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Elham Beyranvand Nejad, Marij J.P. Welters, Ramon Arens & Sjoerd H. van der Burg

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REVIEW

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The importance of correctly timing cancer immunotherapy

Elham Beyranvand Nejad[a,b*], Marij J.P. Welters[a*], Ramon Arens[b] and Sjoerd H. van der Burg[a]

[a]Department of Medical Oncology, Leiden University Medical Center, Leiden, The Netherlands; [b]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

ABSTRACT

Introduction: The treatment options for cancer—surgery, radiotherapy and chemotherapy—are now supplemented with immunotherapy. Previously underappreciated but now gaining strong interest are the immune modulatory properties of the three conventional modalities. Moreover, there is a better understanding of the needs and potential of the different immune therapeutic platforms. Key to improved treatment will be the combinations of modalities that complete each other's shortcomings.

Area covered: Tumor-specific T-cells are required for optimal immunotherapy. In this review, the authors focus on the correct timing of different types of chemotherapeutic agents or immune modulators and immunotherapeutic drugs, not only for the activation and expansion of tumor-specific T-cells but also to support and enhance their anti-tumor efficacy.

Expert opinion: At an early phase of disease, clinical success can be obtained using single treatment modalities but at later disease stages, combinations of several modalities are required. The gain in success is determined by a thorough understanding of the direct and indirect immune effects of the modalities used. Profound knowledge of these effects requires optimal tuning of immunomonitoring. This will guide the appropriate combination of treatments and allow for correct sequencing the order and interval of the different therapeutic modalities.

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Cancer; immunotherapy; timing; combination therapy; immunomonitoring

1. Introduction

The common treatment options for cancer patients so far were surgery, radiotherapy and/or chemotherapy. This is now supplemented with a fourth treatment modality that is called immunotherapy. The latter encompasses several strategies aiming to reinforce the immune system's attack of tumor cells by activation of tumor-specific lymphocytes, alleviation of immune suppressive mechanisms and stimulation of immune effector cell infiltration. Prime examples are vaccination strategies and the adoptive transfer of expanded tumor infiltrating (T-cell receptor engineered or re-educated) lymphocytes to increase the number of tumor-specific T-cells required to control tumor cell growth [1,2]. For instance, a synthetic long peptide (SLP) vaccination against human papillomavirus type 16 (HPV16) resulted in complete clearance of HPV16-induced high-grade premalignant lesions of the vulva in ~50% of the patients [3,4]. Importantly, prolonged survival was found in patients treated with the food and drug administration (FDA) approved autologous cellular vaccine sipuleucel-T for castration-resistant prostate cancer [5]. Furthermore, adoptive transfer of autologous T-cells resulted in clinical objective responses in half of the treated melanoma patients [6,7]. Moreover, cancer regression and improved survival has been achieved in melanoma and lung cancer patients using antibodies to coinhibitory molecules, including anticytotoxic T-lymphocyte-associated protein 4 (CTLA-4; ipilumimab, tremelimumab) antibodies [8,9], antiprogrammed cell death protein 1 (anti-PD-1; nivolumab, pembrolizumab) antibodies [10–13] and antiprogrammed death-ligand 1 (anti-PD-L1; avelumab, atezolizumab; durvalumab) antibodies [14–17]. Clinical success has also been achieved using targeted therapies aiming to inhibit molecular pathways that are important for tumor growth and maintenance, either as a single therapy or in combination with immunotherapeutic strategies [18]. In addition, epigenetic drugs to upregulate immune signaling combined with immunotherapy are currently under investigation [19,20], but this is beyond the scope of the current review.

In spite of all the mentioned immunotherapeutic strategies, there are still numerous cancer patients who do not benefit from these immunotherapeutic drugs. Monotherapy, although successful in a number of cases, is not expected to have a major impact as established tumors use diverse strategies to evade the immune system, a process that is called immunoediting [2,21,22]. Under the attack of the immune system, tumor cells may alter the processes involved in the presentation of antigens to T-cells (i.e. downregulation of major histocompatibility complex (MHC) class I, epigenetic silencing of the antigen processing machinery, loss of tumor associated-antigens) or become more resistant to immune mediated effector mechanisms leading to growth arrest and cell death [2]. Furthermore, the tumor microenvironment may become more immunosuppressive by the attraction and/or induction of suppressive immune cells, i.e. regulatory T-cells (Tregs),

CONTACT Sjoerd H. van der Burg shvdburg@lumc.nl Department of Medical Oncology, Leiden University Medical Center, Building 1, C7-141, PO box 9600, 2300 RC Leiden, The Netherlands

*Authors contributed equally to the study

Article highlights

- A profound understanding of the immune modulatory effects of current cancer therapies allows finding the optimal timing of multiple therapies with most clinical benefit for the cancer patient.
- Single treatment modalities can be successful at an early phase of disease while at later disease stages combinations of several modalities are required.
- Therapies applied before therapeutic vaccination are generally aimed at alleviation of immune suppression.
- Therapies provided concurrently or shortly after vaccination aim to potentiate the vaccine-induced immune response and to prevent normal immune regulation.
- Harmonization of immune monitoring helps paving the way for the rational design of immunotherapeutic combination strategies.

This box summarizes key points contained in the article.

myeloid-derived suppressor cells (MDSCs), type 2 macrophages (M2) [2,23]. This process leads to a less efficient antitumor response. Therefore, there is tremendous demand to develop cancer immunotherapies not only to activate the tumor-specific T-cell response but also to strengthen its force by combatting the immune evasion and suppressive pathways to improve the clinical outcome [2,21,22]. Based on these concepts, wise choices for complementary and synergistic combinations of immunotherapeutic drugs have to be made. Importantly, combinations with more conventional treatments should not be discarded. This selection entails a thorough understanding of the immune-modulating and pharmacological properties as well as the limitations of the agents of choice. Together, this should guide the right combination, dose, and treatment schedule and lead to optimal treatment strategies.

Here we provide an overview of the current literature on the timing of therapeutic vaccination in cancer patients. First, timing applies to the most appropriate stage of disease in which an immunotherapeutic modality can be used for optimal clinical effects. Second, timing concerns the sequence and interval of a combination of drugs for immunotherapy.

2. Immunotherapy efficacy at various stages of the disease

2.1. Treatment of advanced or end-stage cancer may fail due to immune suppression

Immunotherapy is often tested in advanced or end-stage cancer patients. These patients have lost responsiveness to earlier therapies, the tumor has grown to larger extent and general immune suppression is more pronounced [2,21]. In preclinical mouse models this is reflected by the increasing failure to control tumor outgrowth when the time period between tumor engraftment and vaccination is increased. This is mainly due to increased frequencies of Tregs and myeloid suppressor cells [24]. In the human setting this is exemplified by the many vaccine trials that have failed to show an effect [2]. Thus, in these late stages the tumor micro-milieu may frustrate the effector arm of the immune system through different mechanisms. First of all, the influx of tumor-specific T-cells may be hampered by abnormal vascularization [25,26]. In both a xenograft transplant model and an immune refractory spontaneous murine model this problem could be overcome by treatment with low dose of gamma irradiation [25], or by targeting VEGF (vascular endothelial growth factor) [27]. However, even when the effector cells are inside the tumor they may encounter several immune suppressive hurdles before they can reach and kill the tumor cells [28]. The tumor micro-environment contains cells that are helping the tumor to expand and to evade the immune system such as cancer-associated fibroblasts, MDSCs, M2 and Tregs [29]. The important role of macrophages in tumor progression and the possibilities to target these cells were reviewed previously [30,31]. Targeting these tumor-resident immune suppressive myeloid cells could be an option to improve immunotherapy [30–32]. Hence, it is no surprise that specifically mono-immunotherapy – focused on reinforcing the tumor-specific T-cell response – as a last resort therapy is often not successful.

2.2. Success of therapy at early stages of cancer or minimal residual disease

Better clinical outcomes are expected if one can treat patients before recurrences develop, in settings of minimal residual disease, or at early (premalignant) stages of disease. Indeed, as shown in two independent trials, HPV16-SLP vaccination of patients with high-grade premalignant lesions of the vulva resulted in complete regressions of the lesion in almost half of the treated patients [3,33]. Vaccination with a HPV16 E6-E7-L2 fusion protein vaccine in combination with Aldara treatment also achieved clinical success in patients with this disease [34]. Moreover, treatment of high-grade premalignant lesions of the cervix was efficacious when the patients received a DNA vaccine targeting the HPV16 oncoproteins [35]. Moreover, vaccination of patients with HER^{+} breast ductal carcinoma *in situ* resulted in measurable decreases of residual ductal carcinoma [36]. As can be deduced from above, resection of the tumor mass may alleviate immune suppression allowing the use of immunotherapy to prevent new tumors to arise. Indeed, vaccination of patients with completely resected colorectal cancer metastases showed a significant survival advantage when compared to controls [37], whereas HER2 peptide vaccination in disease-free breast cancer patients was associated with a favourable trend for lower recurrences [38]. Unfortunately, this is not always the case as exemplified by a recent report on patients with surgically resected early stage non-small-cell lung cancer whom were vaccinated with MAGE-A3 but failed to show any improvement in disease free survival [39,40].

2.3. Prevention of cancer for patients at risk

Vaccination of individuals to prevent disease has been one of the major achievements in mankind. Current data on the preventive vaccines for cervical cancer and liver cancer support the notion that prevention is key to success [41,42]. Also for non-virally induced cancers, vaccine strategies are being developed for individuals who are at risk to develop cancer. For instance for individuals with BRCA mutations known to

induce breast cancer or for those with Lynch syndrome which is associated with colon cancer [43]. Certainly, there are no cancer-associated hurdles to be overcome when a person is still healthy. This allows the vaccine to induce a protective immune response against the tumor antigens expressed by the type of cancer for which they are at risk [44]. Awareness of the regulatory authorities for this approach is very important to successfully combat cancer [45].

3. Correctly timing of therapeutic vaccination in combination with other therapies

Therapeutic vaccination in combination with other therapies can roughly be divided into three differently timed treatment schedules. Treatments that are given before vaccination are generally aimed at removing tumor-associated immune suppression. Modalities provided close to or in combination with vaccination aim to prevent immune regulation following T-cell activation, thereby improving the quality and efficacy of the vaccine-induced T-cell response. Therapies provided after vaccination generally are to boost the T-cell stimulatory effect of the vaccine or their effector function. An overview of such studies is provided in Table 1.

3.1. Combinations of therapeutic modalities prior to vaccination

3.1.1. Administration of chemotherapy before vaccination alleviates immune suppression

It is known that both local and systemic immune parameters in patients with cancer are associated with the prognosis and response to therapy [93]. The composition, phenotype and activation status of the tumor infiltrating T-cells, DCs and macrophages have a strong impact on clinical outcome. In cancer patients with a higher tumor load, the tumor microenvironment merely is pro-tumorigenic and suppressive for the immune effector cells. In a number of cases this is also reflected by the immune cell markers and function of immune cells in the blood of these patients, as measured by flow cytometry and/or functional immune assays [94,95].

Chemotherapy, radiotherapy and surgery used for the treatment of cancer are applied for tumor reduction or eradication. As a direct result they will remove cancer-derived factors known to induce immune suppression. However, even when unsuccessful as monotherapy, a number of chemotherapeutic compounds may also have direct effects on the immune system [96,97], albeit that for many of these compounds the underlying mechanisms still remain to be elucidated. In HPV16$^+$ TC-1 tumor bearing mice and in advanced stage or recurrent HPV16$^+$ cervical carcinoma patients the number of circulating myeloid cells, including immunosuppressive myeloid cells, is significantly increased when compared to naïve mice and healthy individuals, respectively [48]. The standard chemotherapy treatment (carboplatin combined with paclitaxel) for these advanced cancer patients resulted in normalization of the different myeloid cells in the peripheral blood, starting 2 weeks after the second chemotherapy cycle and coinciding with a stronger general T-cell response and improved antigen presenting capacity. As this chemotherapeutic treatment not only normalized the circulating myeloid cell population in mice but also altered the intratumoral myeloid cell composition and increased the clinical effect of therapeutic vaccination, it is believed that this will also result in a reduced suppressive microenvironment in patients. Indeed, a single vaccination with HPV16-SLP within the correct time window of 2 weeks after the second cycle of chemotherapy resulted in a much stronger HPV-specific T-cell response than observed before, when vaccination was given after chemotherapy failure [4,48]. In line with this data, a similar time window of 12–14 days after combination chemotherapy with carboplatin and paclitaxel has been observed in a study of advanced ovarian cancer patients [47]. Here, they reported that at this time point the number of Tregs was reduced while there were increased percentages of IFNγ-producing CD8$^+$ T-cells, T-helper (Th1) cells, CD45RO memory T-cells and NKT-cells. Also in extensive stage small cell lung cancer, vaccination with DCs transduced with full-length wild-type p53 gene delivered via an adenoviral vector at least 8 weeks after the last dose of chemotherapy with carboplatin or cisplatin demonstrated a high rate of objective clinical responses, and this was associated with the induction of vaccine-induced immune responses [46].

