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

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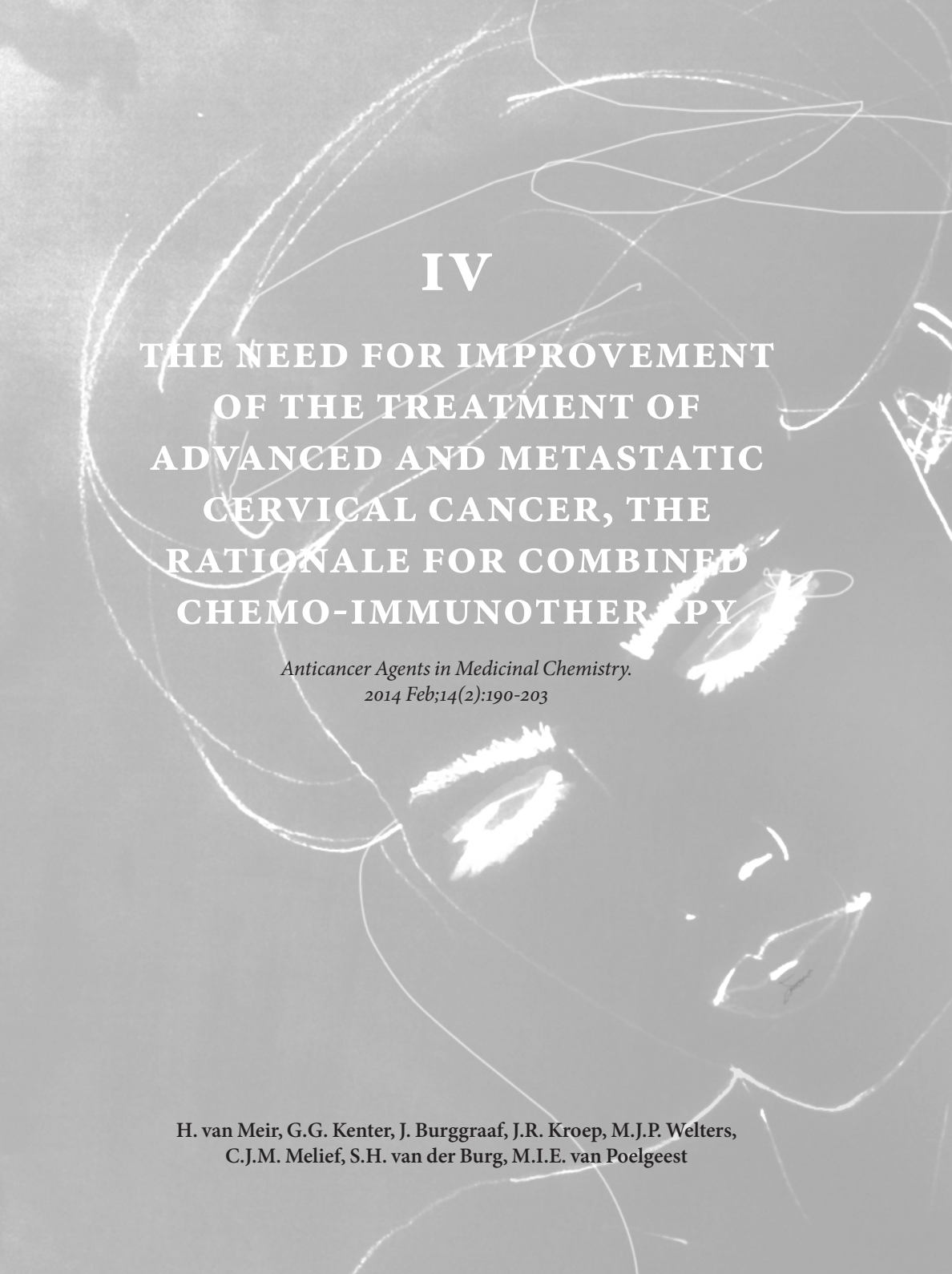

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THE NEED FOR IMPROVEMENT OF THE TREATMENT OF ADVANCED AND METASTATIC CERVICAL CANCER, THE RATIONALE FOR COMBINED CHEMO-IMMUNOTHERAPY

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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.

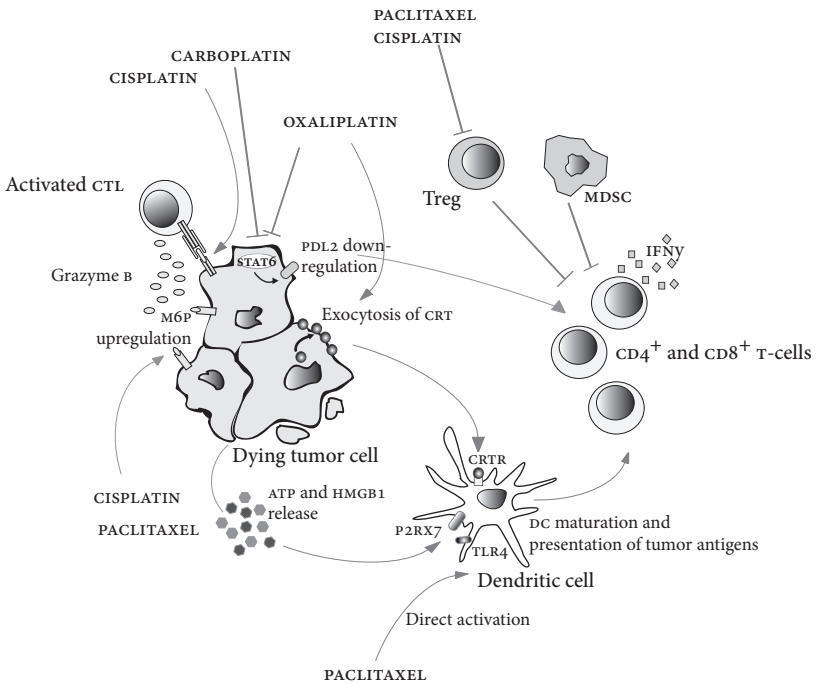

Figure 2 Kaplan-Meier curves suggesting that therapeutic vaccination in patients with advanced cervical cancer has limited clinical effects by itself, but might exert improved therapeutic action if combined with chemotherapy

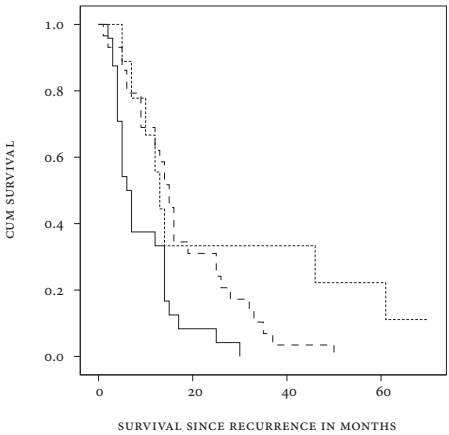

Table 1 Clinicopathological characteristics of advanced cervical cancer patients treated with combined chemo-immunotherapy, isolated immunotherapy or chemotherapy alone.

	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