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Cellular immunotherapy for ovarian cancer

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Abstract

Background—Ovarian cancer is frequently diagnosed at an advanced stage, and although initially responsive to surgery and chemotherapy, has a high rate of recurrence and mortality. Cellular immunotherapy may offer the prospect of treatment to prevent or delay recurrent metastatic disease.

Objective—To provide an overview of current innovations in cellular immunotherapy for ovarian cancer, with an emphasis on dendritic cell vaccination and adoptive T cell immunotherapy.

Methods—Three key areas are explored in this review. First, an appraisal of the current state of the art of cellular immunotherapy for treatment of ovarian cancer. Second, a discussion of the immunological defenses erected by ovarian cancer to prevent immunological attack, with an emphasis on the role of tumor-associated regulatory T cells. Third, an exploration of innovative techniques that may enhance the ability of cellular immunotherapy to overcome ovarian tumor-associated immune suppression.

Results/conclusion—Ovarian cancer is recognized as a paradigm for tumor-associated immune suppression. Innovative approaches for antagonism of tumor-associated regulatory T cell infiltration and redirection of self antigen-driven regulatory T cell activation may provide the key to development of future strategies for cellular immunotherapy against ovarian cancer.

Keywords

Ovarian cancer; Regulatory T cells; $CD4^+$ T cells; Th17 T cells; Dendritic cells; Interleukin-2; Interleukin 1β; Interleukin-15

1. Introduction

The search for effective treatments for ovarian cancer has been a source of frustration for decades, and ovarian cancer remains the disease with the highest mortality rate among gynecological tumors. Patients with advanced disease have a response rate of over 80% following surgery and adjuvant chemotherapy with platinum-taxane, with a median progression-free interval of 18 months [1]. Unfortunately, the majority of these patients will recur, and the overall 5-year survival rate for patients with advanced stage disease is only 23-30% [2]. One of the major reasons why the survival rates are so poor is that as many as three-quarters of patients present with advanced disease at the time of diagnosis [1], indicating that there is a clear need for techniques that would facilitate earlier diagnosis of ovarian cancer. The need for development of novel treatments that prevent disease recurrence or progression following first-line treatment is also a matter of some urgency. Immunotherapy based on the induction of tumor-specific helper T cell and cytotoxic T lymphocyte (CTL) responses may have potential efficacy, but ovarian cancer presents many barriers to the development of successful cellular immunotherapies. However, improved understanding of the mechanisms of tumor-associated immune regulation has raised the prospects for immunological treatment of ovarian cancer, based in part on manipulation of dendritic cells (DC) to facilitate programming of antitumor effector T cell responses. This review will discuss the barriers to

cellular immunotherapy that are imposed by ovarian tumor-associated immunosuppression, and will consider potential strategies by which these barriers may be overcome, with an emphasis on the central role of DC in immune regulation.

2. Dendritic cell immunotherapy

Although DC-based immunotherapy or vaccination has been widely explored for the treatment of many other malignancies, investigation of the potential for DC immunotherapy of ovarian cancer has been relatively limited. Schlienger and colleagues have shown that DC pulsed with killed autologous primary ovarian tumor cells induced antigen-specific T cells that secreted IFNγ upon stimulation with autologous tumor cells [3], suggesting that antigen-pulsed DC may be a viable option for therapeutic vaccination against ovarian cancer. Various reports showing that DC loaded with tumor lysates, DC pulsed with acid-eluted peptides from ovarian cancer cells, DC fused with ovarian tumor cells, or DC loaded with ovarian tumor cells killed by oxidation could induce HLA class I-restricted CTL responses against autologous ovarian tumor cells [4-8] support this position. A limitation of these approaches is that the identity of the tumor antigens recognized by DC-stimulated CTL is not well defined, and it is not clear that T cell responses retain specificity for the tumor. Therapeutic vaccination with DC loaded with defined ovarian tumor antigens of known tissue distribution would be preferable, both in terms of clinical benefit and limitation of autoimmune toxicity, particularly for long-term control of disease progression.