An effect on the number and function of Tregs has specifically been reported for the chemotherapeutic agent cyclophosphamide [98,99]. Several studies suggest that the optimal time point for vaccination is 3–7 days after this type of chemotherapy [50–52]. In a randomized phase II trial, administration of a single dose of cyclophosphamide, followed by vaccination 3 days later with IMA901, a vaccine that consists of multiple-tumor associated peptides (TUMAPs) plus granulocyte-macrophage colony-stimulating factor (GM-CSF), reduced the number of Tregs and was associated with prolonged survival in immune responder patients with advanced renal cell cancer [52]. Similarly, peripheral blood mononuclear cells (PBMCs) analysis of stage II–III melanoma patients, who were vaccinated with HLA-A*0201-modified tumor peptides 7 days after low-dose of cyclophosphamide, showed transient reduction in the frequency of Tregs and an increase in vaccine-induced antigen specific CD8$^+$ T-cells [50]. Other studies in which cyclophosphamide treatment was used, showed that the depletion of Tregs may be associated with the induction of Th17, Th1 and vaccine-induced CD25$^+$CD4$^+$Foxp3-negative effector T-cells [65,66]. Also other chemotherapeutic compounds may affect Tregs and immune suppressive myeloid cells. Gemcitabine is known to reduce both Tregs and MDSCs in mice and in patients with ovarian cancer [53,54,80,100,101]. A selective decrease in MDSCs was also observed after treatment with 5-fluorouracil (5-FU) [102]. The combination of cisplatin plus vinorelbine appears to significantly increase the ratio between effector T-cells and Tregs and to reduce the immunosuppressive activity of the latter in the blood of the majority of non-small cell lung cancer patients [103]. Therefore, modulation of immune suppressive cells by chemotherapeutic agents prior to anticancer vaccine could explain the additive or synergistic antitumor effect of combined chemotherapy and immunotherapy.

3.1.2. Other interventions applied before vaccination that reduce immune suppression

Notably, the reduction of immunosuppressive cells can also be mediated by other methods than chemotherapy. These

Table 1. Classification of different combinatorial strategies based on timing regimen and mechanism.

Timing of the intervention		Intervention	Cancer type	Combined treatment regimen	Efficacy/immunological responses of combined treatments	Mechanisms of intervention		References
Prior to treatment	8 weeks before vaccine	Carboplatin + cisplatin	Extensive stage small cell lung cancer	P53 DC cancer vaccine + carboplatin + cisplatin	High rate of objective clinical responses Association of clinical response with immunologic response	No proposed mechanism	Counteracting suppressive immunity by reducing immunosuppressive cells	Antonia et al. [46]
	2 weeks before vaccine	Carboplatin + paclitaxel	Advanced ovarian cancer	Tumor antigen-loaded DCs + carboplatin + paclitaxel	Immune reconstitution Enhanced antitumor immune response	Decreased percentage of Tregs		Wu et al. [47]
	2 weeks before vaccine	Carboplatin + paclitaxel	HPV 16/17 TC-1 tumor model Advanced cervical cancer	HPV16-SLP vaccination + carboplatin + paclitaxel	Decreased immunosuppressive myeloid cells Increased vaccine-induced T-cell response Improved overall survival of mice	Myeloid cells depletion		Welters et al. [48]
	11 days before vaccine	Irradiation	F10 melanoma tumor model	DC-gp100 tumor vaccine + irradiation	Reduced tumor burden and prolonged mouse survival	Decreased Tregs and increase effector-memory T-cells frequency		Liu et al. [49]
	7 days before vaccine	Cyclophosphamide	Stage II-III melanoma cancer	HLA-A*0201-modified tumor peptide vaccine + cyclophosphamide + IL-2	Increased tumor-specific T-cells	Lower frequency of Tregs		Camisaschi et al. [50]
	4 days before vaccine	Cyclophosphamide	Gastrointestinal, lung, cervical and colorectal cancer	HLA-A2402-restricted tumor-associated antigen epitope peptides + cyclophosphamide	Association of prolonged survival with TAA-specific T-cell responses Safety and correlation of immune responses with vaccine-induced T-cell response in phase I clinical trial	Reduced number of Tregs		Murahashi et al. [51]
	3 days before vaccine	Cyclophosphamide	Advanced renal cell cancer	Multiple tumor associated peptides (TUMAPs) + cyclophosphamide	Prolonged survival in immune responders' patients	Reduced number of Tregs		Walter et al. [52]
	4 and 1 days before treatment	Gemcitabine	Mesothelioma AB12 tumor model	IFN-β cytokine immunogene therapy + gemcitabine	Enhanced antitumor efficacy	Decreased myeloid suppressor cells		Suzuki et al. [53]
	1 day before vaccine	Gemcitabine	Setting without tumor	Particle-mediated epidermal delivery vaccination against NY-ESO-1	Improved the efficacy of vaccine	Reduced percentage of Tregs		Rettig et al. [54]
	4 days before vaccine	Anti-CD25 antibody	B16 melanoma tumor model	Tumor cell-based vaccine + anti-CTLA-4 + anti-CD25	Increased CTL specific response	Depletion of Tregs		Sutmuller et al. [55]
	4 days before prophylactic vaccine	Anti-CD25 antibody	CT26 colorectal carcinoma model	tumor-specific peptide vaccine + anti-CD25	Induction of long-lasting antitumoral immune response	Depletion of Tregs		Casares et al. [56]
	1 day before prophylactic vaccine	Anti-CD25 antibody	B16-F10 tumor model	DC vaccine with stressed tumor cells + anti-CD25	Improved the efficacy of treatment and developed long-lived tumor- protective immune responses	Depletion of Tregs		Prasad et al. [57]
	5 days before ACT and vaccine	PLX3397 kinase inhibitor	B16F10 melanoma tumor model	Adoptive transfer of pmel-1 CD8 T-cells and peptide vaccination + PLX3397 kinase inhibitor	Enhanced CD8-mediated effect of immunotherapy Increase IFNγ- production of tumor-specific $CD8^+$ T-cells	Inhibits CSF-1 R Decreased tumor infiltrating macrophages		Sluijter et al. [58]
	4 days before ACT	PLX3397 kinase inhibitor	Syngeneic mouse model of BRAF (V600E)-driven melanoma	Adoptive cell therapy + PLX3397 kinase inhibitor	Enhanced anti-tumor response Increased IFNγ- producing tumor-infiltrating lymphocytes	Inhibit CSF-1 R Decreasing tumor-infiltrating myeloid cells Skewing $MHCII^{low}$ towards $MHCII^{hi}$ macrophages		Mok et al. [59]
	Prior to vaccination	IL-2 diphtheria toxin conjugate DAB389IL-2	Renal cell carcinoma	Tumor RNA-transfected DC vaccine + IL-2 diphtheria toxin conjugate DAB389IL-2	Improved the stimulation of tumor-specific T-cell response	Deplete Tregs		Dannull et al. [60]

(*Continued*)

Table1. (Continued).

Timing of the intervention		Intervention	Cancer type	Combined treatment regimen	Efficacy/immunological responses of combined treatments	Mechanisms of intervention		References
	2 weeks before vaccine	Chemoradiation	Esophageal squamous cell carcinoma	Multiple epitopes peptide vaccine + cisplatin+ 5FU + radiotherapy	Safety in phase I clinical trial Demonstrate peptide-specific CTL responses	Enhancement of the immunogenicity of the cancer cells and antigen presentation of DCs, thereby increasing CTL responses	Improvement of the T-cell response	Iinuma et al. [61]
	3 days before vaccine	Gemcitabine	Advanced pancreatic cancer	Antigen-pulsed DCs + lymphokine-activated killer cells stimulated with anti-CD3 (CD3-LAKS) + gemcitabine	A synergistic antitumor effect	Induction of tumor antigen-specific CTLs		Hirooka et al. [62]
	2 days before vaccine	Docetaxel	Established lewis lung carcinoma model	GM-CSF-producing tumor vaccine + docetaxel	Tumor regression and prolonged survival	Enhanced survival of antigen-experienced cells Reduced pre-existing memory cells Decreased Tregs		Chu et al. [63]
	2 days before vaccine	Radiation	HPV 16/17 TC-1 tumor model	DNA vaccine encoding calreticulin liked to HPV-E7 + radiation	Induction of antitumor effect Improved survival of tumor-bearing mice Increased E7-specific tumor-infiltrating $CD8^+$ T-cells	Enhancement of tumor cell apoptosis by radiotherapy Increased cell-mediated lysis of tumor cells		Tseng et al. [64]
	1 day before ACT	Cyclophosphamide	Friend leukemia	Adoptive transfer of lymphomonocytes from tumor-immunized mice + cyclophosphamide	Improved antitumor efficacy	Upregulation of various immunomodulatory factors Induction of Th17, Th1 and activated $CD4^+$		Moschella et al. [65]
	1 day before vaccine	Cyclophosphamide	Advanced melanoma	NY-ESO-1/ISCONATRIX vaccine + low dose cyclophosphamide	Improved the immunogenicity of the vaccine	Increase vaccine-induced NY-ESO-1-specific $CD4^+$ T-cell response		Klein et al. [66]
	1 day before vaccine	Cisplatin + 5-FU	MC38 murine colorectal adenocarcinoma tumor model	Intratumoral DCs injection + Low doses of cisplatin + 5-FU	Complete rejection and prolonged survival	Increase cytolytic activity of effector tumor-specific $CD8^+$ T-cells		Tanaka et al. [67]
	1 day before vaccine	Dacarbazine	Melanoma	Vaccine consisting HLA-A2 restricted melanoma antigen A and gp100 analog peptide + dacarbazine	Improved long-lasting memory $CD8^+$ T-cell response	Dacarbazine -induced gene activation involved in cytokine production, Leukocyte activation, immune response and cell motility Widening of TCR repertoire diversity with high avidity and tumor reactivity		Nistico et al. [68] Palermo et al. [69]
	1 day before vaccine	Cisplatin	HPV 16/17 TC-1 tumor model	HPV16 E6E7L2 fusion protein (TA-CIN) with GPI-0100 adjuvant + cisplatin	Reduced tumor out growth and extended survival	Induced tumor antigen-specific $CD8^+$ T-cells		Peng et al. [70]
	5 h before ACT	Cyclophosphamide	Murine lymphomas	Adoptive transfer of tumor-immune cells + cyclophosphamide	Enhanced the antitumor efficacy	Migration of tumor-immune lymphocytes to the tumor Promotion of homeostatic proliferation/activation of transferred lymphocytes Cytokine storm induction		Bracci et al. [71]
	7 days before vaccine	Sunitinib	Colon carcinoma (MC38-CEA)	Poxvirus-based vaccine encoding (CEA) plus 3 costimulatory molecules + sunitinib	Reduced tumor volumes Increased survival	Increased intratumoral infiltration of antigen-specific T-cells Improved type-1 T-cell cytokine response Decreased Tregs and MDSCs	Counteracting suppressive immunity by reducing immunosuppressive cells and Improve T-cell response	Farsaci et al. [72] and Finke et al. [73]
	4–7 days before vaccine	Cisplatin	HPV 16/17 TC-1 tumor model	DNA vaccine encoding calreticulin liked to HPV-E7 + cisplatin	Induce significant anti-tumor effect Improved survival of tumor bearing mice Increase E7-specific tumor-infiltrating $CD8^+$ T-cells	Increased MHC class I expression by tumor cells upon cisplatin treatment Increase cell-mediated lysis of tumor cells Decrease myeloid suppressor cells and Tregs in blood and spleen		Tseng et al. [74]

(*Continued*)

Table1. (Continued).

