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Authors: Kandalaft LE, Singh N, Liao JB, Facciabene A, Berek JS, Powell DJ Jr, Coukos G

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The Emergence of Immunomodulation: Combinatorial Immuno-Chemotherapy Opportunities for the Next Decade

Lana E. Kandalaft[1], **Nathan Singh**[1], **John B. Liao**[1], **Andrea Facciabene**[1], **Jonathan Berek**[2], **Daniel J. Powell Jr.**[1], and **George Coukos**[1,3]

[1] Ovarian Cancer Research Center, University of Pennsylvania, Philadelphia, PA 19104

[2] Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Stanford University School of Medicine, Stanford, CA, USA

Abstract

In the past decade we have witnessed important advances in the treatment of gynecological cancers and have recognized their potential immunogenicity. This has opened the door to explore immune therapy not only in HPV-induced cancers but also in ovarian and endometrial cancers. Here we will review the off target immune effects of select chemotherapy drugs commonly used to treat gynecologic cancers and novel tools that can stimulate both the adaptive and innate immune mechanisms such as novel pleiotropic cytokines, Toll-like receptors, and powerful antibodies that can target inhibitory checkpoints thereby activating effector cellular immune mechanisms and neutralizing suppressor cells. We will also review how existing drugs can be used for combinatorial tumor therapy.

INTRODUCTION

Significant advances have been made in the therapy of gynecologic cancers in the past two decades. Chemotherapy regimens have been optimized with respect to dose, schedule and combinations, and novel targeted therapies have emerged that can selectively neutralize signals that drive or maintain the oncogenic process. Although the cancer cell remains the main target of oncologic therapy, it is becoming progressively clear that the tumor microenvironment provides critical support to tumor growth and therefore opportunities for therapy. Inhibition of tumor angiogenesis is an obvious example of effective biological therapy that has produced clinical results. Importantly, complex mechanisms regulating immune response and inflammation interface with angiogenesis at the tumor microenvironment, and their balance can greatly affect the fate of tumors. The overall balance of tumor inflammatory mechanisms is polarized to promote angiogenesis, tumor cell survival and immune escape, all contributing to tumor growth. However, it is becoming clear that many patients with gynecologic malignancies mount a spontaneous antitumor immune response. Although ineffective to reject tumor, this can be potentially harnessed therapeutically. Here we will review how existing drugs can capitalize on and manipulate natural antitumor immunity and thus be used for combinatorial tumor therapy.

The use of immunomodulatory therapy is predicated on the notion that gynecologic cancers are potentially immunogenic tumors, i.e they can be recognized and attacked by cell based

[3]Corresponding Author: gcks@mail.med.upenn.edu.

CONFLICT OF INTEREST

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immune mechanisms. Cervical and lower genital tract cancers induced by human papillomavirus (HPV) are the prototype of potentially immunogenic tumors that can elicit a spontaneous immune response. HPV xenoantigens expressed by tumor cells are readily recognized by the immune system. Cell-mediated immune responses are important in controlling HPV infections as well as HPV-associated neoplasms (for review, see [1]). The prevalence of HPV-related diseases is increased in patients with impaired cell-mediated immunity, including transplant recipients [2] and HIV-infected patients [3, 4]. Infiltrating $CD4^+$ (T helper cells) and $CD8^+$ (cytotoxic) T cells have been observed in spontaneously regressing warts [5] and, warts often disappear in patients who are on immunosuppressive therapy when treatment is discontinued [6]. In addition, animals immunized with viral proteins are protected from HPV infection or the development of neoplasia, and experience regression of existing lesions [7, 8]. Nevertheless, patients with invasive cervical cancer exhibit exhausted and tolerized T cells that recognize antigen *in vitro* but are unable to reject tumors *in vivo* [9, 10]. The emergence of immunomodulatory therapies revives opportunities to activate and invigorate such T cell immunity and warrants clinical testing.

Although tumor-associated antigens have not undergone rigorous scrutiny in other gynecologic malignancies (reviewed in [11]), similar mechanisms of spontaneous antitumor immune response have been convincingly demonstrated. Tumor-reactive T cells and antibodies have been detected in peripheral blood of patients with advanced stage ovarian cancer at diagnosis [12, 13], while oligoclonal tumor reactive T cells have been isolated from tumors or ascites [14–22]. Importantly, the detection of intratumoral or intraepithelial tumor infiltrating lymphocytes (TIL), i.e. T cells infiltrating tumor islets predicts significantly improved progression survival and overall survival in ovarian cancer. We first reported in an Italian cohort that patients whose tumors had intraepithelial T cells experienced 3.8-fold longer median progression-free survival and 2.8-fold longer overall survival as compared to patients whose tumors lacked intraepithelial T cells. Remarkably, survival rate at five years was 38% in patients whose tumors had intraepithelial T cells (n=102) and 4.5% in patients lacking them (n=72). The impact of intraepithelial T cells was confirmed by multiple independent studies on ethnically diverse populations [23–29]. Similar observations were made in endometrial cancer [30–32] and other solid tumors [33]. Retrospective studies showing that the incidence of many non-virally induced solid tumor types is in fact 4–30 fold increased in immunosuppressed transplant recipients [34–38], provide evidence that immune recognition is probably a universal mechanism in tumors.

