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## Summary Points

- Active versus passive immunization.
- Public versus private antigen targeting.
- Naturally occurring TILs versus engineered T cells.
- Single therapy versus combinational approach.

## Background and Introduction

Although not traditionally considered immunogenic, increasing evidence indicates that ovarian cancers are, in fact, immunogenic tumors and are responsive to immunotherapies. Three distinct categories of data support this claim. First, there is accumulating evidence of spontaneous antitumor immune

responses and of their association with longer survival in a proportion of ovarian cancer patients. Second, and conversely, there is evidence of active tumor immune evasion mechanisms and their association with short survival in some ovarian cancer patients. And finally, preclinical as well as clinical data have now demonstrated that immunotherapy can be efficacious against these cancers.

It is now clear that spontaneous antitumor immune responses exist in many ovarian cancer patients. Tumor-reactive T cells and antibodies have been detected in peripheral blood of patients with advanced stage disease at diagnosis [1, 2], while oligoclonal tumor-reactive T cells have been isolated from tumors or ascites [3–11]. The tumor rejection antigens expressed by ovarian cancer have not been thoroughly characterized. Among the most promising candidates are cdr2, mesothelin, and NY-ESO-1 [12–14]. Several additional well-known tumor-associated antigens are recognized by peripheral blood or tumor-associated lymphocytes of many ovarian cancer patients. These include p53; HER2/neu; folate receptor- $\alpha$ ; cancer-testis antigens such as the MAGE melanoma antigen family members and sperm surface protein Sp17; mucins or glycoproteins such as Lewis(y), sialylated-Tn, CA-125, and MUC-1; and universal tumor antigens such as survivin and hTERT [15]. Importantly, the detection of an antitumor immune response in the form of intraepithelial (also called intratumoral) tumor-infiltrating lymphocytes (TILs), i.e., T cells infiltrating tumor islets, predicts significantly longer survival in ovarian cancer. We first reported in an Italian cohort that patients whose tumors had intraepithelial T cells experienced longer progression-free and overall survival as compared to patients whose tumors lacked intraepithelial T cells [16]. Survival at 5 years was substantial (38 %) in patients whose tumors had intraepithelial T cells ( $n=102$ ) and negligible (4.5 %) in patients lacking them ( $n=72$ ), even after complete response to chemotherapy. A signature of antitumor immune response activation was identified in tumors with intraepithelial T cells [16]. The impact

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of intraepithelial CD3<sup>+</sup> or CD8<sup>+</sup> T cells was confirmed by multiple independent studies on ethnically and geographically diverse populations [17–21]. Importantly, intraepithelial T cells were more prevalent in tumors with increased proliferation, indicating that improved outcome is not due to indolent tumor cell behavior [17].

Significant progress has been made recently in our understanding of immune evasion mechanisms operating in some patients with ovarian cancer. CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> T regulatory (Treg) cells were first demonstrated in ovarian cancer [22, 23], where increased Treg frequency predicts poor patient survival [20, 23]. Immunosuppressive B7-H4 expressing macrophages were recently found to correlate with survival in ovarian cancer [24]. In addition, ovarian cancer cells express programmed death ligand 1 (PD-L1 or B7-H1), a ligand for the immunosuppressive T-cell receptor PD1, which blocks T-cell responses. Expression of PD-L1 by tumor cells predicted paucity of intraepithelial TILs and short overall survival in ovarian cancer [19]. Further, overexpression of the endothelin B receptor (ET<sub>B</sub>R), which suppresses T-cell-endothelial adhesive interactions and T cell homing to tumor, correlated with absence of TIL and short survival in ovarian cancer [25, 26]. Finally, a recent study segregated high- and low-risk ovarian cancer patients based upon their tumor gene signature and found a strong correlation between decreased expression of immune genes and the development of high-risk tumors. In particular, high-risk tumors often displayed downregulation of genes involved in antigen processing and presentation [27].

The association of antitumor immune responses with prolonged survival and, vice versa, the association of immune escape mechanisms with poor survival suggest that ovarian cancers are intrinsically immunogenic. Indeed, ovarian cancers should no longer be considered immunologically inert tumors. Accordingly, pilot clinical data indicate that ovarian cancer patients can, in fact, respond to the same immunotherapy approaches as patients with other immunogenic tumors [28], including interleukin-2 (IL-2) [29, 30], anti-CTLA-4 antibody [31, 32], and adoptive transfer of ex vivo expanded TIL [33, 34]. Notably, each of these therapies is designed to exploit a preexisting endogenous antitumor immune response. Although, insufficient to reject tumor naturally, these responses can potentially be harnessed therapeutically. Here we will review three categories of immunotherapies which can be used to manipulate natural antitumor immunity or to induce new antitumor immune responses. These include cancer vaccines (active immunization), adoptive T-cell therapy (passive immunization), and nonspecific immunomodulation. Each targets immune cells in different ways. They can be used alone, together, or with conventional approaches for combinatorial tumor therapy.