Timing of the intervention		Intervention	Cancer type	Combined treatment regimen	Efficacy/immunological responses of combined treatments	Mechanisms of intervention		References
	4 days before vaccine	Cisplatin	HPV 16/17 TC-1 tumor model	Vaccinia vaccine encoding HPV-16 E7 + cisplatin	Induce significant anti-tumor effect Increase E7-specific tumor-infiltrating $CD8^+$ T-cells	Increase intratumoral $CD11c^+$ Decrease myeloid suppressor cells in spleen		Lee et al. [75]
In combination	Concurrent with vaccine	Cyclophosphamide	Advanced ovarian cancer	Surviving HLA class I peptides (DPX-survivac) + metronomic cyclophosphamide	Enhanced T-cell response associated with differentiation of naïve T-cells into central/ effector memory and late differentiated polyfunctional antigen-specific T-cells	Enhanced vaccine induced T-cell response	Improvement of the T-cell response	Berinstein et al. [76]
	1 day before vaccine (cyclophosphamide + paclitaxel) and 1 week after vaccine (doxorubicin)	Cyclophosphamide + Paclitaxel + doxorubicin	Mammary tumor model in antigen-specific tolerized neu transgenic mice	GM-CSF-secreting HER-2/neu(neu)-expressing whole-cell vaccine + cyclophosphamide + paclitaxel + doxorubicin	Enhanced vaccine anti-tumor effect to delay tumor growth	Enhanced the efficacy of vaccine Increased neu-specific T-cells and Th1 response		Machiels et al. [77]
	Concurrent with vaccine	CD27 antibody	Prostate cancer model	Tumor lysate-pulsed DC vaccine + CD27 antibody	Reduced tumor outgrowth	Enhanced T-cell response		Wei et al. [78]
	Concurrent with vaccine	Cyclophosphamide + Paclitaxel + docetaxel	Hepatocellular carcinoma patients	Multi-peptide cocktail including HCV and tumor antigen vaccine + cyclophosphamide + paclitaxel + docetaxel	Enhanced specific T-cell response	Reduced Treg frequency	Counteracting suppressive immunity by reducing immunosuppressive cells	Tagliamonte et al. [79]
	Concurrent with vaccine	Gemcitabine	Platinum-resistant ovarian cancer	p53 SLP vaccine + pegilated IFN-α + gemcitabine	Safe, feasible, immune stimulatory effect of combined treatment	Reduction in MDSCs by gemcitabine and increase in M1 macrophages		Dijkgraaf et al. [80]
	Concurrent with vaccine	Cisplatin	HPV 16/17 TC-1 tumor model	HPV16-SLP vaccination + cisplatin	A synergistic antitumor effect	Sensitize tumor cells to cisplatin-mediated death by TNFα produced by tumor-specific T-cells	Increased tumor cell apoptosis	Van der Sluis et al. [81]
	Concurrent with vaccine	Nivolumab	Resected stage IIIC and IV melanoma	Tumor antigen multi-peptide vaccine + nivolumab	Demonstrated immunologic activity with promising survival	Increased tumor-specific $CD8^+$ T-cells but also CTLA-4^+ $CD4^+$ and $CD25^+$ Tregs	Counteracting inhibitory immune regulation	Gibney et al. [82]
	Concurrent with vaccine	Anti-PD-1	B16 melanoma	IFNγ-inducing cancer vaccine combined with GM-CSF + TLR agonists + anti-PD-1	Complete tumor regression	Blocking the inhibitory effect of PD-1 induced by vaccine		Fu et al. [83]
	Concurrent with vaccine	Anti-PD-1	Advanced or metastatic HCC patients And RMA lymphoma tumor model	Peptide vaccine + anti-PD-1	Increased tumor specific CTL number and response Synergistic tumor outgrowth reduction	Blocking the PD-1 signaling induced by vaccine on specific CTL Increased TILs and decreased inhibitory receptor on TILs		Sawada et al. [84]
	Concurrent with vaccine	Anti PD-1	TC-1 HPV16/17 tumor model	Listeria monocytogenes based vaccine expressing HPV16 E7 + anti PD-1	Tumor outgrowth inhibition and prolonged survival	Increased Teff/Tregs ratio Increased tumor-specific T-cell response by blocking PD-1/PD-L1 pathway as PD-L1 induced by vaccine		Mkrtichyan et al. [85]
	Concurrent with vaccine	Anti-CTLA-4	Prostate tumor model	GM-CSF-secreting vaccine (GVAX) + anti-CTLA-4	Increased tumor-specific cells and lytic function	Blocking the inhibitory effect of CTLA-4 induced by vaccine on tumor specific $CD8^+$ T-cells		Wada et al. [86]
	Concurrent with vaccine	Anti-IL10R1 monoclonal antibody	Bladder tumor model	Bacillus Calmette-Guérin vaccine + anti-IL10R1 monoclonal antibody	Enhanced anti-bladder cancer immunity and prevention metastasis to lung	Increased CTL specific response	Counteracting suppressive immunity induced by tumor or immunotherapy	Newton et al. [87]
After	1 day after vaccine	Anti- 4–1BB	MCA205 and MCA207 fibrosarcomas	DC vaccine pulsed with tumor lysate + anti-4–1BB	Enhanced tumor regression and improve survival	Increased costimulatory signal of 4-1BB enhanced by vaccine on NK, CD4 and CD8 T-cells Increased T-cell response	Increased costimulatory signals to improve T-cell function	Ito et al. [88]
	2 days after vaccine	Anti-OX40 + anti-4-1-BB	Mammary carcinoma (N202.1A tumor cells in Her-2/neu mice)	DC vaccine pulsed with apoptotic tumor cells + anti-OX40 + anti-4-1-BB	Tumor reduction and rejection	Enhanced CD4 and CD8 T-cell response		Cuadros et al. [89]

(*Continued*)

Table1. (Continued).

Timing of the intervention		Intervention	Cancer type	Combined treatment regimen	Efficacy/immunological responses of combined treatments	Mechanisms of intervention		References
	1 day after vaccine	SM16 (TGFβ blockade)	TC-1 HPV16/17 tumor model	Adenovirus expressing HPV-E7 + SM16	Delayed the tumor outgrowth Increase intratumoral leukocyte infiltration Increase intratumoral antigen-specific $CD8^+$ T-cells	Increased immunostimulatory cytokines and ICAM-1 Increased the percentage and functional status of $CD8^+$ T-cells	Improvement of the T-cell response	Kim et al. [90]
	3–6 days after vaccine	Radiation	Colon adenocarcinoma tumor cells expressing CEA (MC38-CEA)	Recombinant vaccinia/avipox CEA-TRICOM vaccine + radiation	Significant Tumor eradication	Upregulation of Fas on tumor cells by radiation Increased infiltration of T-cells to tumor Induced tumor-specific T-cell response	Induced tumor cell apoptosis and improved T-cell response	Chakraborty et al. [91]
	5 days after vaccine	Radiation	HPV-associated head and neck squamous cell carcinoma	Shiga Toxin B-based HPV vaccine + radiation	Complete tumor clearance	Increased tumor-infiltrating antigen-specific T-cells Induced $CD8^+$ T-cell memory Enhanced intratumoral vascular permeability	Enhanced intratumoral vascular permeability and improved T-cell response	Mondini et al. [92]

ACT: adoptive cell transfer; CEA: carcinoembryonic antigen; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; CTL: cytotoxic T lymphocytes; CSF-1 R: colony stimulating factor-1 receptor; DC: dendritic cell; GM-CSF: granulocyte-macrophage colony-stimulating factor; gp100: glycoprotein 100; HPV: human papillomavirus; HLA: human leukocyte antigen; HER-2: human epidermal growth factor receptor 2; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; IL-2: interleukin-2; IFN-α: interferon-alpha; IFN-β: interferon beta; IFNγ: interferon gamma; IL-10R1: interleukin 10 receptor 1; MHC: major histocompatibility complex; MDSCs: myeloid-derived suppressor cells; NK: natural killer cells; PD-1: programmed cell death protein 1; PD-L1: programmed death-ligand 1; RNA: ribonucleic acid; SLP: synthetic long peptide; Tregs: regulatory T-cells; TAA: tumor-associated antigens; TDLN: tumor-draining lymph node; Th: T helper cells; TCR: T-cell receptor; TLR: Toll-like receptor; TILs: tumor-infiltrating lymphocytes; TNF-α: tumor necrosis factor alpha.

therapies should also be provided prior to administration of immunotherapy or at the time that immunotherapy induces tumor-specific immune responses [104,105]. For instance, in the CT26 tumor model the antibody mediated depletion of $CD25^+$ T-cells (including Tregs) before immunization with tumor antigen AH1 resulted in long-lasting memory T-cell responses and even augmented a tumor-induced $CD4^+$ T-cell response [56]. In another study, vaccination with AH1 tumor antigen in combination with the FOXP3-binding P60 peptide, which reduces the suppressive function of Tregs by preventing the nuclear translocation of FOXP3 and thus its ability to suppress the transcription factor NF-κB and NFAT, efficiently protected mice against CT26 tumor growth [106]. Likewise, antibody-mediated depletion of $CD4^+$ $CD25^+$ Tregs before vaccination with DCs loaded with tumor cells, that had been stressed by heat shock and irradiation, resulted in a delayed tumor outgrowth and long-lived tumor-protective immune responses [57]. Also depletion of $CD25^+$ Tregs prior to tumor cell-based vaccination and CTLA-4 blocking, enhanced the TRP-2-specific $CD8^+$ cytotoxic T lymphocyte (CTL) response in B16 melanoma tumor model [55]. Similarly , application of the CD25-blocking monoclonal antibody daclizumab to patients with metastatic breast cancer, 1 week before multiple injections with a vaccine (consisting of three peptides derived from human telomerase reverse transcriptase [hTERT], survivin, and pp65 of cytomegalovirus [CMV] as a control), resulted in prolonged Treg suppression and robust vaccine-induced IFNγ-producing T-cell responses [107]. However, Treg depletion by using anti-CD25 antibodies may also be performed at the same time with the administration of the vaccine [108]. The elimination of $CD4^+$ $CD25^+$ Tregs by using denileukin diftitox, a diphtheria toxin fragment conjugated to recombinant IL-2 (DAB_{389}IL-2; also known as ONTAK) that is rapidly internalized upon binding to the IL-2 receptor and then releases the apoptosis inducing toxin, significantly enhanced a tumor RNA-transfected DC vaccine-induced tumor-specific T-cell response in renal cell carcinoma (RCC) patients [60].

Small molecule inhibitors were also shown to decrease immune suppression [73]. In a murine colon carcinoma (MC38-CEA) sequential administration of sunitinib, a tyrosine kinase inhibitor, followed by a poxvirus-based vaccine encoding carcinoembryonic antigen (CEA) plus three costimulatory molecules (B7-1, ICAM-1 and LFA-3) resulted in decreased numbers of intratumoral Tregs and MDSCs as well as an increased influx of antigen-specific T-cells, with as consequence a better tumor control and prolonged survival [72]. Last but not least, low-dose total body irradiation therapy also reduced Tregs and increased effector-memory T-cell frequencies. Administration of a DC-gp100 tumor vaccine 11 days after low dose irradiation reduced tumor outgrowth and increased survival of melanoma-bearing mice [49]. Taken together, altering Treg function or depleting Tregs may improve tumor-specific immune responses and expand the efficacy of immunotherapy (reviewed in [105]).