CHEMOTHERAPY AS AN IMMUNE MODULATOR

Although it has been traditionally thought that chemotherapy antagonizes immune mechanisms altogether, recent evidence has challenged this view. Indeed, agents such as cyclophosphamide, doxorubicin and paclitaxel increase the number and function of antigen-specific T cells and thus may enhance cancer immunity [39]. It is becoming progressively clear that conventional chemotherapy has important “off-target” immunologic effects, and in fact, may depend on activation of immune mechanisms to achieve its full efficacy. In mouse models of solid tumors, increased tumor inflammation following administration of chemotherapy predicts better prognosis [40], while tumors grown in immunodeficient mice fail to respond to chemotherapy [41], clearly highlighting a role for the immune system in cancer clearance in the context of cytotoxic therapy. Similar events may occur in humans; tumor-infiltrating lymphocytes predicted complete pathologic response in breast cancer patients after neoadjuvant chemotherapy [42]. Furthermore, neoadjuvant taxol therapy was found to increase TIL [43]. Interestingly, breast cancer patients bearing a loss-of-function Asp299Gly polymorphism of the Toll-like receptor (TLR) 4 receptor (see below) exhibit a higher risk ofrelapse after treatment with chemotherapyand radiation therapy [44].

The immunomodulatory effects of chemotherapy can be broadly grouped in three mechanisms: a) induction of immunogenic cancer cell death, which facilitates tumor antigen presentation (in situ vaccination); b) direct activation of antigen presenting or effector mechanisms; and c) suppression of immune inhibitory cells, thereby releasing regulatory breaks on antitumor immune response (Figure 1). These mechanisms are quite complex and our understanding is still in its infancy, but effects appear to dependent on drug type, dose and schedule as well as the immune cell type. The available evidence is reviewed below.

a) *In situ* Vaccination from Immunogenic Tumor Cell Death

Several recent studies have demonstrated that a number of chemotherapeutic agents are able to induce “immunogenic cell death”. In general, necrotic cell death is considered inflammatory, while apoptotic cell death induces less inflammation. Recently, it was found that doxorubicin, idarubicin, mitoxanthrone, oxaliplatin and other drugs induce apoptotic cell death, which however leads to greatly increased antigen uptake by dendritic cells (DCs), the professional antigen presenting cells, and to DC maturation, leading to *in situ* immunization against tumor antigens [44, 45]. The underlying molecular mechanism is related to the preapoptotic exposure of calreticulin on the cell surface, which mediates DC uptake and maturation [46].

b) Activation of APCs and Effector Mechanisms

Dendritic cells are the most potent antigen-presenting cells in the immune arsenal. High doses of immunogenic chemotherapy may be transiently pro-inflammatory, however they do not promote DC longevity. Cyclophosphamide has interesting immunomodulatory properties, and in combination with cisplatin demonstrated synergy with subcutaneous interferon-gamma in a phase III study in patients with advanced ovarian cancer [47]. Since the 1980s, it has been recognized that cyclophosphamide administered at standard dose prior to cancer vaccines significantly enhanced immune therapy, however the mechanism of this phenomenon remained unclear [48]. A recent study in the mouse reported that cyclophosphamide at myelosuppressive dose induces rebound myelopoiesis and leads to the emergence of tumor-infiltrating DCs that secrete more IL-12 and less IL-10, which are fully capable of priming T cell responses [49]. In addition, metronomic, or low dose non-myelotoxic administration of paclitaxel, doxorubicin, vincristine and other drugs can cause activation and maturation of DCs, comprising increased IL-12 secretion, a critical factor required for T cell priming. Signaling via STAT4 and Rho GTPases may account for these effects [50].

An additional mechanism through which chemotherapy enhances immune mechanisms is by rendering tumor cells more susceptible to killing by immune effector cells. Fas is a membrane-bound protein belonging to the tumor necrosis factor (TNF) family of surface receptors, which, upon ligation with Fas-ligand (FasL), induces apoptosis. FasL is expressed by activated effector immune cells, namely $CD8^+$ T and NK cells. A recent in vitro study showed that treatment of cancer cells with topotecan causesupregulation of Fas expression, and this can lead to significant T cell activation within the tumor microenvironment [51]. Cisplatin has also been shown to increase the susceptibility of tumor cells to tumor-infiltrating lymphocytes or NK cells [52, 53].

Although cytotoxic chemotherapy drugs activate antigen presentation mechanisms and tumor cell susceptibility to immune effector cells, their direct effects on immune effector mechanisms remain poorly understood. High dose cytotoxic chemotherapy could deplete activated tumor-reactive T cells. However, Nowak et al showed that relatively high doses of gemcitabine suppressed IgG antibody production, but did not block T lymphocyte recall responses and were not detrimental to specific anti-tumor immunity [54]. Furthermore,

topotecan at MTD did not impair the frequency or response of virus-reactive lymphocytes in ovarian cancer patients [55]. Finally, cisplatin and paclitaxel did not interfere with T cell function *in vitro*. [56]. However, in the mouse cyclophosphamide, doxorubicin and paclitaxel at maximally tolerated dose (MTD) dampen the efficacy of vaccine if given after the vaccine, although they increase tumor-reactive T cells when given prior to vaccine [39]. Low dose metronomic chemotherapy may be a better approach for combinations with immunotherapy. In a mouse model, metronomic cyclophosphamide provided significantly greater synergism with vaccine than MTD cyclophosphamide. Both metronomic and MTD cyclophosphamide caused deletion of proliferating tumor-specific CTL in blood, but the metronomic dosing schedule caused deletion with slower kinetics and did not delete $CD43^{low}$ memory cells, which maintained potent capacity to respond to repeat tumor challenge [57].

c) Suppression of Inhibitory Cells

T regulatory (Treg) cells are characterized as $CD25^+CD4^+Foxp3^+$ T cells which function to suppress immune responses and maintain peripheral tolerance. We have shown significantly increased proportions of TGF-β secreting Treg in patients with ovarian cancer [58], capable of suppressing tumor-specific T cell immunity and contributing to growth of human tumors in vivo [59]. Increased frequency of Treg in ovarian tumors predicts reduced survival [23, 59].