In the clinical setting, a phase I trial of autologous tumor antigen-loaded DC vaccination in 6 patients with ovarian cancer revealed no significant toxicity and 3 of 6 patients showed stable disease lasting 25 to 45 weeks [9]. Lymphoproliferative responses to tumor antigen were detected in 2 patients. The most durable clinical response to DC vaccination was described in a case report of a patient with recurrent metastatic ovarian cancer, who received 10 vaccinations of autologous DC loaded with mRNA encoding folate receptor-α [10]. Follow-up CT at 5 months after the last vaccination showed a partial response, and CT at 16 months showed greater than 50% remission of lymph node metastases. CA-125 levels were greatly reduced after the 1^{st} vaccination (from 640 U/mL to 60 U/mL) and remained at baseline 11 months after completion of vaccination. A clinical trial of MUC1 and HER2/neu peptide-pulsed DC vaccination in patients with advanced ovarian or breast cancer reported peptide-specific CTL responses in 5 of 10 patients [11], and also showed evidence of epitope spreading. In one patient vaccinated with MUC1 peptides, carcinoembryonic antigen and MAGE3 peptide-specific T-cell responses were detected, and in a second patient, MUC1-specific T-cell responses were detected after seven vaccinations with HER2/neu peptide-pulsed DC [11]. These results are interesting and encouraging, notwithstanding the limitations of HER2/neu as a target antigen for ovarian tumor immunotherapy, since this antigen is expressed in only about 10% of ovarian cancers.

Probably the best known ovarian tumor antigen is CA125, but it has received surprisingly little attention as a target for cellular immunotherapy. Possible reasons may have included a lack of information on the primary sequence, and the consequent difficulty of producing CA125 in recombinant form. However, the primary sequence and structure of CA125 has now been elucidated, revealing a core protein with a mass exceeding 2 million Da [12]. Knowledge of the sequence has facilitated identification of HLA class I-restricted epitopes, resulting in DC-driven generation of CA-125-specific $CD8^+$ CTL responses capable of killing ovarian tumors that express CA125 [13].

Serine proteases represent a group of antigens with potential as targets for cellular immunotherapy of ovarian cancer [14]. Of these antigens, stratum corneum chymotryptic enzyme (SCCE) in particular holds promise, based on a high frequency of expression by

ovarian tumors and limited distribution in normal tissues. Quantitative RT-PCR analysis indicated that SCCE was overexpressed in >88% of serous ovarian tumors, and 100% of endometrioid and clear cell tumors, respectively [15]. Using computer analysis of algorithms derived for estimation of epitope binding affinities to HLA DR molecules [16], peptide sequences with multiple DR-binding motifs have been identified within SCCE. DC loaded with one such peptide, SCCE 110-139, were capable of stimulating $CD4^+$ Th1 T cell responses from individuals of diverse HLA class II backgrounds, and were also capable of cross-priming $CD8^+$ CTL responses restricted by HLA A2.1 and other class I molecules [17]. This approach thus has the virtue of stimulating both $CD4^+$ helper T cell responses and $CD8^+$ CTL responses in a broad patient population, without suffering from the limitations of HLA type that are usually associated with peptide-based strategies.

3. Adoptive T cell immunotherapy

The presence of tumor-infiltrating T cells in ovarian cancer has been associated with improved survival [18], suggesting that T cell immunotherapy may afford clinical benefit. This idea is reinforced by studies demonstrating expansion of tumor-infiltrating lymphocytes (TIL) for treatment of ovarian cancer patients [19,20] and in vitro activation of tumor-specific T cells derived from peripheral blood or TIL [4,5,21]. More recent studies have explored the potential of adoptive transfer with T cells engineered to express receptors that endow antitumor activity. Drawing on the observation that over 80% of ovarian tumors express receptors for NKG2D (an agonist NK receptor), Barber and colleagues transduced T cells with a chimeric receptor linking NKG2D with the CD3ζ chain [22], and showed that NKG2D/CD3ζ T cells expressed inflammatory cytokines when cultured with autologous tumor cells from ovarian cancer patients. They further showed that chimeric receptor-expressing T cells could be used therapeutically in a mouse model of ovarian cancer [22]. Another promising strategy embodies the use of T cells engineered to express chimeric receptors composed of tumor antigen-specific single chain variable antibody fragments linked to T cell signaling domains. A very recent report has described genetically redirected T cells expressing a single chain antibody specific for mesothelin (an important ovarian tumor antigen) coupled to signaling domains from the T cell receptor (CD3) ζ chain, CD28 and CD137. These mesothelin-specific T cells could lyse mesothelin-expressing ovarian and mesothelioma tumor cells *in vitro*, and could control established mesothelioma tumor xenografts in NOD/SCID mice [23]. T cells engineered to express a single chain antibody specific for folate receptor have been tested in a phase I clinical trial for treatment of metastatic ovarian cancer [24]. Some patients experienced grade 3 to grade 4 toxicity, probably attributable to co-administration of IL-2, but otherwise treatment was well tolerated. The transferred T cells did not persist in the circulation, being barely detectable one month after transfer, and tracking studies with In^{111}-labeled T cells showed a failure of T cells to home to the tumor [24]. From this experience, one may conclude that improved T cell persistence and homing ability are needed for adoptive T cell transfer to offer the prospect of clinical benefit. However, it is possible that adoptively transferred T cells may still afford some benefit through recruitment of more durable endogenous anti-tumor immune responses, as suggested by the studies of Sentman and colleagues [22].