As already eluded to in the above section, some modalities may also affect MDSC or M2 function and number [109]. However, others are specifically designed to impact on myeloid cells. For instance, the CSF-1 receptor (CSF-1R) is a key regulator for monocyte differentiation from progenitors of the bone marrow and for monocyte activation and migration. It has been shown that macrophages induction by CSF-1 could lead to polarization towards an immunosuppressive and tumor-promoting phenotype [110]. Blocking of CSF-1R signaling by using recombinant CSF-1 antibodies against the ligand and the receptor, or specific inhibitors of the CSF-1R kinase activity might be effective in combination with other immunotherapies. In the B16F10 mouse melanoma model, inhibition of CSF-1R (PLX3397 kinase) in combination with CD8 T cell-mediated immunotherapy, consisting of the transfusion of pmel-1 $CD8^+$ T-cells and peptide vaccination, could efficiently remove intratumoral $F4/80^+$ macrophages, increase IFNγ production of tumor-specific $CD8^+$ T-cells and delay tumor outgrowth [58]. In another study, PLX3397 given 4 days before adoptive T-cell therapy improved the efficacy of this immunotherapy in a syngeneic mouse model of BRAF (V600E)-driven melanoma by decreasing tumor-infiltrating myeloid cells, skewing macrophages towards $MHCII^{hi}$ type 1 macrophages (M1) and by increasing the number of IFNγ-producing tumor-infiltrating lymphocytes (TIL) [59]. Therefore, targeting myeloid cells either to prevent their recruitment to the tumor or to inhibit their pro-tumor polarization may foster immune control of tumor cells. Such a strategy, used as a standalone therapy or in combination with other immunotherapies has been shown successful to enhance antitumor immune responses [109,111].

3.1.3. Chemoradiation applied prior to vaccination can induce optimal T-cell responses

Chemotherapy not only acts through the alleviation of immune suppression, but can also have beneficial effects on the immune system through other mechanisms. For example, cyclophosphamide also can induce the infiltration of immune lymphocytes to the tumor as well as promote homeostatic proliferation/activation of B and T lymphocytes due to a cytokine storm (GM-CSF, IL-1B, IL-7, IL-15, IL-2, IL-21 and IFNα) after drug-induced lymphodepletion [71]. Moreover, many other immunomodulatory factors such as danger signals, pattern recognition receptors, and chemokines receptors are upregulated after cyclophosphamide treatment. These alterations may explain the improved antitumor immunity observed after cyclophosphamide treatment in those cases where an overt effect of cyclophosphamide on the levels and function of Tregs could not be detected [112,113]. The pharmacokinetic analysis of gene and protein expression and anti-tumor efficacy in different therapeutic regimens indicate that the optimal time point to apply adoptive immunotherapy is 1 day after cyclophosphamide treatment [65].

Above all, in cases where the treatment modalities stimulate the functionality or stability of tumor-specific $CD8^+$ T-cells, administration of immunotherapy following conventional therapy can improve the antitumor immune responses [63,67]. In an established 3LL lung tumor model, administration of docetaxel before but not after vaccination with a GM-CSF-producing tumor vaccine could significantly induce tumor regression and prolonged survival [63]. This is due to the docetaxel-associated enhanced survival of activated antigen-experienced T-cells induced by the vaccine over that of pre-existing memory $CD8^+$ T-cells and Tregs [63]. Similar results

have been observed in melanoma patients who received dacarbazine (DTIC) 1 day before tumor–antigen vaccination [68]. Dacarbazine induces activation of genes involved in cytokine production, leukocyte activation, immune response and cell motility, thereby enhancing $CD8^+$ memory T-cell response [68]. It also broadens the TCR repertoire used and this is accompanied by high T-cell avidity and tumor reactivity [69]. Furthermore, in a HPV-induced tumor model, vaccination with HPV16 E6E7L2 fusion protein (TA-CIN) with GPI-0100 adjuvant 1 day after administration of cisplatin reduced tumor outgrowth and extended the survival of TC-1 tumor-bearing mice compared to vaccination alone [70]. In similar studies, cisplatin treatment of mice 4–7 days before vaccination with a DNA vaccine encoding calreticulin linked to HPV16 E7 antigen or with a vaccinia virus vaccine encoding this E7 antigen significantly increased the survival of TC-1 tumor-bearing mice [74,75]. Interestingly, this effect has only been observed when cisplatin was given before and not after the vaccination, emphasizing the importance of timing of the given therapies [74]. The combination induced significantly higher frequencies of E7-specific $CD8^+$ T-cells, both in blood and tumor, compared to each treatment alone. Reason for this is that cisplatin on one hand increases the expression of MHC class I on tumor cells, thereby enhancing their susceptibility to be lysed by specific CTLs and on the other hand, increases the influx of intratumoral $CD11c^+$ while decreasing the number of myeloid suppressor cells and Tregs in blood and spleen [74,75]. Similarly, gemcitabine could also increase the percentage of circulating monocytes, M1, and DCs [52,114]. Administration of this chemotherapy alone or in poly-chemotherapy regimens before immunotherapy may also enhance the clinical efficacy through these mechanisms [62,114,115].

Similar observations have been made with low-dose radiation 2 days before vaccination, although the highest frequency of E7-specific $CD8^+$ T-cells in tumor and spleen was reached when radiation was given in combination with a second DNA vaccination [64]. Moreover, in a phase I clinical study two courses of cisplatin and 5-FU concurrent with radiotherapy and before vaccination with a multiple epitope peptide vaccine increased the vaccine-specific CTL response in patients with unresectable chemo-naïve esophageal squamous cell carcinoma [61]. Thus, both chemotherapy and radiotherapy or their combination (chemoradiation) given prior to vaccination can mediate immune stimulating and clinical effects.

In conclusion, there are a number of modalities that are able to relief immune suppression as well as to condition the immune system to optimally respond to immunotherapy. Therefore, immunotherapy properly timed after these treatments provides a platform to increase the clinical response of patients to immunotherapy.

3.2. Concurrent combination of therapeutic modalities with vaccination

3.2.1. Simultaneous combination of chemotherapy with vaccination increases vaccine efficacy

Given the immune stimulatory capacity of certain chemotherapeutic agents, administration of these cytotoxic drugs not only before but also during vaccination can result in improved antitumor responses. Concurrent administration of metronomic doses of cyclophosphamide in combination with an HLA class I-binding survivin peptide vaccine (DPX-Survivac) increased the antigen-specific immune response. This was associated with the differentiation of naïve T-cells into central/effector memory cells (CM/EM) and late differentiated poly-functional antigen-specific $CD8^+$ T-cells in advanced ovarian cancer patients [76]. Similarly, metronomic administration of a high dose cocktail of chemotherapeutic agents (including cyclophosphamide, paclitaxel and docetaxel) during vaccination with a multi-peptide cocktail of peptides (comprising hepatitis C virus-derived antigens and tumor-associated antigens) resulted in an enhanced specific T-cell response and a reduced Treg frequency in hepatocellular carcinoma (HCC) patients [79]. Coadministration of gemcitabine with DC-based vaccines in a pancreatic carcinoma model showed a synergistic antitumor effect [116]. Treatment of patients with ovarian cancer with a p53 SLP vaccine in combination with pegintron (pegylated IFNα) 7 days before the first cycle of gemcitabine and repeated at day 22, during gemcitabine treatment, resulted in a strong p53-specific T-cell response. In this study gemcitabine treatment was associated with reduced levels of MDSCs, and increased percentages of M1 macrophages, circulating proliferating $CD4^+$ and $CD8^+$ T-cells but not Tregs [80].

In addition, there are some reports suggesting that immunotherapy may provide the correct environment for chemotherapy to become more effective. In a poly-chemotherapy regimen, administration of cyclophosphamide or paclitaxel 1 day before and doxorubicin 7 days after vaccination with GM-CSF-secreting tumor cells, enhanced the antitumor efficacy of these chemotherapeutics in HER-2/neu tolerized mice. Doxorubicin was provided to augment the vaccine-induced antitumor efficacy but the mechanism was unclear [77]. Potentially, immunotherapy operates via increasing the number of cytokine-producing tumor-specific T-cells thereby sensitizing tumor cells for chemotherapy-induced cell death. In a HPV16 preclinical tumor model, vaccination with HPV16 E6 and E7 SLPs combined with cisplatin displayed a synergistic antitumor effect [81]. The vaccination resulted in increased numbers of intratumoral IFNγ and TNFα producing $CD8^+$ T-cells. The TNFα produced by these HPV-specific $CD8^+$ T-cells not only had a direct effect on tumor cell proliferation but also increased the sensitivity of the tumor cells to cisplatin-induced tumor cell death in a JNK-dependent fashion [81]. Thus, even at this phase there is a place for chemotherapy to improve vaccine-induced antitumor immunity.

3.2.2. Concurrent combination of immunomodulatory antibodies with vaccination to curtail inhibitory immune regulation

Another reasonable strategy to improve the efficacy and maintenance of tumor-specific T-cell responses is by counteracting immune regulation that acts when effector cells start to express coinhibitory molecules as a normal response to activation. Occasionally immunotherapy can alter the

tumor microenvironment or the expression of regulatory molecules in a way that is not favorable for the therapeutic outcome. Therefore, the appropriate combination of treatments and the correct timing of each treatment modality can be crucial for the final outcome. The demonstration of high numbers of PD-1 and PD-L1 expressing $CD8^+$ T-cells at the invasive tumor margin and inside tumors of patients with metastatic melanoma, suggested the application of anti-PD-1 therapy in melanoma patients [117]. Indeed, treatment with a multi-peptide tumor-epitope vaccine and PD-1 antibody (nivolumab) was well tolerated in patients with resected high-risk metastatic melanoma. Furthermore, immunological activity, such as increased antigen-specific $CD8^+$ T-cells but also increased frequencies of $CD25^+$ or CTLA-4^+ $CD4^+$ T-cells, was associated with promising survival results [82]. Preclinical evidence has shown that TEGVAX vaccine, which is an IFNγ-inducing cancer vaccine combined with GM-CSF and TLR agonists, is associated with DC activation and an increase in the number of tumor-specific IFNγ-producing tumor-infiltrating T-cells [83]. However, as a consequence of this influx, cells in the tumor microenvironment upregulate PD-L1 resulting in incomplete tumor cell elimination. Furthermore, analysis of PBMCs following administration of a glypcian-3 (GPC3) peptide vaccine in advanced or metastatic HCC revealed a high expression of PD-1 on peptide-specific $CD8^+$ T-cells [84]. Similarly, immunotherapy using Listeria monocytogenes (Lm)-LLO can be improved by reduction of the Tregs and MDSCs, but as it is also associated with the upregulation of PD-L1 on immune cells, in particular macrophages, the use of antibodies blocking the PD-1:PD-L1 axis is justified [85]. Indeed, administration of the PD-1 blocking antibody in combination with the cancer vaccine caused increased tumor regression. Another example is the combined use of a cell-based GM-CSF-secreting vaccine (GVAX) with CTLA-4 blocking antibody in an autochronous prostate cancer model (Pro-TRAMP), which enhanced the tumor antigen-specific $CD4^+$ and $CD8^+$ effector T-cells to Treg ratio and the tumor-antigen directed lytic function [86]. Interestingly, the timing of administration of the CTLA-4 blocking antibody greatly influenced the outcome as the maximum effect has been observed when the blocking antibody was applied after, but not before the vaccination. The mechanism behind this crucially timed application of the blocking antibody is that the GVAX vaccine not only increases tumor-specific $CD8^+$ T-cells but also upregulates the CTLA-4 expression on these effector cells, thus by blocking this inhibitory molecule and its pathway the effector function is not abrogated. Interestingly, in patients with advanced small-cell lung cancer similar results have been observed [118]. Administration of four doses of ipilimumab after two doses of paclitaxel/carboplatin improved the immune-related progression-free survival and overall survival of patients compared to concurrent administration of ipilimumab and paclitaxel/carboplatin [118]. However, the detailed mechanisms still need to be investigated. Thus, vaccination followed by specific treatments to release the brakes on T-cells, instigated by coinhibitory receptors, may form a powerful strategy to improve tumor-specific T-cell efficacy.

In addition to coinhibitory receptor regulation, one might also provide antibodies to known immune regulatory cytokines or other (soluble) factors, which potentially can hamper the induction of or weaken the antitumor immune response. Among them are known tumor-derived factors such as VEGF, transforming growth factor-β (TGFβ), prostaglandin E_2 (PGE_2), IL-6, IL-10 and indoleamine-pyrrole 2,3-dioxygenase (IDO). For instance, the combination of the standard therapy- Bacillus Calmette-Guérin (BCG)- with a blocking anti-IL-10 receptor 1 monoclonal antibody (anti-IL10R1) resulted in enhanced antitumor immunity by inducing Th1 immune response in bladder cancer mouse model [87]. Therefore, these strategies to dampen inhibitory immune regulation can be applied in combination or even after vaccination as will be described further in this review.