Intravenous cyclophosphamide has been used in humans to augment cancer vaccines [60, 61]. In addition, intravenous cyclophosphamide, doxorubicin and paclitaxel at maximally tolerated dose (MTD) were shown to help break tolerance to “self” cancer antigens and enhanced cancer vaccine efficacy [39]. The exact molecular function of these drugs on particular immune cells subsets has recently begun to be clarified. In mouse models, cyclophosphamide enhanced tumor immunotherapy by elimination of tumor-induced suppressor T cells [62] independently of cytotoxic reduction in tumor burden [63], and enhanced the magnitude of secondary but not primary cytotoxic lymphocyte responses induced by cancer vaccines [64, 65]. In the human, oral administration of metronomic cyclophosphamide was shown to induce a profound and selective reduction of circulating $CD4^+CD25^+$ regulatory T cells and restored T and NK effector functions in end-stage cancer patients [66]. Cyclophosphamide may have additional effects contributing to restoration of the immune response. For example, it can enhance IFN-γ production by splenocytes in a mouse model [67]. Conventional paclitaxel therapy also caused a significant decline in both numbers and activity of Treg, enhancing $CD4^+$ and $CD8^+$ activity systemically in patients with non-small cell lung cancer [68].

Myeloid-derived suppressor cells (MDSCs) is a recently described cell population characterized phenotypically as $CD11b^+Gr\text{-}1^+$ in the mouse. In the human, $CD11b^+Gr^+$ MDSC have not been clearly identified, but $HLA\text{-}DR^{neg}$ monocytes have functional features of suppressor cells and may be MDSC. A high frequency of $HLA\text{-}DR^{neg}$ monocytes secreting IL-10 have been described in ovarian cancer ascites in the human [69]. The main mechanism by which MDSC suppress T effector function is through the production of L-arginase, which reduces the availability of L-arginine, an essential amino acid fundamental for the function of T lymphocytes [70]. Importantly, gemcitabine used at conventional doses was found to significantly reduce the number of MDSCs in the spleens of treated mice, whereas other leukocytes were not affected [71].

In summary, chemotherapy drugs commonly used in gynecologic malignancies have specific immunomodulatory properties and could be used rationally to harness preexisting or induce a *de novo* antitumor immune response. It is thus expected that rational combination of these drugs with other immunomodulatory drugs may produce important clinical results,

especially in patients with gynecologic malignancies who show pre-existing antitumor immune response. Below we will review some of the *bona fide* immunomodulatory tools already in the clinic that are available for clinical testing in immuno-chemotherapy combinations.

NON-SPECIFIC IMMUNE ACTIVATION

Multifaceted, pleiotropic immune activation can be achieved with cytokines and Toll-like receptor agonist therapy and is suitable for combination with immunomodulatory chemotherapy. Below we summarize experience accumulated to date in gynecologic cancers with older, more traditional cytokines, and will review newer cytokines and TLR agonists.

a) Interferons

Interferons were first described as antiviral cytokines, but have since been shown to be secreted in response to a vast number of stimulatory factors other than viruses. They are divided into two broad categories; type I and type II interferons. Type I interferons are subdivided into two classes, known as alpha and beta. Interestingly, 12 forms of IFN-α have been identified, while only one form of IFN-β has been isolated. Signaling through their corresponding receptors on target cells is mediated by a series of Jak/STAT proteins and results in several antiviral activities. Additionally, they have potent effects on cell proliferation. Mouse models have demonstrated that gene therapy with IFN-β can greatly enhance tumor cell death in the context of several different malignancies [72]. Many clinical trials have demonstrated the efficacy of type I interferon therapy in the treatment of hematologic malignancies [73–75], melanoma [76–80] and renal cell carcinoma [81–83]. Phase I/II clinical studies have examined the therapeutic value of type I IFNs in ovarian cancer. Intraperitoneal recombinant IFN-α alone or combined with cisplatin as salvage therapy for persistent ovarian cancer after primary chemotherapy has shown clinical efficacy in small volume disease [84, 85], but there was no significant effect in a cohort of patients with recurrent, platinum-resistant disease [86]. Although encouraging, these results did not support additional clinical development of type I interferon in ovarian cancer.

One of the limitations of interferon therapy relates to the high intratumoral cytokine levels required to induce antitumor responses, which cannot be achieved without eliciting systemic toxicity and cannot be sustained owing to the short half-life of recombinant proteins. Cytokine gene therapy using recombinant viral vectors can achieve much higher and sustained cytokine levels at the tumor site than those resulting from systemic or regional administration of recombinant cytokine proteins without engendering systemic toxicity [87]. A trial of intrapleural adenovirus delivering human IFN-β was recently completed at the University of Pennsylvania. Toxicity was minimal. One patient with recurrent, platinum-resistant low-grade ovarian carcinoma achieved complete objective and cytologic response of both pleural and intraperitoneal disease following a single intrapleural injection of adenovirus vector in this trial [88]. Disease stability or objective responses were also observed in patients with malignant pleural mesothelioma enrolled in the study [89]. These data present promising evidence that IFN-β can serve as a potent anticancer agent, and its use in combination with other forms of chemo and immunotherapy certainly warrants further consideration.