## Cancer Vaccines

As with many other tumor types, vaccines have been the primary approach to ovarian cancer immunotherapy so far [15, 35–37]. Consistent with experience in other immunogenic tumors [38], vaccines have shown limited efficacy as monotherapy in patients with advanced recurrent disease. Clearly, much work is required to improve their performance. Current efforts to improve vaccines are directed broadly towards (a) optimizing the choice of antigens, (b) improving vaccine delivery systems to maximize the magnitude and quality (phenotype and polarization) of T-cell response, and (c) developing combinatorial approaches with adoptive T cell or immunomodulation therapy to maximize activation and function of vaccine-primed T cells in vivo.

## Pros

The results of some studies provide encouragement for further vaccine development. In a retrospective review of patients treated in the adjuvant setting after secondary complete response, Sabbatini and colleagues noted that patients vaccinated with monovalent or heptavalent vaccines against carbohydrate epitopes experienced significantly longer time to progression and higher progression-free survival rates relative to controls from the same institutions treated with alternative consolidation therapies [39]. In addition, vaccination with anti-idiotypic ACA-125, an analogue of CA-125, resulted in CA-125-specific antibodies and was associated with prolonged survival [40]. Another study was performed using CEA-MUC-1-TRICOM poxviral-based vaccines in 16 patients including 3 ovarian cancer patients. Immune responses to MUC-1 and/or CEA were seen following vaccination in 9 patients. A patient with clear cell ovarian cancer and symptomatic ascites had a radiographically and biochemically durable (18-month) clinical response [41]. In another study, vaccination against HER2 has resulted in sustained antigen-specific T-cell and humoral immunity as well as epitope spreading in ovarian cancer patients [42].

An alternative to vaccines directed towards specific antigens is whole tumor antigen vaccines created using tumor cells, autologous tumor lysate, or tumor-derived RNA [43–45]. Tumor antigen preparations can be injected into patients directly, or they can be first loaded onto autologous dendritic cells. Advantages of these vaccines include the opportunity to induce immunity to a personalized and broad range of antigens, which could minimize the development of tumor escape variants, the inclusion of yet unidentified tumor rejection antigens, no HLA haplotype restriction, and the simultaneous administration of MHC class I and class II epitopes, which could prove beneficial for immunologic memory. In a pilot study using mature DCs pulsed with whole

autologous tumor lysate, three of six subjects demonstrated remission inversion, i.e., their progression-free survival post-vaccination was longer than the interval between pre-vaccine recurrence and prior chemotherapy treatment [46]. The use of DC/tumor cell fusion approach is a viable alternative whereby autologous DCs are fused with tumor cells, which allows DCs to express the entire antigen repertoire of the tumor cells to CD4<sup>+</sup> and CD8<sup>+</sup> T cells. DC/ovarian tumor cell fusions have been generated and demonstrated to be able to induce antitumor CTL activity *in vitro* [47].

Several groups have used viruses to increase tumor cell immunogenicity for whole tumor cell vaccination. Objective responses have been seen after intracavity delivery of a viral oncolysate vaccine generated with ovarian cancer cell lines infected with influenza A virus [48, 49] or with autologous tumor cells infected with Newcastle disease virus [50]. We also performed preclinical studies using replication-restricted herpes simplex virus (HSV) 1 to infect autologous tumor cells for vaccine preparation. HSV-infected tumor cells used directly or pulsed on dendritic cells elicited potent antitumor immune response in the mouse, which was superior to the use of UV-irradiated tumor cells [51–53]. Thus, whole tumor antigen vaccines can produce objective response if immunogenicity is increased through the use of pathogens.

## Cons

A major limitation of cancer vaccines presently stems from the inability to elicit a rapid and overwhelming T-cell response, which is required to reject established tumors. This problem is magnified in ovarian cancer by the paucity of well-characterized rejection antigens to target and by the significant molecular heterogeneity of the disease [54]. Even when a defined target is available, and vaccination successfully induces an immune response, the long-term benefit can be limited by tumor evolution. In a recent study, one patient experienced complete objective response to NY-ESO-1 peptide vaccine, but later recurred with an NY-ESO-1-negative tumor, proving that single-target immunization can result in immune escape tumor variants following initial response [55].

A recent meta-analysis of 173 published, peer-reviewed immunotherapy trials revealed the low success rate of cancer vaccines to date. The trials involved patients with a variety of tumor types, including melanoma, renal cell and hepatocellular carcinomas, lung, prostate, breast, colorectal, cervical, pancreatic, and ovarian cancers. Patients received either molecular-defined antigens (synthetic peptides or proteins and viral or plasmid vectors encoding peptides or proteins; 1,711 patients) or whole tumor antigen (autologous or allogeneic tumor cells, dendritic cells pulsed with tumor extracts or mRNA; 1,733 patients). Overall, the authors calculated that 8.1 % of patients vaccinated with whole tumor antigen

had objective clinical responses while 3.6 % of patients vaccinated with molecularly defined tumor antigens had objective clinical responses ( $p < 0.0001$ , chi-square test) [56].