3.3. Administration of therapeutic modalities after vaccination

3.3.1. Combination of vaccines and immunomodulatory antibodies enhances the antitumor response

To enhance antitumor immunity induced by vaccination, other treatments such as agonistic antibodies to costimulatory receptors on T-cells, the provision of cytokines and the stimulation of antigen-presenting cells (APCs) by toll-like receptor (TLR) agonists can play a major role as cotreatments. In these cases the correct timing depends on the mechanism of action of the immunomodulatory compound used, but most likely they will be given in combination or after vaccination. Indeed, immunization with vaccines that stimulate a broad immune response and transfer of costimulatory agonist antibodies potentially improves the antitumor response by enhancing the T-cell response. Using DCs pulsed with apoptotic tumor cells, which stimulate a broad immune response in Her-2/neu mice, in combination with agonist anti-OX40 (CD134) or anti-4-1BB (CD137) monoclonal antibodies showed substantial tumor size reduction [89]. Mechanistically, administration of these agonistic antibodies given 2 days after DC immunization could improve the induced T-cell response as the effect of this combined treatment is abrogated in the absence of $CD4^+$ and $CD8^+$ T-cells. Likewise, combination of agonistic anti-4-1BB one day after vaccination with tumor lysate-pulsed DCs following another administration after 3 days enhanced tumor regression in established pulmonary and subcutaneous tumor models [88]. The rationale behind this therapeutic strategy is that tumor lysate-pulsed DC vaccines induce transient upregulation of 4-1BB on T-cells and NK cells in vaccine-primed lymph nodes. Therefore, treatment with agonistic 4-1BB antibody could polarize effector T-cells towards a type 1 (IFNγ) response to tumor antigen in a $CD4^+$, $CD8^+$ and NK cell dependent manner. In a similar fashion, treatment with tumor lysate-pulsed DCs upregulate CD27 on T-cells in RM-1 prostate cancer tumor model and administration of CD27 agonistic antibodies after vaccination with these DCs could reduce tumor outgrowth [78]. Thus, administration of immunomodulatory compounds which stimulate DC or T-cell function, either by providing cytokines or costimulatory signals, following vaccination strategies is a convenient way to promote the vaccine-induced antitumor efficacy.

3.3.2. Postvaccination interventions to dampen the immune regulatory response

Intervention drugs to abrogate the immune suppressive effect of regulatory cytokines or molecules can be applied not only in combination with vaccination but also after immunotherapy. For instance, systemic blocking of TGFβ signaling after vaccination with adenovirus expressing HPV-E7, or prior to immunotherapy by adenovirus expressing IFN-β, increased the antitumor efficacy compared to each treatment alone [90]. TGFβ receptor blockade enhanced the intratumoral production of immunostimulatory cytokines and chemokines as well as the expression of ICAM-1 and led to the increased infiltration of antigen-specific and functional $CD8^+$ T-cells.

Another example is IDO, which catalyzes essential amino acids such as tryptophan that are required for proliferation and activation of immune cells, in particular T-cells. IDO is overexpressed in tumor cells and tumor-associated APCs. To improve the capacity of a DC-based vaccine, the IDO inhibitor 1-MT was administered after vaccination in a prophylactic Lewis lung carcinoma murine model. Interestingly, IDO was not expressed by tumor cells but only by the myeloid cells within the tumor, tumor draining lymph nodes and spleen. Moreover, a combination of the IDO inhibitor with other modalities such as chemotherapy or blockade of coinhibitory molecules, was shown to potentiate the antitumor efficacy of therapy [119,120].

Postvaccination radiation may also aid by interfering with immune regulating mechanisms. In a mouse colon adenocarcinoma tumor model of CEA, vaccination in a prime-boost strategy with vaccinia and avipox recombinants, expressing CEA and a triad of T-cell costimulatory molecules, in combination with local tumor irradiation induced synergistic antitumor effects [91]. Radiation 3 days after vaccination induced upregulation of the death receptor Fas on tumor cells, leading to Fas/Fas ligand pathway mediated cell death. Therefore, the combined treatment led to increased infiltration of T-cells and $CD4^+$ and $CD8^+$ T-cell responses not only specific for CEA but also for other overexpressed antigens in tumor. Similarly, local radiation 5 days after vaccination with Shiga Toxin B-based HPV vaccine in HPV-associated head and neck squamous cell carcinoma induced synergistic tumor eradication in mice by normalizing the tumor vasculature and thus improving tumor infiltration by immune cells [92]. Thus, to improve the vaccine-induced antitumor response one should also alleviate immune regulatory mechanism that act after the activation of tumor-specific T-cells.

4. Immunoguiding is important for the development of immunotherapeutic strategies

The increasing reports on the immune modulatory properties of chemotherapy and radiotherapy, the debate on the exact working mechanism of CTLA-4-blocking antibodies [121–123] and the unexpected outcomes of myeloid cell depletion by CSF1R inhibition during immunotherapy [58,124] exemplify the need for immunomonitoring studies that analyze the effect of drugs on the immune system, and in particular beyond the expected mechanisms of action. This immunological knowledge then can be used to guide the rational design of combination therapies, which in the past were more based on empirical testing [23,95]. It is well known that there are many immunological differences between all the different animal models used – and sometimes even between what used to be the same tumor cell lines – to develop immunotherapy. Moreover, the number of analyses, required to study the many permutations possible, is too high for single consortia to address. Therefore, an important aspect is to ensure that the measurement and reporting of the results obtained are comparable between laboratories so that it is easier to interpret and value the data generated. In the last decade a huge effort, undertaken by the Association of Cancer ImmunoTherapy's (CIMT's) immunoguiding program [125] and Cancer Immunotherapy Consortium (CIC) [126], has focused on this in the realm of human studies, and this has successfully led to: (i) a strong awareness of the need to harmonize or standardize measurements, (ii) to technical improvements and harmonization of the assays used, and (iii) to more transparent reporting [127–129]. It can be envisaged that similar efforts by scientists performing studies in mouse tumor models will lead to a quicker design of immunotherapeutic strategies, which expedite the translation to the treatment of patients.

5. Conclusions

In conclusion, the field is moving rapidly towards understanding the requirements for optimal immunotherapy of cancer. Combinations of treatment modalities are designed and tested to accommodate the most effective tumor-specific immune response. Surgery, chemotherapeutic compounds, radiotherapy schedules, antibodies and targeted therapies have successfully been administrated before applying immunotherapy with the aim to reduce immunosuppressive cells, or during and after immunotherapy to potentiate the immune response and to prevent immune regulation. A full understanding of the immunological effects of the compounds used, their pharmacological dynamics, and the optimal sequence during treatment will result in clinical benefit for the majority of patients.

6. Expert opinion

Cancer may be cured by immunotherapy but the development of cancer is associated with increased immune suppression. Furthermore, the induction of tumor-specific immunity is difficult and regulated afterwards. Hence, for an optimal antitumor response it is important to provide the most appropriate therapeutic modality at the right time. The different schedules and various modalities, and combinations thereof, have been discussed in this review and are recapitulated in Figure 1. In our opinion, the prevention of tumor development in high-risk patients, treatment of premalignant lesions or prevention of recurrences after curative treatments, is the ideal time period to stop cancer and vaccines are ideal to induce protective tumor-specific immunity. However, in general practice immunotherapy is applied to cancer patients with more advanced stage of disease, and as such its success is determined by the level of immunosuppression

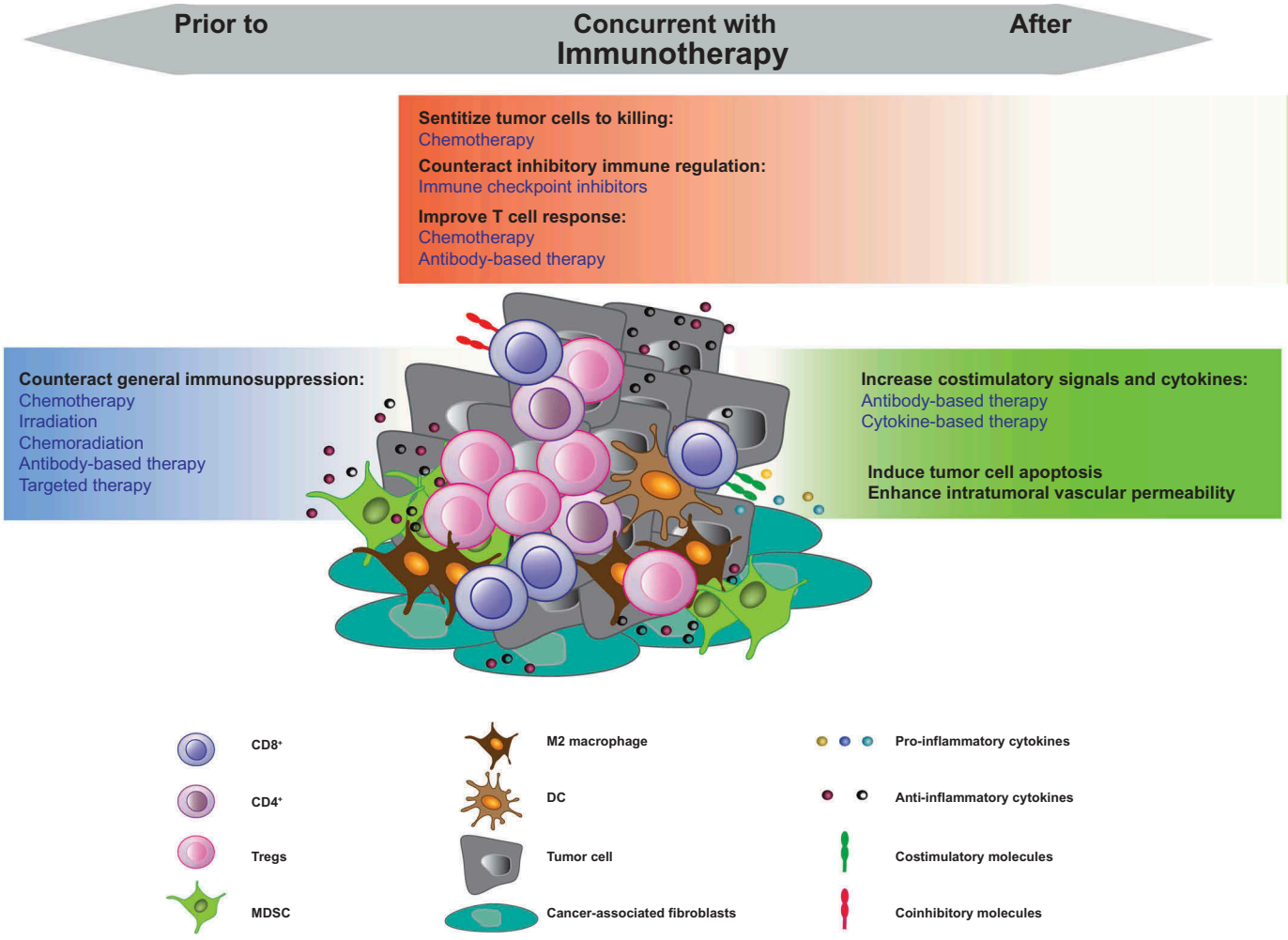

Figure 1. Illustration of different timing schedules of various therapies in combination with immunotherapy based on their mechanisms. The individual examples of each combination therapy are summarized in Table 1. The colour gradients show the time points (prior, concurrent and/or after immunotherapy) at which the therapies are mostly applied. The blue box shows the therapies which are mostly applied prior to immunotherapy and less often concurrent with immunotherapy. The orange box shows the therapies which are often applied concurrent with immunotherapy and less after immunotherapy. The green box shows the therapies which are used mainly after immunotherapy and less concurrent with immunotherapy. Abbreviations: Tregs: Regulatory T-cells, MDSC: Myeloid-derived suppressor cells, M2 macrophages: type 2 macrophages, DC: dendritic cell.