Structurally unrelated to type I interferons, IFN-γ is secreted by activated effector T cells and NK cells in response to target recognition. IFN-γ has been shown to have direct anti-proliferative activity on ovarian cancer cells *in vitro*, which proved to be synergistic with cisplatin and doxorubicin [90–92]. *In vitro* and *in vivo*, IFN-γ upregulates HLA class I and class II molecules and antigen presentation in ovarian tumor cells [93], a requisite for recognition by T cells. In fact, HLA class I expression by the tumor correlates with the

intensity of T cell infiltration [94], a predictor of longer survival. Furthermore, IFN-γ has antiangiogenic effects [95].

Encouraging results have been reported with recombinant human (rh)IFN-γ either as intraperitoneal monotherapy or in combinations in early phase trials [96–99]. Theoretically, the effects are likely to be greatest in women who are also receiving chemotherapy because of IFN-γ's non-specific immunomodulatory effects [100]. Confirming expectations, a three-fold prolongation of progression-free survival was observed in a phase-III multi-center study from Europe with subcutaneous administration of rhIFN-γ combined with MTD cisplatin and cyclophosphamide chemotherapy, with minimal added toxicity [47]. However, in a subsequent randomized phase-III trial conducted in the U.S., addition of subcutaneous rhIFN-γ to carboplatin and paclitaxel did not improve survival [101]. Although one cannot exclude that racial and other demographic differences may account for opposite results, this data may indicate that the choice of chemotherapy drugs is in fact critical in combinatorial approaches with immune therapy. Indeed, whereas cyclophosphamide has potent immunomodulatory effects on many cell subsets including suppressing T regulatory (Treg) cells (see below), high dose steroids, which are necessarily given with paclitaxel to prevent acute hypersensitivity reactions, are immunosuppressive and induce Treg in the setting of antigen presentation.

b) Interleukins

Interleukin-2 (IL-2) promotes expansion and enhances the cytotoxicity of effector immune cells [102]. In addition, IL-2 can restore T cell function following suppression by negative regulatory receptors such as PD-1 (see below). IL-2 represents the most widely investigated cytokine for use in cancer therapy, having shown clinical efficacy in malignant melanoma and renal cell carcinoma [103, 104], for which it is now FDA approved. Additionally, it has been used to enhance the efficacy of immunotherapy including vaccines and adoptive T-cell therapy [105]. However, its use has several limitations. In monotherapy and in the context of adoptive immunotherapy, IL-2 is used at MTD, which induces a systemic inflammatory response, with significant morbidity including multiple organ toxicities, most significantly the heart, lungs, kidneys, and central nervous system. Other manifestation of IL-2 toxicity is capillary leak syndrome, resulting in a hypovolemic state and fluid accumulation in the extravascular space [106].

Because ovarian cancer patients exhibit spontaneous antitumor immune response, IL-2 therapy may be a rational approach to activate preexisting immunity or enhance immunomodulatory therapy. Intraperitoneal IL-2 was used in a phase I/II study in 41 patients with laparotomy-confirmed persistent or recurrent ovarian cancer. Weekly IL-2 infusion was relatively well tolerated and demonstrated evidence of long-term efficacy in a modest number of patients. Twenty-percent of patients had a negative third look, i.e. exhibited pathologic evidence of complete response and no residual disease at repeat abdominal exploration [107]. Reflecting pre-therapy T cell activity, low expression of the CD3-zeta chain in peripheral blood T cells prior to therapy, a biomarker of T cell functional suppression by tumor-derived factors, predicted poor of response to IL-2 therapy [108]. Importantly, IL-2 is essential for the peripheral homeostasis of $CD4^+CD25^+Foxp3^+$ Treg cells, and it is now known that IL-2 is also an important activator of Treg suppressive activity in vivo [109]. After IL-2 cessation, the number of Treg cells more efficiently dropped in patients who experienced a clinical response than in non-responders [110]. Together, this data indicate that patients with pre-existing tumor-reactive, functional T cells and low prevalence of Treg are those likely to benefit from IL-2 monotherapy. In another phase II study, 44 patients with EOC responding to primary chemotherapy were treated with subcutaneous low dose IL-2 and oral retinoic acid for one year and with intermittent

schedules for up to five years. Patients experienced significantly improved progression-free and overall survival relative to 82 well-matched controls treated with standard therapy [111].

Alternate cytokines that selectively support activation of effector cells without promoting Treg cells may prove even more effective. IL-7, IL-15, IL-18 and IL-21 provide possible alternatives to IL-2, however their function and clinical use are still under investigation. The function of IL-7 has not been completely appreciated until recently. It serves an essential role not only in lymphopoeisis, but also T cell activity and maintenance and can promote antitumor immunity [112, 113]. A recent study using a mouse model of lung cancer examined the effects of IL-7 administration and found significant reduction in tumor burden, with a correlating increase in $CD4^+$ and $CD8^+$ T cells [114, 115]. IL-15 has similar functions to IL-2 in its effects of T cells, but also potentiates NK cell maturation and activity [116]. IL-21 is a promising cytokine as it enhances the cytolytic activity of $CD8^+$ T cells and NK cells, but also modulates the activity of CD4 T cells, B cells and suppresses Treg cells [117]. A recent phase II trial demonstrated that administration of IL-21 was associated with antitumor activity in patients with unresectable metastatic melanoma [118].