Although whole tumor vaccines offer distinct advantages, some drawbacks warrant consideration. First, surgical procurement of large numbers of autologous tumor cells may not be possible in many patients. Alternatives to this limitation exist, including use of allogeneic cell lines or the use of tumor mRNA. RNA electroporation of DCs is a convenient approach to generate a potent tumor vaccine [52]. An additional concern with whole tumor vaccination relates to the inclusion of a large number of “self” antigens, which could potentially drive tolerogenic responses, i.e., expand Treg rather than cytotoxic lymphocyte responses. Recent work has demonstrated that DCs can be polarized *ex vivo* with the use of interferons, Toll-like receptor agonists, or p38 mitogen-activated protein kinase (MAPK) inhibitors to drive cytotoxic lymphocytes and Th17 effector cells at the expense of Treg [57]. On the other hand, if immunization is successful, there may be increased concern for breaking tolerance to “self” antigens, leading to immunopathology. To date, pilot studies with whole tumor vaccines have reported no autoimmunity in patients with ovarian cancer.

There is a controversy in the choice of target antigen with cancer vaccines and adoptively transferred T cells, as well. In the past few decades, shared (also known as “public”) tumor-associated antigens have been the favored target of various immunotherapy strategies. This approach has been based largely on studies with melanoma [58]. This leads to the concept of “dispensable tissues,” meaning that in order to achieve tumor eradication, it was necessary to expect tissue-specific toxicity damaging normal tissues [59]. As the expression of these antigens was shared between most individuals, this would make the manufacturing of a universal vaccine a possibility. However, recent advances in the clinical application of immunotherapy suggest that immunotherapy with “personalized” antigens (that arise from mutations) with preexisting immunity, which are designed to stimulate antigen-specific memory T cells, could also be expected to induce rapid and strong secondary immune responses (reviewed in [60, 61]). The current view is that both approaches, targeting public or targeting private antigens, can be beneficial either in cancer vaccines or adoptive T-cell therapy, but to increase the clinical benefits, special attention should be paid to the immunological status of each patient by characterizing the preexisting immune responses to the targeted antigens before immunotherapy.

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## Adoptive T-Cell Therapy

Effective cancer immunotherapy is dependent on the presence of large numbers of antitumor lymphocytes with appropriate homing and effector functions that enable them to seek out and destroy cancer cells *in vivo*. The adoptive transfer of

ex vivo expanded tumor-reactive T cells holds the potential of achieving this condition in a short period of time. Clinical trials testing spontaneous or induced polyclonal or oligoclonal T cells conducted in the past two decades have provided crucial lessons that can guide further optimization. The use of ex vivo expanded TILs has yielded promising clinical results. Based on animal studies showing that host lymphodepletion prior to T-cell transfer enhances persistence of T cells and antitumor responses, a scheme of incremental lymphodepletion through high dose non-myeloablating chemotherapy and added whole-body radiation was tested. Infused cells were both long lived and highly penetrating, showing regression of voluminous metastatic tumors, with up to 16 % complete response and 72 % overall objective response rates in recent reports with maximal lymphodepletion and radiation. T-cell persistence correlated with long lasting responses [38, 62]. Although these are phase I studies involving a highly selected cohort of patients with metastatic melanoma with preexisting antitumor immunity, whose tumors yield tumor-reactive TILs, the results clearly demonstrate the power of adoptive immunotherapy and dispel the assumption that immunotherapy can only control small tumors [28]. Furthermore, although the role of CD8<sup>+</sup> T cells has been well established in adoptive immunotherapy [38, 62], CD4<sup>+</sup> cells can also produce objective responses [63].

Currently, attempts to improve the efficacy of adoptive TIL therapy are focused on two areas: (a) optimizing methods to select tumor-reactive TIL and expand them under optimal costimulation conditions and (b) optimizing host and/or tumor conditioning. Findings from melanoma trials argue that use of memory rather than effector cells may be more efficacious for adoptive transfer [64]. In these key studies, although infused cells dominantly displayed a highly differentiated effector cell phenotype (CD27<sup>-</sup> CD28<sup>-</sup> CD45RA<sup>-</sup> CD62L<sup>-</sup> CCR7<sup>-</sup>), TILs persisting 2 months after infusion in patients who exhibited tumor regression were characterized by a less differentiated phenotype (CD27<sup>+</sup> CD28<sup>+</sup> CD45RA<sup>+</sup> but CD62L<sup>-</sup> CCR7<sup>-</sup>) and longer telomeres [65–69]. Mouse models confirm these findings [70]. Because TILs comprise a large number of tumor-reactive effector cells, identification of culture conditions that preferentially expand memory phenotypes is a priority. Recent technological advances with the development of artificial antigen-presenting cells (aAPCs) expressing a variable repertoire of costimulatory molecules and cytokines have generated new opportunities to provide the desired costimulatory molecules and cytokines to reeducate TILs, improving their potency and function in vivo. Carl June and colleagues have described the development of a next-generation K562-based aAPC platform capable of expressing multiple gene inserts, including human lymphocyte antigen (HLA)-A2; CD64 (the high-affinity Fc receptor), CD80, CD83, CD86, CD137L (4-1BBL), and CD252 (Ox40L); and a variety of T-cell supporting cytokines [71].