encountered in microenvironment. The treatment of premalignant lesions [3,33], but certainly that of patients with cancer, demonstrates that the increase in general and local immune suppression requires additional modalities to curtail these suppressive mechanisms and to optimally activate and expand tumor-specific T-cells. At this phase a correct sequence and interval of drugs is essential, as it will be required to use prime combinations of several modalities to obtain clinical success in the majority of patients. Based on the observations in mouse tumor models and in patients it is clear that myeloid suppressor cells and Tregs stifle the induction of a strong and effective antitumor response and that alleviation of this immune suppression is required before the activation of the antitumor T-cell response. In addition, extra support is needed to ensure the strong expansion and appropriate effector function of the tumor-specific T-cells. Finally, as the activated effector T-cells will start to express coinhibitory molecules, the ligands of which can be expressed at the tumor site or will be as a consequence of T-cell produced IFNγ, there is a need to counteract immune regulation at this point during therapy. As described in this review, for each of the stages (before, during and after vaccination) help can be provided via either surgical tumor reduction, chemotherapy, radiation therapy and/or a number of other immune modulators. However, before such combinations are designed it will be essential to establish the dynamics and exact immune modulatory properties of the agents used. Chemotherapeutic agents may affect different immune cell populations and this effect may be transient [48]. While its seems logical to understand the dynamics and immune modulating properties of chemotherapeutic compounds, this is also required for other compounds such as targeted therapy of macrophages [58,124] and antibodies targeting costimulatory and coinhibitory molecules. This is well illustrated by different timing schedules of anti-CTLA-4 and anti-OX40 in a murine colorectal carcinoma model in combination with radiation therapy [130]. Antibodies against CTLA-4 and OX40 can only improve the survival of tumor bearing mice when applied before and after radiation therapy, respectively. Therefore, not only the mechanism of each immunotherapeutic modality but also the

sequence of administration should be tested and considered in the design of the most optimal combined-therapeutic strategy. The drawbacks of this approach of course are the costs and the time needed to perform these interrogations with as result that such well-designed studies are scarce, but they are coming. Immunomonitoring is an extremely important aspect in understanding these matters. It should be applied in the broadest sense, certainly not restricted to the expected mechanism of action, and also take into account (unexpected) negative effects of the treatment. As many of the studies will be performed (only) in mouse models it becomes important that laboratories using mouse models will focus on increasing the comparability of immunomonitoring and reporting of results, similar to current efforts in the monitoring of human immune responses. Notably, one should be aware that while we can learn a lot from murine experiments, they still not fully reflect the human situation. Therefore, effort should be undertaken to properly investigate the sequence and timing of the most promising combinations in clinical studies. Fortunately, the field is moving forward towards rationally designed combination therapy and a better understanding of the patient population that is to be treated. Finally, we envisage that the ideal combination aims to: (1) increase the number of tumor-specific T-cells; (2) prevent their subsequent regulation in the tumor bed; (3) decrease the number of immune suppressive myeloid cells and Tregs; and (4) activate intratumoral antigen presenting cells. In theory, this should lead to major improvements in clinical outcome, well above the levels that is currently reached.

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References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Melief CJ, van Hall T, Arens R, et al. Therapeutic cancer vaccines. J Clin Invest. 2015;125:3401–3412. DOI:10.1172/JCI80009
2. van der Burg SH, Arens R, Ossendorp F, et al. Vaccines for established cancer: overcoming the challenges posed by immune evasion. Nat Rev Cancer. 2016;16:219–233. DOI:10.1038/nrc.2016.16.
•• **This is a comprehensive review about cancer immune evasion strategies that needs to be considered to design a therapeutic vaccine for established cancer**
3. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med. 2009;361:1838–1847. DOI:10.1056/NEJMoa0810097
4. van Poelgeest MI, Welters MJ, van Esch EM, et al. HPV16 synthetic long peptide (HPV16-SLP) vaccination therapy of patients with advanced or recurrent HPV16-induced gynecological carcinoma, a phase II trial. J Transl Med. 2013;11:88. DOI:10.1186/1479-5876-11-88
5. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med. 2010;363:411–422. DOI:10.1056/NEJMoa1001294
6. Rosenberg SA, Yang JC, Sherry RM, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res. 2011;17:4550–4557. DOI:10.1158/1078-0432.CCR-11-0116
7. Verdegaal EM. Adoptive cell therapy: a highly successful individualized therapy for melanoma with great potential for other malignancies. Curr Opin Immunol. 2016;39:90–95. DOI:10.1016/j.coi.2016.01.004
8. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363:711–723. DOI:10.1056/NEJMoa1003466
9. Ribas A, Kefford R, Marshall MA, et al. Phase III randomized clinical trial comparing tremelimumab with standard-of-care chemotherapy in patients with advanced melanoma. J Clin Oncol. 2013;31:616–622. DOI:10.1200/JCO.2012.44.6112
10. Homet MB, Parisi G, Robert L, et al. Anti-PD-1 therapy in melanoma. Semin Oncol. 2015;42:466–473. DOI:10.1053/j.seminoncol.2015.02.008
11. Robert C, Schachter J, Long GV, et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. N Engl J Med. 2015;372:2521–2532. DOI:10.1056/NEJMoa1503093
12. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366:2443–2454. DOI:10.1056/NEJMoa1200690
13. Wolchok JD, Kluger H, Callahan MK, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med. 2013;369:122–133. DOI:10.1056/NEJMoa1302369
14. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012;366:2455–2465. DOI:10.1056/NEJMoa1200694
15. Forde PM, Reiss KA, Zeidan AM, et al. What lies within: novel strategies in immunotherapy for non-small cell lung cancer. Oncologist. 2013;18:1203–1213. DOI:10.1634/theoncologist.2013-0171
16. Garon EB. Current Perspectives in Immunotherapy for Non-Small Cell Lung Cancer. Semin Oncol. 2015;42(Suppl 2):S11–S8. DOI:10.1053/j.seminoncol.2015.09.019
17. Lipson EJ, Forde PM, Hammers HJ, et al. Antagonists of PD-1 and PD-L1 in Cancer Treatment. Semin Oncol. 2015;42:587–600. DOI:10.1053/j.seminoncol.2015.05.013
18. Hughes PE, Caenepeel S, Wu LC. Targeted Therapy and Checkpoint Immunotherapy Combinations for the Treatment of Cancer. Trends Immunol. 2016;37:462–476. DOI:10.1016/j.it.2016.04.010
19. Chiappinelli KB, Zahnow CA, Ahuja N, et al. Combining Epigenetic and Immunotherapy to Combat Cancer. Cancer Res. 2016;76:1683–1689. DOI:10.1158/0008-5472.CAN-15-2125
20. Saleh MH, Wang L, Goldberg MS. Improving cancer immunotherapy with DNA methyltransferase inhibitors. Cancer Immunol Immunother. 2016;65:787–796. DOI:10.1007/s00262-015-1776-3
21. Arina A, Corrales L, Bronte V. Enhancing T-cell therapy by overcoming the immunosuppressive tumor microenvironment. Semin Immunol. 2016;28:54–63. DOI:10.1016/j.smim.2016.01.002
22. Wolchok JD, Hoos A, O'Day S, et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. Clin Cancer Res. 2009;15:7412–7420. DOI:10.1158/1078-0432.CCR-09-1624
23. van der Burg SH. Therapeutic vaccines in cancer: moving from immunomonitoring to immunoguiding. Expert Rev Vaccines. 2008;7:1–5. DOI:10.1586/14760584.7.1.1

24. Berraondo P, Nouze C, Preville X, et al. Eradication of large tumors in mice by a tritherapy targeting the innate, adaptive, and regulatory components of the immune system. Cancer Res. 2007;67:8847–8855. DOI:10.1158/0008-5472.CAN-07-0321
25. Klug F, Prakash H, Huber PE, et al. Low-dose irradiation programs macrophage differentiation to an iNOS(+)/M1 phenotype that orchestrates effective T-cell immunotherapy. Cancer Cell. 2013;24:589–602. DOI:10.1016/j.ccr.2013.09.014
26. Motz GT, Santoro SP, Wang LP, et al. Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. Nat Med. 2014;20:607–615. DOI:10.1038/nm.3541
27. Samant RS, Shevde LA. Recent advances in anti-angiogenic therapy of cancer. Oncotarget. 2011;2:122–134. DOI:10.18632/oncotarget.234
28. Fox BA, Schendel DJ, Butterfield LH, et al. Defining the critical hurdles in cancer immunotherapy. J Transl Med. 2011;9:214. DOI:10.1186/1479-5876-9-214
29. Sharma SH, Thulasingam S, Nagarajan S. Chemopreventive agents targeting tumor microenvironment. Life Sci. 2016;145:74–84. DOI:10.1016/j.lfs.2015.12.016
30. Mills CD, Lenz LL, Harris RA. A breakthrough: macrophage-directed cancer immunotherapy. Cancer Res. 2016;76:513–516. DOI:10.1158/0008-5472.CAN-15-1737
31. Panni RZ, Linehan DC, DeNardo DG. Targeting tumor-infiltrating macrophages to combat cancer. Immunotherapy. 2013;5:1075–1087. DOI:10.2217/imt.13.102
32. Weigert A, Sekar D, Brune B. Tumor-associated macrophages as targets for tumor immunotherapy. Immunotherapy. 2009;1:83–95. DOI:10.2217/1750743X.1.1.83
33. van Poelgeest MI, Welters MJ, Vermeij R, et al. Vaccination against oncoproteins of HPV16 for non-invasive vulvar/vaginal lesions: lesion clearance is related to the strength of the T-cell response. Clin Cancer Res. 2016. DOI:10.1158/1078-0432.CCR-15-2594
    - **Together with reference 35, these studies show clinical efficacy of vaccine in premalignant phase.**
34. Daayana S, Elkord E, Winters U, et al. Phase II trial of imiquimod and HPV therapeutic vaccination in patients with vulval intraepithelial neoplasia. Br J Cancer. 2010;102:1129–1136. DOI:10.1038/sj.bjc.6605611
35. Trimble CL, Morrow MP, Kraynyak KA, et al. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. Lancet. 2015;386:2078–2088. DOI:10.1016/S0140-6736(15)00239-1.
    - **Together with reference 33, these studies show clinical efficacy of vaccine in premalignant phase**
36. Czerniecki BJ, Koski GK, Koldovsky U, et al. Targeting HER-2/neu in early breast cancer development using dendritic cells with staged interleukin-12 burst secretion. Cancer Res. 2007;67:1842–1852. DOI:10.1158/0008-5472.CAN-06-4038
37. Morse MA, Niedzwiecki D, Marshall JL, et al. A randomized phase II study of immunization with dendritic cells modified with poxvectors encoding CEA and MUC1 compared with the same poxvectors plus GM-CSF for resected metastatic colorectal cancer. Ann Surg. 2013;258:879–886. DOI:10.1097/SLA.0b013e318292919e
38. Holmes JP, Gates JD, Benavides LC, et al. Optimal dose and schedule of an HER-2/neu (E75) peptide vaccine to prevent breast cancer recurrence: from US Military Cancer Institute Clinical Trials Group Study I-01 and I-02. Cancer. 2008;113:1666–1675. DOI:10.1002/cncr.23772
    - **Together with reference 40, these studies show that the clinical efficacy of vaccination in adjuvant setting varies between studies.**
39. Cuppens K, Vansteenkiste J. Vaccination therapy for non-small-cell lung cancer. Curr Opin Oncol. 2014;26:165–170. DOI:10.1097/CCO.0000000000000052
40. Vansteenkiste JF, Cho BC, Vanakesa T, et al. Efficacy of the MAGE-A3 cancer immunotherapeutic as adjuvant therapy in patients with resected MAGE-A3-positive non-small-cell lung cancer (MAGRIT): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2016;17:822–835. DOI:10.1016/S1470-2045(16)00099-1
    - **Together with reference 38, these studies show that the clinical efficacy of vaccination in adjuvant setting varies between studies.**
41. Chang MH. Hepatitis B virus and cancer prevention. Recent Results Cancer Res. Fortschritte der Krebsforschung Progres dans les recherches sur le cancer. 2011;188:75–84. DOI:10.1007/978-3-642-10858-7_6
42. Chow EP, Danielewski JA, Fehler G, et al. Human papillomavirus in young women with Chlamydia trachomatis infection 7 years after the Australian human papillomavirus vaccination programme: a cross-sectional study. Lancet Infect Dis. 2015;15:1314–1323. DOI:10.1016/S1473-3099(15)00055-9
43. Saletta F, Dalla PL, Byrne JA. Genetic causes of cancer predisposition in children and adolescents. Transl Pediatr. 2015;4:67–75. DOI:10.3978/j.issn.2224-4336.2015.04.08
44. Finn OJ, Beatty PL. Cancer immunoprevention. Curr Opin Immunol. 2016;39:52–58. DOI:10.1016/j.coi.2016.01.002
45. Finn OJ, Khleif SN, Herberman RB. The FDA guidance on therapeutic cancer vaccines: the need for revision to include preventive cancer vaccines or for a new guidance dedicated to them. Cancer Prev Res (Phila). 2015;8:1011–1016. DOI:10.1158/1940-6207.CAPR-15-0234
46. Antonia SJ, Mirza N, Fricke I, et al. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. Clin Cancer Res. 2006;12:878–887. DOI:10.1158/1078-0432.CCR-05-2013
47. Wu X, Feng QM, Wang Y, et al. The immunologic aspects in advanced ovarian cancer patients treated with paclitaxel and carboplatin chemotherapy. Cancer Immunol Immunother. 2010;59:279–291. DOI:10.1007/s00262-009-0749-9
48. Welters MJ, van der Sluis TC, van Meir H, et al. Vaccination during myeloid cell depletion by cancer chemotherapy fosters robust T-cell responses. Sci Transl Med. 2016;8:334ra52. DOI:10.1126/scitranslmed.aaf0746
    - **In this study, the vaccination has been given at a specific time point during chemotherapy based on preclinical immunomonitoring of chemotherapeutic impact on immune system.**
49. Liu R, Xiong S, Zhang L, et al. Enhancement of antitumor immunity by low-dose total body irradiationis associated with selectively decreasing the proportion and number of T regulatory cells. Cell Mol Immunol. 2010;7:157–162. DOI:10.1038/cmi.2009.117
50. Camisaschi C, Filipazzi P, Tazzari M, et al. Effects of cyclophosphamide and IL-2 on regulatory CD4+ T-cell frequency and function in melanoma patients vaccinated with HLA-class I peptides: impact on the antigen-specific T-cell response. Cancer Immunol Immunother. 2013;62:897–908. DOI:10.1007/s00262-013-1397-7.
    - **Together with references 57 and 58, it was shown that the dose and timing of cyclophosphamide administration prior to peptide vaccination to affect regulatory T-cells determines the vaccine-induced immunity and clinical outcome**
51. Murahashi M, Hijikata Y, Yamada K, et al. Phase I clinical trial of a five-peptide cancer vaccine combined with cyclophosphamide in advanced solid tumors. Clin Immunol. 2016. DOI:10.1016/j.clim.2016.03.015.
    - **Together with references 56 and 58, it was shown that the dose and timing of cyclophosphamide administration prior to peptide vaccination to affect Tregs determines the vaccine-induced immunity and clinical outcome**
52. Walter S, Weinschenk T, Stenzl A, et al. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med. 2012;18:1254–1261. DOI:10.1038/nm.2883.
    - **Together with references 56 and 57, it was shown that the dose and timing of cyclophosphamide administration prior to peptide vaccination to affect Tregs determines the vaccine-induced immunity and clinical outcome**
53. Suzuki E, Kapoor V, Jassar AS, et al. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-