IL-18 is a novel cytokine that has been shown to have very potent immunostimulatory effects, including induction if IFN-γ, TNF-α, IL-1α, and GM-CSF, augmentation of NK cell cytotoxicity, activation of effector T cells, and promotion of Th1 responses, which are critical for tumor rejection. In a recent study, rhIL-18 was found to expand human effector T cells and reduce human Treg in a mouse model transplanted with human peripheral blood lymphocytes [119]. Clearly this biology points to a strong potential for the use of IL-18 in cancer immunotherapy. The immunostimulatory activity of IL-18 *in vivo* has been demonstrated in non-human primates [120] and humans [121]. In phase I clinical evaluation, recombinant human (rh)IL-18 was safely administered as monotherapy to 28 patients with solid tumors, with minimal dose-limiting toxicities and two partial tumor responses [121]. Toxicity has generally been mild to moderate even with repeat administration and a maximum tolerated dose has not been reached to date [122]. IL-18 enhanced activation of peripheral blood $CD8^+$ T cells, NK cells and monocytes and induced a transient increase in the frequency and expression level of Fas ligand (FasL) in peripheral blood $CD8^+$ T cells and NK cells [122]. The relatively minor toxicity of rhIL-18, compared with other immunostimulatory cytokines that have undergone clinical development, is remarkable and renders IL-18 a well suited drug for combinatorial approaches with chemotherapy. In mice with established ovarian carcinoma, administration of IL-18 alone was shown to have modest effects on tumor immunity, but when combined with pegylated liposomal doxorubicin chemotherapy its effects were greatly enhanced (Coukos et al, unpublished data). Clearly the use of IL-18 therapy with other immunogenic chemotherapy warrants further investigation. A phase I study is currently under way to test this hypothesis.

c) Toll-like Receptor Agonists

One of the most basic mechanisms for activation of the immune system is through the Toll-like receptors (TLRs). TLRs belong to the type I transmembrane receptor family. Their expression is ubiquitous, from epithelial to immune cells. The TLR-family members are pattern-recognition receptors that collectively recognize lipid, carbohydrate, peptide and nucleic-acid structures that are broadly expressed by different groups of microorganisms. Some TLRs are expressed at the cell surface, whereas others are expressed on the membrane of endocytic vesicles or other intracellular organelles. There are at least 10 known TLRs in humans grouped in six major families, based on their phylogenetic background [123]. Each family is attributed to a general class of PAMPs. TLRs 3, 7, 8 and 9 are located mainly in endosomes; double-stranded RNA are ligands for TLR3 [124], while TLRs 7 and 8 recognize single-stranded viral RNA [125]. The other TLRs are located on the cell surface

[126]; TLRs 1, 2, 5, 6 and 10 respond to bacterial, fungal and viral PAMPs [127–129]. Lipopolysaccharides are TLR4 ligands [130].

TLR engagement alerts the immune system and leads to the activation of innate immune cells. Two major signaling pathways are generally activated in response to a TLR ligand [131]. One pathway involves the MyD88-independent production of type I interferons. The second uses MyD88 to activate nuclear factor-kappa B (NF-κB), JUN kinase (JNK) and p38, finally resulting in the production of proinflammatory cytokines such as TNF-α, IL-12 and IL-1 and induction of innate effector mechanisms [132, 133]. Additionally, TLR triggering induces DC maturation, which leads to the upregulation of costimulatory molecules such as CD40, CD80 and CD86, and secretion of immune modulatory cytokines and chemokines. In addition, TLRs can directly stimulate the proliferation of $CD4^+$ and $CD8^+$ T cells as well as reverse the suppressive function of Treg cells [126][134][135]. Adding TLR 3, 4, 7 or 9 ligands was shown to activate $CD8^+$ cytotoxic T cells with increased IFN-γ production and promote a stimulatory cytokine milieu at the tumor microenvironment [136, 137]. Optimal antitumor immunity requires robust enhancement of the effector T cell response induced by tumor antigenic peptides and control or elimination of Treg suppressive function. Thus, the combination of peptide-based vaccines with TLR agonists, in particular a TLR8 agonist, may greatly improve the therapeutic potential of cancer vaccines.

Several clinical trials have demonstrated that administration of agonists for TLRs 3,4,7 and 9 can enhance activity of cancer vaccines in the context of non-small cell lung cancer [138], non-Hodgkins lymphoma [139, 140], glioblastoma [141], and superficial basal cell carcinoma [142]. Multiple TLR agonists have also been explored in melanoma. TLR 7 or 9 agonists were used in combination with melanoma antigen vaccine in advanced melanoma [31, 143, 144]. In addition, the TLR ligand Ribomunyl has been used in conjunction with a dendrtic cell vaccine in a phase I/II trial, which reported a median survival of 10.5 months in patients with advanced melanoma [145].

The use of TLR agonists in the clinic requires careful preclinical evaluation. For example, in the absence of specific cell-mediate antitumor immunity, non-specific activation of inflammation could in fact promote tumor growth rather than reducing it, because of the potent tumor-promoting effects of inflammation [146]. Thus, combinations with active immunization or adoptive immunotherapy seem ideal, as these approaches greatly benefit from concomitant activation of innate immune response. If combination with chemotherapy is designed, it seems rational to combine TLR agonists with chemotherapy drugs that can activate cellular immune mechanisms. Finally, the choice of TLR agonists may matter. Whereas TLR 3 and 9 agonists induce apoptosis of TLR-expressing tumor cells [147]. TLR4 agonists were shown to promote tumor cell survival, tumor growth and paclitaxel resistance in a proportion of ovarian cancer cells [148, 149].