Cell-based aAPCs have proven to be more efficient at activating and expanding CD8<sup>+</sup> CD28<sup>-</sup> T cells, and antigen-specific T cells, than the magnetic bead-based aAPC [71].

TIL therapy is only possible for a fraction of patients. To generate TIL, a tumor mass must first be resected, which is not always possible. Additionally, that tumor mass must contain TIL, and those TIL must be responsive to the existing ex vivo expansion protocols. For many patients, these limitations make TIL therapy impossible. One strategy to make adoptive therapy available to a larger patient population involves engineering polyclonal T cells to redirect their specificity towards tumor antigens. This can be accomplished by transducing lymphocytes with a cloned T-cell receptor (TCR) of high affinity to tumor-associated epitopes. In this case, the cloned heterodimeric TCR is transduced to mixed peripheral blood T cells isolated from the patient, creating a large population of bispecific T cells, which are polyclonal with respect to their original TCR, but potentially monoclonal for the cloned TCR [72].

A second strategy to generate novel tumor-targeted T cells is to transduce the polyclonal population with receptors that recognize antigens in an MHC-unrestricted fashion. These so-called chimeric antigen receptors (CARs) are fusion genes encoding an extracellular domain that specifically binds to tumor epitopes through a single-chain variable fragment (scFv) linked to intracellular signaling modules (such as the CD3 zeta chain, TCRz) that mediate T-cell activation [72–74]. The scFv contains the V<sub>H</sub> and V<sub>L</sub> chains of an antitumor antibody joined by a peptide linker of about 15 residues in length, and it confers the parental antibody's specificity to the transduced T cells. In principle, universal targeting vectors can be constructed, because the scFvs bind to native cell surface epitopes and bypass the requirement for MHC restriction [75, 76]. Thus, in comparison to TCRs, CARs have two major advantages: (a) their HLA-independent recognition of antigen, which makes them broadly applicable regardless of the subject's HLA and regardless of the level of HLA expression on tumor cells, and (b) their signaling, which redirects T-cell cytotoxicity and permits T-cell proliferation and survival upon repeat antigen exposure. A potential drawback stems from their potential immunogenicity, if scFv are nonhuman. This can be averted by using human scFv.

## Pros

There is evidence that TIL-based adoptive therapy is an important opportunity in ovarian cancer. In the early 1990s, ovarian cancers were found to yield reactive TILs after IL-2 culturing in vitro [77, 78]. Moreover, in pilot clinical trials, patients who received adjuvant therapy with adoptive transfer of tumor-derived lymphocytes expanded ex vivo with IL-2, following surgical debulking and frontline chemotherapy,

showed a survival advantage [33, 34]. Stage III EOC patients treated with consolidation adoptive transfer of expanded TILs after completion of cisplatin-based frontline chemotherapy ( $n=13$ ) had a 3-year overall survival rate of 100 %, while that of a control group of patients ( $n=10$ ) receiving only chemotherapy was 67.5 % ( $p<0.01$ ). The 3-year disease-free survival rate of the patients in the TIL group and in the control group was 82.1 and 54.5 %, respectively. While these results can be limited by the lack of randomization, they nevertheless support the feasibility of adoptive therapy for ovarian cancer [33].

TCR-based engineering represents a potentially powerful strategy for ovarian cancer therapy as TCRs that recognize HLA-A2-restricted epitopes from known ovarian cancer antigens such as NY-ESO-1 and p53 are available for clinical testing as well [79–82]. Optimization through selection of naturally occurring or recombinant high-affinity receptors, engineering to prevent recombination with endogenous TCR, and the use of lentiviral vectors developed in the June lab with transfection efficiency above 90 % are poised to improve this approach significantly [83].

Adoptive transfer of T cells engineered to express chimeric receptors is also expected to be useful for ovarian cancer patients once the tools are refined. Some of the CARs investigated *in vitro* and *in vivo* target ovarian cancer antigens including FBP [84, 85], MUC-1 [86], HER-2, and mesothelin [87].