bearing animals and enhances antitumor immune activity. Clin Cancer Res. 2005;11:6713–6721. DOI:10.1158/1078-0432.CCR-05-0883

54. Rettig L, Seidenberg S, Parvanova I, et al. Gemcitabine depletes regulatory T-cells in human and mice and enhances triggering of vaccine-specific cytotoxic T-cells. Int J Cancer. 2011;129:832–838. DOI:10.1002/ijc.25756
55. Sutmuller RP, van Duivenvoorde LM, van Elsas A, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T-cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med. 2001;194:823–832.
56. Casares N, Arribillaga L, Sarobe P, et al. CD4+/CD25+ regulatory cells inhibit activation of tumor-primed CD4+ T-cells with IFN-gamma-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. J Immunol. 2003;171:5931–5939.
57. Prasad SJ, Farrand KJ, Matthews SA, et al. Dendritic cells loaded with stressed tumor cells elicit long-lasting protective tumor immunity in mice depleted of CD4+CD25+ regulatory T-cells. J Immunol. 2005;174:90–98.
58. Sluijter M, van der Sluis TC, van der Velden PA, et al. Inhibition of CSF-1R supports T-cell mediated melanoma therapy. PLoS One. 2014;9:e104230. DOI:10.1371/journal.pone.0104230
59. Mok S, Koya RC, Tsui C, et al. Inhibition of CSF-1 receptor improves the antitumor efficacy of adoptive cell transfer immunotherapy. Cancer Res. 2014;74:153–161. DOI:10.1158/0008-5472.CAN-13-1816
60. Dannull J, Su Z, Rizzieri D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T-cells. J Clin Invest. 2005;115:3623–3633. DOI:10.1172/JCI25947
    - **This report demonstrates that proper depletion of regulatory T-cells prior to vaccination significantly improved the efficacy of the vaccine in renal cell cancer patients.**
61. Iinuma H, Fukushima R, Inaba T, et al. Phase I clinical study of multiple epitope peptide vaccine combined with chemoradiation therapy in esophageal cancer patients. J Transl Med. 2014;12:84. DOI:10.1186/1479-5876-12-84
62. Hirooka Y, Itoh A, Kawashima H, et al. A combination therapy of gemcitabine with immunotherapy for patients with inoperable locally advanced pancreatic cancer. Pancreas. 2009;38:e69–e74. DOI:10.1097/MPA.0b013e318197a9e3.
    - •• **Together with references 61 and 97, these studies reveal different mechanisms of gemcitabine all resulting in enhanced efficacy of the immunotherapy**
63. Chu Y, Wang LX, Yang G, et al. Efficacy of GM-CSF-producing tumor vaccine after docetaxel chemotherapy in mice bearing established Lewis lung carcinoma. J Immunother. 2006;29:367–380. DOI:10.1097/01.cji.0000199198.43587.ba
64. Tseng CW, Trimble C, Zeng Q, et al. Low-dose radiation enhances therapeutic HPV DNA vaccination in tumor-bearing hosts. Cancer Immunol Immunother. 2009;58:737–748. DOI:10.1007/s00262-008-0596-0
65. Moschella F, Valentini M, Arico E, et al. Unraveling cancer chemoimmunotherapy mechanisms by gene and protein expression profiling of responses to cyclophosphamide. Cancer Res. 2011;71:3528–3539. DOI:10.1158/0008-5472.CAN-10-4523
66. Klein O, Davis ID, McArthur GA, et al. Low-dose cyclophosphamide enhances antigen-specific CD4(+) T-cell responses to NY-ESO-1/ISCOMATRIX vaccine in patients with advanced melanoma. Cancer Immunol Immunother. 2015;64:507–518. DOI:10.1007/s00262-015-1656-x
67. Tanaka F, Yamaguchi H, Ohta M, et al. Intratumoral injection of dendritic cells after treatment of anticancer drugs induces tumor-specific antitumor effect in vivo. Int J Cancer. 2002;101:265–269. DOI:10.1002/ijc.10597
68. Nistico P, Capone I, Palermo B, et al. Chemotherapy enhances vaccine-induced antitumor immunity in melanoma patients. Int J Cancer. 2009;124:130–139. DOI:10.1002/ijc.23886.
    - **This report demonstrates a chemotherapy-induced enhancement of the vaccine-induced $CD8^+$ (memory) T-cell immune response**
69. Palermo B, Del BD, Sottini A, et al. Dacarbazine treatment before peptide vaccination enlarges T-cell repertoire diversity of melan-a-specific, tumor-reactive CTL in melanoma patients. Cancer Res. 2010;70:7084–7092. DOI:10.1158/0008-5472.CAN-10-1326
70. Peng S, Wang JW, Karanam B, et al. Sequential cisplatin therapy and vaccination with HPV16 E6E7L2 fusion protein in saponin adjuvant GPI-0100 for the treatment of a model HPV16+ cancer. PLoS One. 2015;10:e116389. DOI:10.1371/journal.pone.0116389
71. Bracci L, Moschella F, Sestili P, et al. Cyclophosphamide enhances the antitumor efficacy of adoptively transferred immune cells through the induction of cytokine expression, B-cell and T-cell homeostatic proliferation, and specific tumor infiltration. Clin Cancer Res. 2007;13:644–653. DOI:10.1158/1078-0432.CCR-06-1209.
    - **Next to effect of cyclophoshamide on regulatory T-cells this report shows a different mechanism of enhancing the antitumor response**
72. Farsaci B, Higgins JP, Hodge JW. Consequence of dose scheduling of sunitinib on host immune response elements and vaccine combination therapy. Int J Cancer. 2012;130:1948–1959. DOI:10.1002/ijc.26219
73. Finke JH, Rini B, Ireland J, et al. Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. Clin Cancer Res. 2008;14:6674–6682. DOI:10.1158/1078-0432.CCR-07-5212
74. Tseng CW, Hung CF, Alvarez RD, et al. Pretreatment with cisplatin enhances E7-specific CD8+ T-Cell-mediated antitumor immunity induced by DNA vaccination. Clin Cancer Res. 2008;14:3185–3192. DOI:10.1158/1078-0432.CCR-08-0037
75. Lee SY, Kang TH, Knoff J, et al. Intratumoral injection of therapeutic HPV vaccinia vaccine following cisplatin enhances HPV-specific antitumor effects. Cancer Immunol Immunother. 2013;62:1175–1185. DOI:10.1007/s00262-013-1421-y
76. Berinstein NL, Karkada M, Oza AM, et al. Survivin-targeted immunotherapy drives robust polyfunctional T-cell generation and differentiation in advanced ovarian cancer patients. Oncoimmunology. 2015;4:e1026529. DOI:10.1080/2162402X.2015.1008371
77. Machiels JP, Reilly RT, Emens LA, et al. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. Cancer Res. 2001;61:3689–3697.
78. Wei SM, Fei JX, Tao F, et al. Anti-CD27 antibody potentiates antitumor effect of dendritic cell-based vaccine in prostate cancer-bearing mice. Int Surg. 2015;100:155–163. DOI:10.9738/INTSURG-D-14-00147.1
79. Tagliamonte M, Petrizzo A, Napolitano M, et al. Novel metronomic chemotherapy and cancer vaccine combinatorial strategy for hepatocellular carcinoma in a mouse model. Cancer Immunol Immunother. 2015;64:1305–1314. DOI:10.1007/s00262-015-1698-0
80. Dijkgraaf EM, Santegoets SJ, Reyners AK, et al. A phase 1/2 study combining gemcitabine, Pegintron and p53 SLP vaccine in patients with platinum-resistant ovarian cancer. Oncotarget. 2015;6:32228–32243. DOI:10.18632/oncotarget.4772.
    - •• **Together with references 95 and 96, these studies reveal different mechanisms of gemcitabine all resulting in enhanced efficacy of the immunotherapy**
81. van der Sluis TC, van Duikeren S, Huppelschoten S, et al. Vaccine-induced tumor necrosis factor-producing T-cells synergize with cisplatin to promote tumor cell death. Clin Cancer Res. 2015;21:781–794. DOI:10.1158/1078-0432.CCR-14-2142
82. Gibney GT, Kudchadkar RR, DeConti RC, et al. Safety, correlative markers, and clinical results of adjuvant nivolumab in combination with vaccine in resected high-risk metastatic melanoma. Clin Cancer Res. 2015;21:712–720. DOI:10.1158/1078-0432.CCR-14-2468
83. Fu J, Malm IJ, Kadayakkara DK, et al. Preclinical evidence that PD1 blockade cooperates with cancer vaccine TEGVAX to elicit