ACTIVATION OF CELLULAR IMMUNITY

Generation of a successful antitumor adaptive immune response requires first and foremost the primary signal provided by the binding of T cell receptor to cognate tumor antigen. However, multiple secondary signals can activate or suppress this response. Characterization of these pathways in tumors and the development of specific agonistic or antagonistic antibodies or ligands has created new opportunities for powerful stimulation of antitumor immune response (Figure 2).

a) DC Activation via CD40

The CD40 receptor is a member of the TNF receptor family expressed by antigen presenting cells and B cells. Its ligand, CD40L, is transiently upregulated on activated T cells, activated

B cells and platelets; and under inflammatory conditions is also induced on monocytes and other innate immune cells. CD40 is a potent stimulator of antigen presenting cells and cellular immunity, and CD40/CD40L interaction is critical in the development of protective anti-tumor immunity. Mice deficient in CD40 fail to mount a protective anti-tumor immune response following vaccination. In addition, neutralizing anti-CD40L monoclonal antibody can abrogate the therapeutic value of potent tumor vaccines [150]. Vice versa, a CD40 agonistic antibody was shown to be able to overcome peripheral tolerance and generate antitumor immunity able to reject tumors [151]. The main mechanism of immune stimulation by CD40 ligands is activation of DCs resulting in increased survival, upregulation of costimulatory molecules, and secretion of critical cytokines for T cell priming such as IL-12. This promotes antigen presentation, priming and cross-priming of $CD4^+$ and $CD8^+$ effector T cells [152]. However, agonistic anti-CD40 antibody alone can have adverse effects on antitumor immunity as in the mouse it can ultimately impair the development of tumor-specific T cells [153] or accelerate the deletion of tumor-specific cytotoxic lymphocytes in the absence of antigen vaccination [154]. CD40 ligation could thus be best used in combinatorial approaches including vaccines and TLR agonists [152, 155]. Based on the imunomodulatory effects of select chemotherapy agents, the combination of CD40 ligands with chemotherapy is also a rational approach that warrants thorough investigation. For example, in mice with established solid tumors, the administration of gemcitabine with CD40L triggered potent antitumor immune response that eliminated tumor burden, and these mice became also resistant to repeated tumor challenge [156].

Interestingly, the CD40 receptor is expressed on a variety of tumors including melanoma, lung, bladder and prostate cancers [152], but also cervix [157] and the majority of ovarian cancers [158–162]. Because tumor cells also express the CD40L, it is likely that low-level constitutive engagement of CD40 facilitates malignant cell growth. However, transient potent activation of CD40 on carcinomas with ligand results in direct anti-proliferative effects and apoptosis. CD40 agonists promoted apoptosis and resulted in growth inhibition of ovarian carcinoma lines expressing CD40. CD40 ligation also induced NF-κB activation, and TNF-α, IL-6 and IL-8 production in most EOC cell lines [158, 163]. In vivo, administration of rhuCD40L inhibited the growth of several ovarian adenocarcinoma xenografts in severe combined immunodeficient mice through a direct effect causing apoptosis, fibrosis and tumor destruction. The antitumor effect of rhuCD40L was further increased by cisplatin [164]. Interestingly, rIFN-γ enhanced expression of CD40 on tumor cells and the efficacy of on EOC cell lines [159]. Thus, CD40 agonists can have a direct cytotoxic effects on tumors, even in the absence of any additional immune responses and cells.

Early clinical experience with monoclonal IgG agonistic antibodies is encouraging. In a recent phase I study, patients with advanced solid tumors received single doses of CD40 agonistic antibody CP-870,893 intravenously. CP-870,893 was well tolerated; the most common adverse event being cytokine release syndrome including chills, rigors, and fever; 14% of all patients and 27% of melanoma patients had objective partial responses [165].

b) Activation of T Effector Cells via Blockade of Inhibitory Checkpoints

T-cell activation is triggered through the T-cell receptor by recognition of the cognate antigen complexed with MHC. T cell activation is regulateD by complex signals downstream of the diverse family of CD28 family immune receptors, which includes costimulatory (CD28 and ICOS) and inhibitory receptors (CTLA-4, PD-1 and BTLA). CD28 and CTLA-4 share the same ligands, B7-1 (CD80) and B7-2 (CD86), whereas PD-1 interacts with PD ligand 1 (PD-L1), also named B7-H1, and PD-L2, also named B7-DC. Simultaneous recognition of the cognate MHC-peptide complex by the TCR (signal 1) and CD80 or CD86 by CD28 (signal 2) results in T cell activation, proliferation, and

differentiation, as well as effector cytokine production. PD-1 and CTLA-4 are induced on T cells following a TCR signal, and result in cell cycle arrest and termination of T cell activation. The importance of the PD-1 and CTLA-4 pathways in the physiologic regulation of T cell activation is demonstrated by the autoimmune diseases occurring in CTLA-4 and PD-1 knockout mice [166] and further illustrated by the inflammatory side effects that can result from a therapeutic blockade of CTLA-4 in vivo, both in animal models and in humans [167–170]. The use of blocking CTLA-4or PD-1mAbs can sustain the activation and proliferation of tumor-specificT cells, preventing anergy or exhaustion and thereby allowing the development of an effectivetumor-specific immune response.

b.1) CTLA-4 Blockade—CTLA-4 is the best characterized among the inhibitory B7 receptors; like CD28, CTLA-4 also binds CD80 and 86 expressed by APCs, but provides inhibitory signals to theT cell, serving as a negative feedback loop. In animal models, CTLA-4 proved critical in the normal functioning immune system; CTLA-4 knockout mice developed uncontrolled lymphoproliferation with early lethalities. Furtermore, administration of CTLA-4 blockade significantly enhanced antitumor immunity in a variety of mouse models and with various therapeutic combinations including vaccine, TLR agonist, chemotherapy and radiation therapy. The mechanism by which CTLA-4 blockade induces tumor regression is not fully understood. Although Treg constitutively express CTLA-4, CTLA-4 blockade had no effect on the number or function of Treg in cancer patients [171]. It seems that CTLA-4 blockade activates directly CD4 and $CD8^+$ T effector cells by removing an inhibotory checkpoint on proliferation and function [172], and combination of direct enhancement of Teff cell function and inhibition of T reg activity is essential for mediating the full therapeutic effects of anti-CTLA-4 antibodies during cancer immunotherapy [173].