## Cons

So far, there has only been a single study of adoptive transfer of CAR T cells in ovarian cancer [88]. Patients received autologous T cells which had been transduced with an FR $\alpha$ -specific CAR. While this study demonstrated safety, the results were disappointing. There were no clinically evident tumor responses—most likely due to low expression of the transgenic CAR and poor persistence of the transferred T cells [88]. However, strategies to address these issues are being developed. For instance, T-cell persistence can be dramatically improved by using human scFv and by adding costimulatory signaling capabilities to the intracytoplasmic domain of CARs. Indeed, one issue needing to be addressed with CARs is that signaling through the cytosolic domain of the usual scFv-TCRz construct does not fully replicate the multichain TCR signaling complex. This can be solved by incorporating additional signaling modules in the cytoplasmic domain of the chimeric receptor. The value of such innovations was recently demonstrated in a mouse xenograft model. Similar to the unsuccessful clinical trial, T cells were transduced with a CAR targeting FR $\alpha$ . However, in this study, the signaling domain of costimulatory molecule CD137 was added to the CAR's intracellular tail. When transferred into mice, these CAR T cells demonstrated enhanced *in vivo*

persistence and tumor infiltration and achieved tumor regression superior to that seen in mice treated with T cells lacking the CD137 signaling domain [89].

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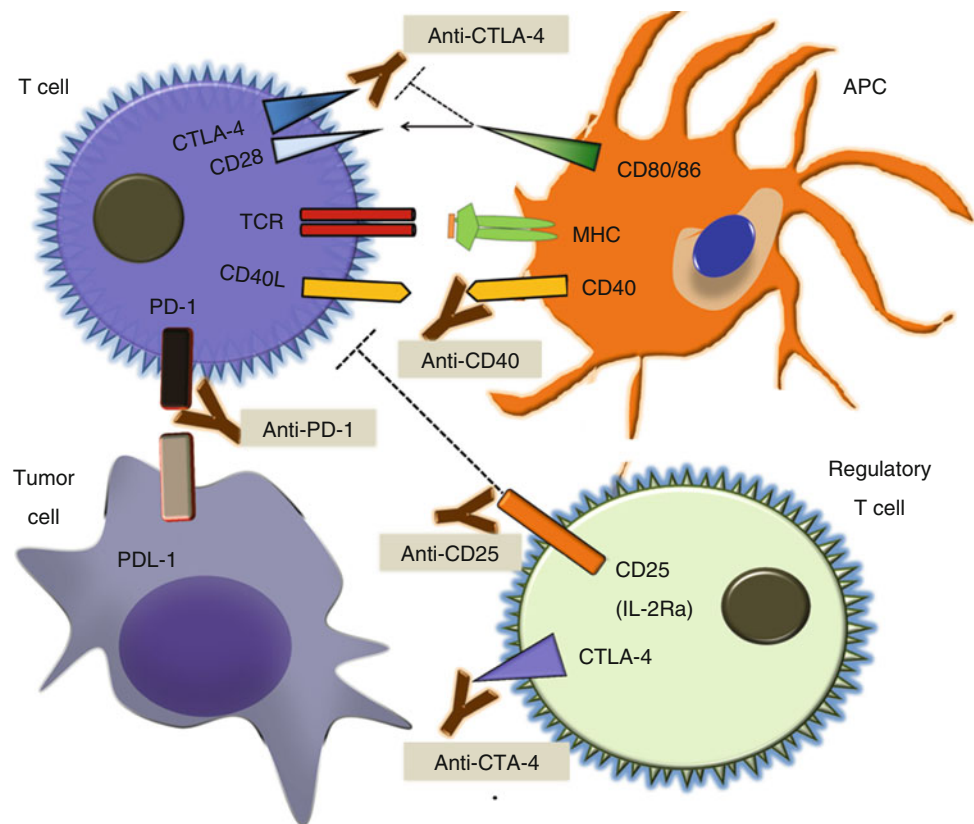
## Nonspecific Immunomodulation

Given the limitations of immunotherapy, there is a reason to hope that modulating immune checkpoints (Fig. 29.1) by activation of effector cells, depletion of Tregs, or activation of professional APCs could substantially improve the therapeutic efficacy of vaccines or adoptively transferred T cells. Certain chemotherapy regimens promote antitumor immunity through each of these mechanisms. Additionally, a number of nonspecific immunotherapies, including immunomodulatory cytokines, Toll-like receptor (TLR) agonists, and functional antibodies, are being developed to achieve these goals. Many of these nonspecific therapies may prove to be valuable adjuvants to more targeted immunotherapies, including vaccination and adoptive T-cell therapy.