regression of established tumors. Cancer Res. 2014;74:4042–4052. DOI:10.1158/0008-5472.CAN-13-2685
84. Sawada Y, Yoshikawa T, Shimomura M, et al. Programmed death-1 blockade enhances the antitumor effects of peptide vaccine-induced peptide-specific cytotoxic T lymphocytes. Int J Oncol. 2015;46:28–36. DOI:10.3892/ijo.2014.2737
85. Mkrtichyan M, Chong N, Abu ER, et al. Anti-PD-1 antibody significantly increases therapeutic efficacy of Listeria monocytogenes (Lm)-LLO immunotherapy. J Immunother Cancer. 2013;1:15. DOI:10.1186/2051-1426-1-15
86. Wada S, Jackson CM, Yoshimura K, et al. Sequencing CTLA-4 blockade with cell-based immunotherapy for prostate cancer. J Transl Med. 2013;11:89. DOI:10.1186/1479-5876-11-89
87. Newton MR, Askeland EJ, Andresen ED, et al. Anti-interleukin-10R1 monoclonal antibody in combination with bacillus Calmette–Guerin is protective against bladder cancer metastasis in a murine orthotopic tumour model and demonstrates systemic specific anti-tumour immunity. Clin Exp Immunol. 2014;177:261–268. DOI:10.1111/cei.12315
88. Ito F, Li Q, Shreiner AB, et al. Anti-CD137 monoclonal antibody administration augments the antitumor efficacy of dendritic cell-based vaccines. Cancer Res. 2004;64:8411–8419. DOI:10.1158/0008-5472.CAN-04-0590
89. Cuadros C, Dominguez AL, Lollini PL, et al. Vaccination with dendritic cells pulsed with apoptotic tumors in combination with anti-OX40 and anti-4-1BB monoclonal antibodies induces T-cell-mediated protective immunity in Her-2/neu transgenic mice. Int J Cancer. 2005;116:934–943. DOI:10.1002/ijc.21098
90. Kim S, Buchlis G, Fridlender ZG, et al. Systemic blockade of transforming growth factor-beta signaling augments the efficacy of immunogene therapy. Cancer Res. 2008;68:10247–10256. DOI:10.1158/0008-5472.CAN-08-1494
91. Chakraborty M, Abrams SI, Coleman CN, et al. External beam radiation of tumors alters phenotype of tumor cells to render them susceptible to vaccine-mediated T-cell killing. Cancer Res. 2004;64:4328–4337. DOI:10.1158/0008-5472.CAN-04-0073
• **Together with reference 120, these studies demonstrate the importance of radiation therapy given after immunotherapy in a mouse model to increase the anti-tumor efficacy.**
92. Mondini M, Nizard M, Tran T, et al. Synergy of Radiotherapy and a Cancer Vaccine for the Treatment of HPV-Associated Head and Neck Cancer. Mol Cancer Ther. 2015;14:1336–1345. DOI:10.1158/1535-7163.MCT-14-1015
• **Together with reference 119, these studies demonstrate the importance of radiation therapy given after immunotherapy in a mouse model to increase the antitumor efficacy.**
93. Fridman WH, Pages F, Sautes-Fridman C, et al. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012;12:298–306. DOI:10.1038/nrc3245
94. Gouttefangeas C, Walter S, Welters M, et al. Flow cytometry in cancer immunotherapy: applications, quality assurance and future. In: Cancer Immunology: Translational Medicine from Bench to Bedside (N. Rezaei *editor*). Springer-Verlag Berlin Heidelberg. Chapter 25: pages 471–486. DOI: 10.1007/978-3-662-44006-3
95. Welters MJ, Van Der Burg SH. Comprehensive immunomonitoring to guide the development of immunotherapeutic products for cancer. In: Prendergast GC, Jaffee EM, editors. Cancer Immunotherapy second edition: Immune suppression and tumor growth. 2013. Elsevier Academic Press London UK, Chapter 16, p. 241–258.
96. Galluzzi L, Buque A, Kepp O, et al. Immunological Effects of Conventional Chemotherapy and Targeted Anticancer Agents. Cancer Cell. 2015;28:690–714. DOI:10.1016/j.ccell.2015.10.012
97. Vacchelli E, Aranda F, Eggermont A, et al. Trial watch: chemotherapy with immunogenic cell death inducers. Oncoimmunology. 2014;3:e27878. DOI:10.4161/onci.27878
98. Ghiringhelli F, Larmonier N, Schmitt E, et al. CD4+CD25+ regulatory T-cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol. 2004;34:336–344. DOI:10.1002/eji.200324181
99. Lutsiak ME, Semnani RT, De PR, et al. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood. 2005;105:2862–2868. DOI:10.1182/blood-2004-06-2410
100. Homma Y, Taniguchi K, Nakazawa M, et al. Changes in the immune cell population and cell proliferation in peripheral blood after gemcitabine-based chemotherapy for pancreatic cancer. Clin Transl Oncol. 2014;16:330–335. DOI:10.1007/s12094-013-1079-0
101. Shevchenko I, Karakhanova S, Soltek S, et al. Low-dose gemcitabine depletes regulatory T-cells and improves survival in the orthotopic Panc02 model of pancreatic cancer. Int J Cancer. 2013;133:98–107. DOI:10.1002/ijc.27990
102. Vincent J, Mignot G, Chalmin F, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T-cell-dependent antitumor immunity. Cancer Res. 2010;70:3052–3061. DOI:10.1158/0008-5472.CAN-09-3690
103. Roselli M, Cereda V, di Bari MG, et al. Effects of conventional therapeutic interventions on the number and function of regulatory T-cells. Oncoimmunology. 2013;2:e27025. DOI:10.4161/onci.27025
104. Vieweg J, Su Z, Dahm P, et al. Reversal of tumor-mediated immunosuppression. Clin Cancer Res. 2007;13:727s–32s. DOI:10.1158/1078-0432.CCR-06-1924
105. Welters MJ, Piersma SJ, van der Burg SH. T-regulatory cells in tumour-specific vaccination strategies. Expert Opin Biol Ther. 2008;8:1365–1379. DOI:10.1517/14712598.8.9.1365
106. Casares N, Rudilla F, Arribillaga L, et al. A peptide inhibitor of FOXP3 impairs regulatory T-cell activity and improves vaccine efficacy in mice. J Immunol. 2010;185:5150–5159. DOI:10.4049/jimmunol.1001114
107. Rech AJ, Mick R, Martin S, et al. CD25 blockade depletes and selectively reprograms regulatory T-cells in concert with immunotherapy in cancer patients. Sci Transl Med. 2012;4:134ra62. DOI:10.1126/scitranslmed.3003330
108. Kudo-Saito C, Schlom J, Camphausen K, et al. The requirement of multimodal therapy (vaccine, local tumor radiation, and reduction of suppressor cells) to eliminate established tumors. Clin Cancer Res. 2005;11:4533–4544. DOI:10.1158/1078-0432.CCR-04-2237
109. Ruffell B, Coussens LM. Macrophages and therapeutic resistance in cancer. Cancer Cell. 2015;27:462–472. DOI:10.1016/j.ccell.2015.02.015
110. Hume DA, MacDonald KP. Therapeutic applications of macrophage colony-stimulating factor-1 (CSF-1) and antagonists of CSF-1 receptor (CSF-1R) signaling. Blood. 2012;119:1810–1820. DOI:10.1182/blood-2011-09-379214
111. Srivastava MK, Dubinett S, Sharma S. Targeting MDSCs enhance therapeutic vaccination responses against lung cancer. Oncoimmunology. 2012;1:1650–1651. DOI:10.4161/onci.21970
112. Moschella F, Torelli GF, Valentini M, et al. Cyclophosphamide induces a type I interferon-associated sterile inflammatory response signature in cancer patients' blood cells: implications for cancer chemoimmunotherapy. Clin Cancer Res. 2013;19:4249–4261. DOI:10.1158/1078-0432.CCR-12-3666
113. Vermeij R, Leffers N, Hoogeboom BN, et al. Potentiation of a p53-SLP vaccine by cyclophosphamide in ovarian cancer: a single-arm phase II study. Int J Cancer. 2012;131:E670–E80. DOI:10.1002/ijc.27388
114. Kaida M, Morita-Hoshi Y, Soeda A, et al. Phase 1 trial of Wilms tumor 1 (WT1) peptide vaccine and gemcitabine combination therapy in patients with advanced pancreatic or biliary tract cancer. J Immunother. 2011;34:92–99. DOI:10.1097/CJI.0b013e3181fb65b9.
•• **Together with references 61 and 96, these studies reveal different mechanisms of gemcitabine all resulting in enhanced efficacy of the immunotherapy**
115. Correale P, Cusi MG, Tsang KY, et al. Chemo-immunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. J Clin Oncol. 2005;23:8950–8958. DOI:10.1200/JCO.2005.12.147

116. Bauer C, Bauernfeind F, Sterzik A, et al. Dendritic cell-based vaccination combined with gemcitabine increases survival in a murine pancreatic carcinoma model. Gut. 2007;56:1275–1282. DOI:10.1136/gut.2006.108621
117. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature. 2014;515:568–571. DOI:10.1038/nature13954
118. Reck M, Bondarenko I, Luft A, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line therapy in extensive-disease-small-cell lung cancer: results from a randomized, double-blind, multicenter phase 2 trial. Ann Oncol. 2013;24:75–83. DOI:10.1093/annonc/mds213.
•• **Not the concurrent administation but chemotherapy prior to checkpoint blockade results in improved clinical outcome**
119. Muller AJ, DuHadaway JB, Donover PS, et al. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med. 2005;11:312–319. DOI:10.1038/nm1196.
120. Bjoern J, Iversen TZ, Nitschke NJ, et al. Safety, immune and clinical responses in metastatic melanoma patients vaccinated with a long peptide derived from indoleamine 2,3-dioxygenase in combination with ipilimumab. Cytotherapy. 2016;18:1043–1055. DOI:10.1016/j.jcyt.2016.05.010
121. Kvistborg P, Philips D, Kelderman S, et al. Anti-CTLA-4 therapy broadens the melanoma-reactive CD8+ T-cell response. Sci Transl Med. 2014;6:254ra128. DOI:10.1126/scitranslmed.3008918
122. Peggs KS, Quezada SA, Chambers CA, et al. Blockade of CTLA-4 on both effector and regulatory T-cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. J Exp Med. 2009;206:1717–1725. DOI:10.1084/jem.20082492
123. Romano E, Kusio-Kobialka M, Foukas PG, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T-cells ex vivo by nonclassical monocytes in melanoma patients. Proc Natl Acad Sci U S A. 2015;112:6140–6145. DOI:10.1073/pnas.1417320112
124. van der Sluis TC, Sluijter M, van Duikeren S, et al. Therapeutic Peptide Vaccine-Induced CD8 T Cells Strongly Modulate Intratumoral Macrophages Required for Tumor Regression. Cancer Immunol Res. 2015;3:1042–1051. DOI:10.1158/2326-6066.CIR-15-0052
125. Association of Cancer Immunotherapy's (CIMT's) immunoguiding program. Available from: http://www.cimt.eu/workgroups/cip/
126. Cancer Immunotherapy Consortium (CIC). http://www.cancerresearch.org/cic
127. Britten CM, Gouttefangeas C, Welters MJ, et al. The CIMT-monitoring panel: a two-step approach to harmonize the enumeration of antigen-specific CD8+ T lymphocytes by structural and functional assays. Cancer Immunol Immunother. 2008;57:289–302. DOI:10.1007/s00262-007-0378-0
128. Filbert H, Attig S, Bidmon N, et al. Serum-free freezing media support high cell quality and excellent ELISPOT assay performance across a wide variety of different assay protocols. Cancer Immunol Immunother. 2013;62:615–627. DOI:10.1007/s00262-012-1359-5
129. Britten CM, Janetzki S, Butterfield LH, et al. T-cell assays and MIATA: the essential minimum for maximum impact. Immunity. 2012;37:1–2. DOI:10.1016/j.immuni.2012.07.010
130. Young KH, Baird JR, Savage T, et al. Optimizing Timing of Immunotherapy Improves Control of Tumors by Hypofractionated Radiation Therapy. PLoS One. 2016;11:e0157164. DOI:10.1371/journal.pone.0157164
•• **This report shows the importance of specific timing in the combination therapy using agonistic antibody therapy and immune checkpoint blockade.**