Based on these data, several clinical studies have been undertaken to assess the efficacy of a CTLA-4 antagonist antibodies in the setting of human cancer. The majority of clinical data to date have emerged from studies inpatients with melanoma (reviewed in [174]). Phase II studies with ipilimumab monotherapy showed objective responses at a dose of 3 to 9 mg/kg every3 weeks, with 10 mg/kg showing the best risk-benefit profile. Ipilimumab (3 mg/kg every 4 weeks × 4) administeredin combination with *chemotherapy (*dacarbazine) resulted in enhanced objective response rates without added toxicity. Ipilumimab has also been combined successfully with IL-2 (720,000 U/kg every8 hours × 15 (ORrate of 22% - three CRs) or melanoma vaccine (OR rateof 13% - two complete responses). In addition, tremelimumab has also produced OR and CRs in melanoma patients. To date, CTLA-4 antibody has not shown superiority over standard chemotherapy trial in treatment-naive melanoma patients in a phase III randomized clinical, which was discontinued after interim analysis. In melanoma patients, the most common grade 3 to 4 adverse events were colitis/ enterocolitis (increased frequency of diarrhea/stools), dermatitis, andhypophysitis.

Eleven patients with ovarian carcinoma, previously vaccinated with GM-CSF modified, irradiated autologous tumor cells (GVAX), received ipilumimab (1 month to 3 years following GVAX). Significant antitumor effects were observed in a minority of the patients. One patient achieved a dramatic fall in CA-125 levels several months after an initial dose of ipilumimab; although the response was not maintained, a second infusion resulted in a more rapid decline in CA-125 values. Nine additional infusions of the anti-CTLA-4 antibody spaced at 3- to 5-month intervals over nearly 4 years have maintained profound disease control, with grade 1 rash as the only adverse event. An objective radiographic response was noted in parallel with the reduction in CA125 Moreover, this patient's course was initially characterized by a remarkable periodicity to the rise and fall of CA-125 levels, but this gradually dampened as a function of time and/or prolonged therapy, demonstrating a lack of acquired resistance to ipilimumab commonly observed with cytotoxic chemotherapy [169].

In contrast to the melanoma cohort, a single dose of 3 mg/kg Ipilumimab triggered two cases of grade 3 gastrointestinal inflammation among the 9 ovarian carcinoma patients, consistent of significant diarrhea. Endoscopic biopsies revealed mucosal damage associated with abundant granulocytes, macrophages, CD4+ and CD8+ T cells, and FoxP3$^+$ Treg. The remaining seven patients showed only minor inflammatory toxicities including papular rash, or urticarial-like reactions at sites of prior vaccination [169]. Tumor regression in these patients correlated with the CD8$^+$/Treg ratio, suggesting that other forms of therapy that target Treg depletion may provide a highly effective form of treatment when combined with the tumor vaccine and CTLA-4 antibody arsenal [169].

b.2) PD-1 Blockade—Programmed death receptor-1 is a negative regulator of cell activation, primarily expressed on both B and T lymphocytes, which binds PD-L1 and PD-L2 ligands. PD-L2 is restricted to professional antigen presenting cells, while PD-L1 is expressed on many tissues, and plays a pivotal role in maintaining peripheral tolerance. Importantly, cancer cells have adopted expression of PD-L1 into their immunosuppressive arsenal [175]; a variety of epithelial cancers express PD-L1 [176–178]. It has also recently been shown that ovarian carcinoma cells as well as tumor-infiltrating tolerogenic DCs and myeloid derived suppressor cells express PD-L1 [179, 180], and indeed expression levels correlate with disease course.[1] Although PD-1 signaling can occur at very low levels of receptor expression, elevated levels of PD-1 have recently been attributed functional significance; during conditions of chronic antigen persistence (such as in chronic viral infections), antigen specific effector T cells expressing high levels of PD-1 were demonstrated to be functionally exhausted, i.e. unable to proliferate, secrete IL-2 or kill target cells [181]. It has been recently shown that tumor infiltrating lymphocytes in metastatic melanoma and other tumors expresses higher levels of PD-1 than CD4$^+$ and CD8$^+$ cells in the peripheral blood of healthy patients and exhibit phenotypic and functional characteristics of exhausted T cells with impaired effector function. These findings suggest that the tumor microenvironment can lead to up-regulation of PD-1 on tumor-reactive T cells and contribute to impaired antitumor immune responses [182]. Indeed, constitutive or inducible expression of PD-L1by tumors conferred resistance to immunotherapy in mice related to failure of antigen-specific CD8$^+$ CTLs to destroy tumor cells but without impairment of CTL function [183].

Following evidence that PD-1 blockade through gene therapy approaches in the tumor microenvironment activates antitumor immunity [184], antibodies blocking PD-L1 or PD-1 were found to profoundly enhance the efficacy of immune therapy [183, 185]. Anti-PD-1 antibody has been combined with cell-based vaccines and demonstrated increased infiltration, potency and duration of activity of CD8 T cells in the tumor microenvironment in the setting of B16 melanoma and CT26 colon carcinoma in mice.