## Pros

The immunomodulatory effects of chemotherapy can be broadly grouped into three mechanisms. First, chemotherapy-induced tumor cell death can result in *in situ* vaccination. Drugs such as doxorubicin, idarubicin, mitoxantrone, and oxaliplatin induce immunogenic tumor cell death, which facilitates tumor antigen uptake by professional antigen-presenting cells and subsequent antigen presentation to antitumor T cells. Second, some chemotherapy drugs can also induce direct activation of antigen-presenting cells. Since the 1980s, it has been recognized that cyclophosphamide administered at standard dose prior to cancer vaccines significantly enhanced immunotherapy. However, the mechanism of this phenomenon was initially unclear [90]. A recent study in the mouse reported that a myelosuppressive dose of cyclophosphamide induces rebound myelopoiesis and leads to the emergence of tumor-infiltrating DCs that secrete more IL-12 and less IL-10 and are fully capable of priming T-cell responses [91]. In addition, metronomic or low-dose, non-myelotoxic administration of paclitaxel, doxorubicin, vincristine, and other drugs can cause activation and maturation of DCs, including increased IL-12 secretion, a critical factor required for T-cell priming. Signaling via STAT4 and Rho GTPases may account for these effects [92]. The third mechanism by which chemotherapy achieves immunomodulation is through suppression of immune inhibitory cells. For instance, oral administration of metronomic cyclophosphamide was shown to induce a profound and selective reduction of circulating CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells and restored T and NK effector functions in end-stage cancer patients [93]. Cyclophosphamide may also have additional effects contributing to restoration of

**Fig. 29.1** Modulation of immune checkpoints can lead to improved therapeutic efficacy of vaccines or adoptively transferred T cells



the immune response; it can enhance IFN- $\gamma$  production by splenocytes in a mouse model [94]. Conventional paclitaxel therapy also caused a significant decline in both numbers and activity of Treg, enhancing CD4<sup>+</sup> and CD8<sup>+</sup> activity systemically in patients with non-small cell lung cancer [95]. The mechanisms behind each of these immunomodulatory mechanisms are quite complex, and our understanding is still in its infancy. But effects appear to be dependent on drug type, dose, and schedule as well as the immune cell type.

Pleiotropic immune activation can also be achieved with cytokines and Toll-like receptor agonist therapy. Type I and II interferons and IL-2 are the most extensively studied cytokines for tumor therapy.

IFN- $\gamma$  has been shown to have direct antiproliferative activity on ovarian cancer cells in vitro, which proved to be synergistic with cisplatin and doxorubicin [96–98]. In vitro and in vivo, IFN- $\gamma$  upregulates HLA class I and class II molecules and antigen presentation in ovarian tumor cells [99], a requisite for recognition by T cells. In fact, HLA class I expression by the tumor correlates with the intensity of T-cell infiltration [100], a predictor of longer survival. Furthermore, IFN- $\gamma$  has antiangiogenic effects [101].

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). 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 of 24 h duration was relatively well tolerated and demonstrated evidence of long-term efficacy in a modest number of patients. The toxicities of systemic IL-2 are significant; however, the peritoneal delivery method appeared to reduce the number and severity of the toxicities until the concentration in the intraperitoneal infusion reached the point where serum IL-2 became detectable. The appearance of systemic toxicity such as hypotension and thrombocytopenia, as well as locoregional dose-limiting toxicity (catheter infection), was associated with the highest doses. 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 [29]. Recently, the therapeutic potential of several additional cytokines has been of increasing interest. 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 [103–112].

Like cytokines, TLR agonists have multifaceted stimulatory effects on the immune system. TLR triggering induces DC maturation, which leads to the upregulation of costimulatory molecules, including CD40, CD80, and CD86, and secretion of immunomodulatory cytokines and chemokines. In

addition, TLRs can directly stimulate the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as reverse the suppressive function of Treg cells [113–115]. 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 [116], non-Hodgkins lymphoma [117, 118], glioblastoma [119], superficial basal cell carcinoma [120], and melanoma [121–124]. Adding TLR 3, 4, 7, or 9 ligands was shown to activate CD8<sup>+</sup> cytotoxic T cells with increased IFN- $\alpha$  production and promote a stimulatory cytokine milieu at the tumor microenvironment [125, 126].

The use of antibodies to block T-cell inhibitory receptors such as CTLA-4 and PD-1 can lead to sustained activation and proliferation of tumor-specific T cells, preventing anergy or exhaustion and thereby allowing the development of an effective tumor-specific immune response. The majority of clinical data to date have emerged from studies in patients with melanoma [127], where CTLA-4 blockade has yielded objective responses. In a small study of ovarian cancer patients, one patient experienced a durable objective radiographic response. Multiple infusions of anti-CTLA-4 antibody every 3–5 months maintained disease control over 4 years [31]. The toxicities of CTLA-4 treatment showed similar pattern compared with those shown in melanoma patients, namely, grade I, rash in most of the patients (8/9); grade I or II, constitutional symptoms in 33 % (3/9) and sweet's syndrome in 22 % (1/9); and grade III, diarrhea in 22 % of the patients (2/9). Tumor regression correlated with the CD8<sup>+</sup>/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 [31].

