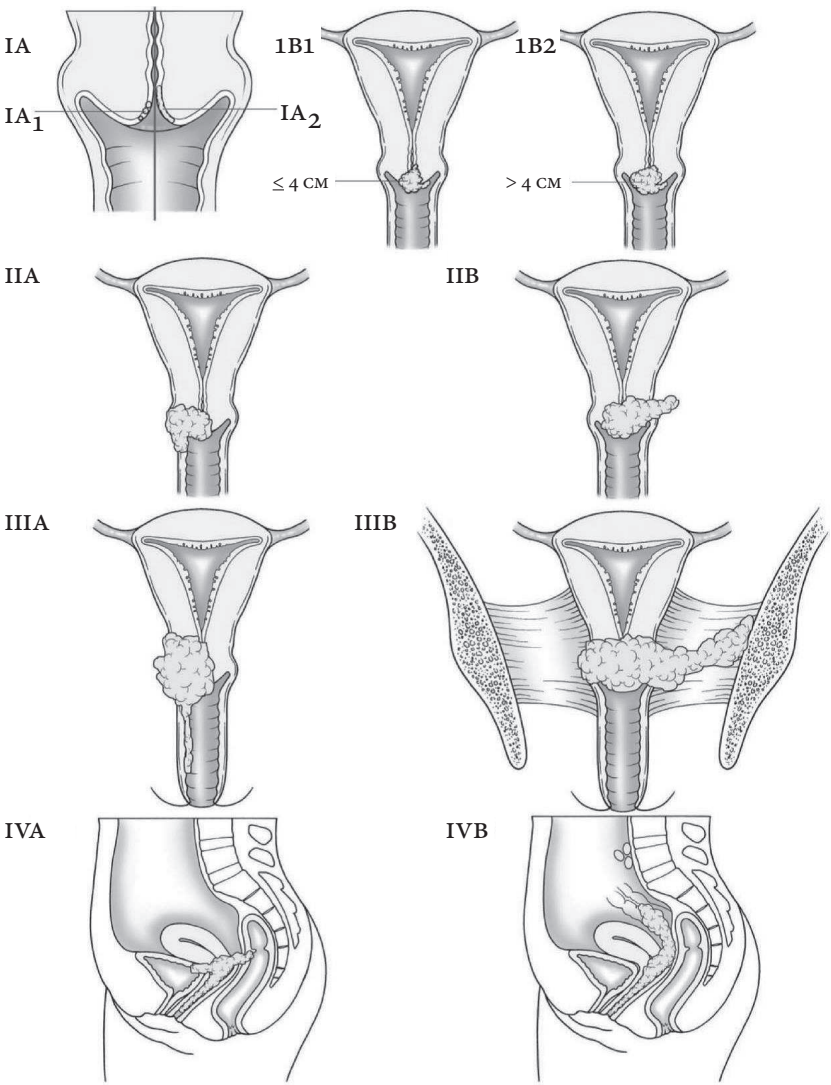
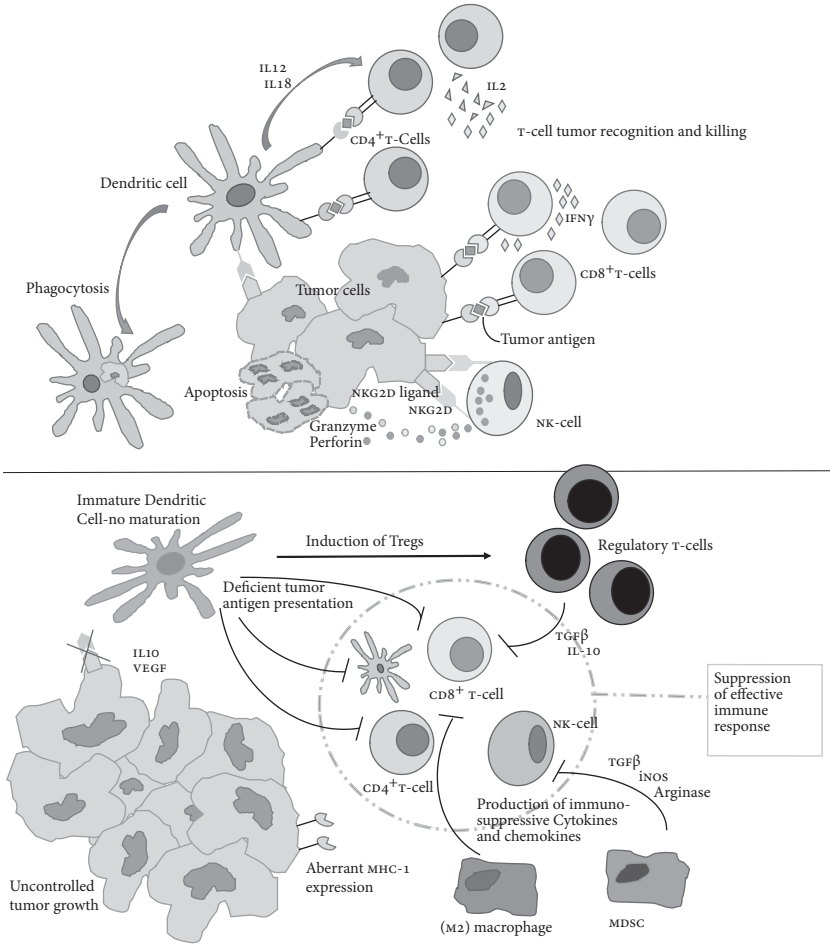


Figure 2 Tumor elimination phase.



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