Despite their similarities, the regulatory roles of CTLA-4 and PD-1 may differ substantially in cancer patients. First, Even though PD-1 and CTLA-4 ligation efficiently block T-cell responses, the homeostatic function of the two inhibitory receptors differs. For example, CTLA-4 ligation does not inhibit PI3-kinase signaling, while PD-1 engagement blocks the induction of PI3K activity. PD-1-mediated signaling blocks T-cell activation more effectively than CTLA-4-mediated signaling. In addition, PD-1 signaling may be related to T cell apoptosis. Second, whereas the ligands for CTLA-4, CD80 and CD86, are primarily expressed by mature antigen presenting cells, PD-L1 (on tumor cells or myeloid cells) and PD-1 (on effector T cells) are significantly upregulated in the tumor microenvironment. Thus, the effects of PD-1 blockade may be more pronounced in the tumor than in periphery. Indeed, a phase I study using PD-1 blocking antibody showed the antibody to be safe and well tolerated in patients with hematologic malignancies. No single maximum tolerated dose

was defined in this study. Clinical benefit was observed in 33% of the patients with one complete remission [186].

c) Depletion of T Regulatory Cells

As mentioned previously, CD4+CD25+Foxp3+ T regulatory cells are responsible for maintaining peripheral tolerance by inhibiting T cell activity therefore forming an obstacle for cancer immunotherapy. Depletion of Treg in vivo enhances tumor immunity in numerous animal models [187–189]. In clinical trials, a number of Treg-depleting strategies have been investigated [39, 105, 190–192]. An example is the use of cyclophosphamide in combination with vaccines [61, 193]. Other strategies for Treg depletion is through targeting the IL-2 receptor alpha chain, also known as CD25. In mouse models, the use of anti-CD25 monoclonal antibody before vaccination led to complete tumor rejection and establishment of long-lasting tumor immunity with essentially no autoimmune complications [194, 195]. Preclinical studies have shown that administration of anti-CD25 antibody linked to a potent pro-inflammatory toxin can selectively reduce Treg concentrations *in vitro* while maintaining other T cell populations [196]. This same molecule was used in patients with metastatic melanoma and showed consistent reduction in CD4+CD25+ Treg cells [197]. However, the effect was transient, with a half-life of the immunotoxin of only 2 hours. Another clinical appproach of targeting CD25 is through Denileukin diftitox, a fusion protein of IL-2 and diphtheria toxin that targets CD25-expressing cells used in patients with melanoma, ovarian cancer and renal cell carcinoma [198–201]. Although effective in short-term infusions, these conjugates are quite immunogenic and induce neutralizing antibodies, which hamper their long-term application.

Another agent is Daclizumab (Roche), which is an FDA-approved humanized IgG1-kappa mAb that binds specifically to CD25 [202]. Daclizumab inhibits T cell proliferation to recall antigens, but does not inhibit IL-15 effects. It has been used in autoimmune disorders [203, 204], acute graft-versus-host disease [205], and in cancer patients with $CD25^+$ T cell malignancies [206]. The advantage of Daclizumab is that it extremely well tolerated, and has a half-life of 20 days [207]. Furthermore, It is significantly less immunogenic than immunotoxins and can be given for longer periods of time. In a recent study, Daclizumab was used in a single dose of 1 mg/m^2 prior to hTERT peptide vaccine for metastatic breast cancer. Total $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ cells remained suppressed for several weeks during vaccination. There have been no significant toxicities. Importantly, administration of CD25 antibody was compatible with effective vaccination (Robert Vonderheide, unpublished observations).

CONCLUSIONS

In the past decade we have witnessed important advances. First, gynecologic cancers were proposed as potentially immunogenic tumors, a characterization formerly reserved only for melanoma and renal cell cancer. This has opened the door to explore immune therapy not only in HPV-induced cancers but also in ovarian and endometrial cancers. Second, the *a priori* notion that chemotherapy drugs antagonize immune mechanisms altogether was challenged by evidence that select chemotherapy drugs commonly used to treat gynecologic cancers have important immunomodulatory effects. This has opened the door to explore interactions of these drugs with natural antitumor immunity. Third, over the past decade several mechanisms of tumor immune escape, accounting for failure of immunotherapy, have been deciphered, and the importance of combinatorial immunotherapy targeting both adaptive and innate, effector and suppressor mechanisms has been proven. Fourth, this decade has produced novel and potent *bona fide* stimulants of innate and adaptive immunity, such as novel pleiotropic cytokines and Toll-like receptors, as well as powerful antibodies activating cellular immune mechanisms. Finally, the development of several syngeneic

mouse models of ovarian and other gynecologic cancers in immunocompetent animals is an important step forward in the preclinical development of these agents. The next decade will be the time to test and optimize these combinations to maximize efficacy and decrease toxicity. Rational combinations of agents will require understanding of their precise mechanism of action in order to select combinations yielding positive interactions. Careful preclinical evaluation in well-characterized animal models will be necessary to screen combinations before undertaking clinical studies.

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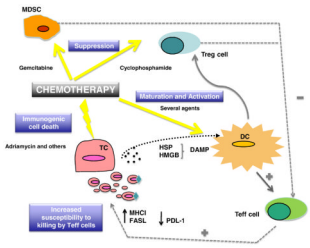

Figure 1.

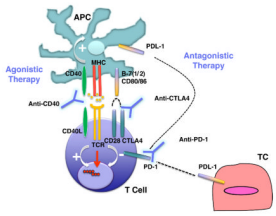

Figure 2.