Another way to enhance antitumor T-cell activity is through blockade of the PD-1 pathway. PD-1, expressed on activated T cells, binds PD-L1 and PD-L2 ligands. PD-L2 is restricted to professional antigen-presenting cells, while PD-L1 is expressed on many tissues. Importantly, ovarian carcinoma cells as well as tumor-infiltrating tolerogenic DCs and myeloid-derived suppressor cells express PD-L1 [128, 129], and expression levels correlate with disease course. Constitutive expression of PD-L1 by tumors conferred resistance to immunotherapy in mice [130], while antibodies blocking PD-L1 or PD-1 profoundly enhanced the efficacy of immunotherapy [130, 131]. A phase I study using PD-1 blocking antibody showed the antibody to be safe and well tolerated in patients with hematologic malignancies. Clinical benefit was observed in 33 % of the patients, with one complete remission [132].

Antibodies targeting the IL-2 receptor  $\alpha$  chain (also known as CD25) can be used to deplete Tregs. 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 no autoimmune complications [133, 134]. Daclizumab, which is an FDA-approved humanized IgG1- $\kappa$  mAb that binds specifically to CD25

[135], has been used in autoimmune disorders [136, 137], acute graft-versus-host disease [138], and in cancer patients with CD25<sup>+</sup> T-cell malignancies [139]. The advantage of daclizumab is that it is well tolerated and has a half-life of 20 days [140]. In a recent study, daclizumab was used in a single dose of 1 mg/m<sup>2</sup> prior to hTERT peptide vaccine for metastatic breast cancer. Total CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells remained suppressed for several weeks after a single infusion. Importantly, administration of anti-CD25 antibody was compatible with effective vaccination [141].

The main mechanism of immune stimulation by CD40 agonists (including recombinant CD40 ligand and agonistic anti-CD40 antibodies) is activation of CD40-expressing DCs, resulting in increased survival, upregulation of costimulatory molecules, and secretion of critical cytokines for T-cell priming, such as IL-12. In vitro human cell studies have also been conducted to evaluate whether recombinant CD40L is able to stimulate maturation of DCs derived from ovarian cancer patients. In one study, autologous DCs from ten ovarian cancer patients were pulsed with killed primary tumors as a source of tumor antigens. DCs were then cultured in the presence of TNF, TRANCE (tumor necrosis factor-related activation-induced cytokine), and CD40L to induce maturation. These mature whole lysate-pulsed DCs were able to stimulate CD8<sup>+</sup> T cells that secreted IFN- $\gamma$  in responses to ovarian tumor antigens. Similar results were also obtained in another study where DCs derived from ovarian cancer patients who were in remission were first loaded with HOCI-SKOV-3 tumor lysate and subsequently matured with activating anti-CD40 antibody [142]. In this study, mature DCs were able to stimulate both CD8<sup>+</sup> and CD4<sup>+</sup> antitumor T-cell responses. All these results highly suggested a potential benefit of using CD40L or anti-CD40 activating antibody as an adjuvant in DC-based whole tumor cell immunotherapy. Additional value of administering CD40 agonists in vivo is provided by the fact that ovarian cancers, like many tumors, express the CD40 receptor [143–146] and respond to CD40 ligation with apoptosis and growth inhibition in vitro and in vivo [145, 147, 148].

## Cons

The usefulness of IL-2, although FDA approved for treatment of melanoma and renal cell carcinoma, has several limitations. Alone or 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. Another manifestation of IL-2 toxicity is capillary leak syndrome, resulting in a hypovolemic state and fluid accumulation in the extravascular space [149]. Additionally, IL-2 is essential for the

peripheral homeostasis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, and it is now known that IL-2 is also an important activator of Treg suppressive activity in vivo [150].

Many clinical trials have demonstrated the efficacy of type I interferon therapy in the treatment of hematologic malignancies [151–153], melanoma [154–158], and renal cell carcinoma [159–161]. In contrast, trials in ovarian carcinoma were less encouraging. Intraperitoneal recombinant IFN- $\alpha$  alone or combined with cisplatin as salvage therapy for persistent ovarian cancer after primary chemotherapy has shown clinical efficacy in small volume disease [162, 163], but there was no significant effect in a cohort of patients with recurrent, platinum-resistant disease [164]. A large randomized, phase III trial ( $n=300$ ) conducted in patients with epithelial ovarian cancer concluded that INF- $\alpha$ 2a as maintenance therapy following surgery and/or chemotherapy is not effective alone [165].

Conflicting results from trials involving IFN- $\gamma$  administration highlight the difficulty in designing immunomodulation therapies. In one instance, a threefold prolongation of progression-free survival was observed in a phase III multicenter study from Europe with subcutaneous administration of rhIFN- $\gamma$  combined with MTD cisplatin and cyclophosphamide chemotherapy, with minimal added toxicity [166]. However, in a subsequent randomized phase III trial conducted in the USA, addition of subcutaneous rhIFN- $\gamma$  to carboplatin and paclitaxel did not improve survival [167]. Although one cannot exclude that racial and other demographic differences may account for opposite results, these data may indicate that the choice of chemotherapy drugs is in fact critical in combinatorial approaches with immunotherapy. Indeed, whereas cyclophosphamide has potent immunomodulatory effects on suppressive Tregs, 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.

Similarly, the use of TLR agonists in the clinic requires careful preclinical evaluation. For example, in the absence of specific cell-mediated antitumor immunity, nonspecific activation of inflammation might in fact promote tumor growth rather than reducing it [168]. TLR4 agonists were shown to promote tumor cell survival, tumor growth, and paclitaxel resistance in a proportion of ovarian cancer cells [169, 170].

Meanwhile, agonistic anti-CD40 antibody is best used in combination with vaccines or TLR agonists [171, 172]. This is because, when used alone, it can accelerate the deletion of tumor-specific cytotoxic lymphocytes [173].

## Conclusions

In the past decade, we have witnessed important advances in the development of immunotherapies for gynecologic cancers. First, ovarian cancers are now seen as potentially immunogenic tumors, a characterization formerly reserved only for melanoma and renal cell cancer. 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, 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. 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.

## Future Directions

Evidence now convincingly shows that ovarian cancers are immunogenic tumors. The dramatic advances in laboratory technology and clinical procedures in cellular immunotherapy, along with the development of powerful immunomodulatory antibodies, create new opportunities in ovarian cancer therapeutics. The challenge for the next decade will be to test rational combinations that offer maximal clinical benefit at the lowest cost.

Selection of appropriate patients for clinical trial participation will also be quite influential. Additional biomarkers are needed to maximize selection of patients who may benefit from immunotherapy. Evidence to date indicates that many ovarian cancer patients display a spontaneous antitumor immune response. These patients may be best suited for vaccine therapy or TIL-based therapy as they are the most likely to harbor a natural repertoire of tumor-reactive T cells with tumor rejecting potential that can be expanded in vivo or ex vivo. In addition, patients whose tumors exhibit intraepithelial T cells may be most likely to respond to immunotherapy as the tumor microenvironment is already conducive to T-cell homing and engraftment. Finally, more work will be necessary to develop strategies to integrate immunotherapy with current standard of care. We have previously demonstrated that patients with advanced ovarian cancer whose tumors exhibit low frequency of intraepithelial CD8<sup>+</sup> T cells or high Ki67 expression are more likely to draw benefit from aggressive surgical cytoreduction, while debulking did not significantly affect the survival of patients with brisk CD8<sup>+</sup> T cells or low Ki67 expression [17]. It is possible that immunotherapy with adoptive transfer of TILs and/or vaccine plus immunomodulation could be a rational



adjuvant therapy for patients with intraepithelial T cells following conventional debulking surgery and chemotherapy. Based on the observation that VEGF antibody blockade enhances T-cell infiltration in tumors and that its efficacy depends on antitumor CD8 T-cell response [174], it is possible that patients with intraepithelial T cells may also respond better to bevacizumab or other VEGF inhibitors. On the other hand, our data suggest that maximal debulking efforts should be undertaken in tumors with low T cells and it is possible that these patients are not the best candidates for adjuvant immunotherapy that exploits natural antitumor immune response. Personalized adoptive therapy with engineered T cells redirected against known tumor epitopes might be the most efficient approach to adjuvant immunotherapy in patients with low level of naturally occurring TILs. Careful preclinical evaluation in well-characterized animal models will be necessary to evaluate combinations before undertaking clinical studies. However, the major challenge facing the field at present is to conduct randomized clinical trials demonstrating sufficient clinical benefit to justify the logistics and expense of customized cellular therapies. A positive outcome from immunotherapy trials in terms of effective therapy, extension of progression free, and overall survival would represent a major advancement for patients with advanced ovarian cancer.

#### Concluding Comments

- Appropriate diagnostic methods are needed to identify patients suitable for immunotherapy.
- Novel pharmacodynamics biomarkers need to be implemented to provide proper metrics for effectiveness of immune therapies.
- New strategies need to be developed to integrate immunotherapy with standard of care or targeted therapies to offer long-term and durable benefit.
- Formulation of effective, scalable, and reproducibly manufactured vaccines, T-cell products and immunomodulatory agents will be necessary to make these therapies commercially viable.
- Randomized clinical trials need to be conducted to demonstrate sufficient clinical benefit in order to justify the expense of immunotherapies and the integration of them into standard of care.

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