3.1 The increasing significance of $CD4^+$ T cells in cellular immunotherapy

Although the conventional wisdom of cellular immunotherapy dictates that an emphasis should be placed on stimulation of tumor-specific $CD8^+$ CTL responses, accumulating evidence points to a critical, and possibly dominant, role for tumor antigen-specific $CD4^+$ T cell responses in effective anti-tumor immunity [25-28]. A notable recent study showed that $CD4^+$ T cells could eliminate tumors that are resistant to $CD8^+$ T cell-mediated rejection, even when the tumors expressed MHC class I, but not MHC class II, suggesting that $CD4^+$ T cell responses could outperform CD8+ CTL in mediating antitumor effector function [29]. The significance of these

findings is underlined by a novel report of successful treatment of metastatic melanoma by tumor antigen-specific $CD4^+$ T cell immunotherapy [30]. From these observations, we propose that, in contrast with most prior studies, cellular immunotherapy or antitumor vaccination should place an emphasis on activation of ovarian tumor antigen-specific $CD4^+$ T cell responses. In addition to the above noted work on HLA class I and class II-binding epitopes within an extended peptide from SCCE [17], this principle has been recognized by two recent studies, the first of which describes activation of both $CD4^+$ and $CD8^+$ T cell responses following vaccination of ovarian cancer patients with a peptide containing both HLA class I and class II-binding epitopes from NY-ESO [31]. Extending this work, these investigators have identified two novel HLA class I-restricted NY-ESO epitopes embedded within a region containing promiscuous HLA DR-binding epitopes, thus broadening the potential number of ovarian cancer patients that may benefit from vaccination [32].

4. Barriers to cellular immunotherapy for ovarian cancer

4.1 Tumor-infiltrating regulatory T cells

The first evidence that ovarian tissues may be immunogenic was provided by a study published in 1969, revealing that neonatal day 3 thymectomy of female mice resulted in loss of the ovaries [33]. This curious result was first interpreted as evidence for an endocrine link between the thymus and ovarian development, but subsequent studies revealed heavy lymphocytic infiltration and autoimmune destruction of the ovaries, and further showed that if the mice were reconstituted with normal T cells, the autoimmune process was inhibited [34]. The most probable mechanism is that neonatal thymectomy resulted in loss of production of innate, thymus-derived regulatory T cells (Treg) that would normally block autoimmune T cell responses to ovarian antigens. However, more recent studies have shown that day 3 thymectomized mice in fact possess functional Treg in ovarian draining lymph nodes, but they are not sufficient to control fully the development of autoimmune ovarian disease [35]. A second, and non-exclusive, mechanism is that day 3 thymectomy results in enrichment of autoreactive, pathogenic $CD4^+$ T cells that escape deletion [36].

An important conclusion is that ovarian tissues, and by inference ovarian tumors, are intrinsically immunogenic, but that homeostatic mechanisms effectively block immune reactivity. The clinical significance of tumor-associated $CD4^+$ Treg in ovarian cancer is highlighted by the work of Curiel and colleagues, who showed that Treg are recruited to ovarian tumors by the chemokine CCL22 (predominantly expressed by ovarian tumors), and that the presence of Treg confers immune privilege and is associated with a poor prognosis and increased mortality [37]. These observations are underlined by further studies showing that high expression of the forkhead box transcription factor foxp3, which is preferentially expressed by $CD4^+$ Treg, is an independent prognostic factor for reduced overall survival in ovarian cancer [38]. Such findings lend credence to the notion that strategies for depletion of tumor-associated Treg, or inhibition of Treg function, may be of therapeutic benefit, particularly in conjunction with active, tumor-specific immunotherapy.

4.2 Inhibitory B7 family receptors

The role of the classical B7.1 and B7.2 receptors in regulation of T cell activation, either delivering a positive costimulatory signal through CD28 on T cells, or delivering an anergic signal through engagement of CTLA-4, has been established for over 20 years. More recently, new members of the B7 family have been described, at least two of which, B7-H1 and B7-H4, are likely to play important roles in regulation of antitumor immunity in ovarian cancer.

B7-H1 (also known as PD-L1) is expressed on ovarian cancer cells and infiltrating myeloid DC in the tumor microenvironment, and can promote T cell anergy or apoptosis through

engagement of PD-1 (or possibly CD80) expressed by T cells [39-41]. B7-H1 expression by DC has also been implicated in DC-driven conversion of naïve $CD4^+$ T cells to adaptive $foxp3^+$ Treg, a process that can be blocked by DC maturation with anti-CD40 and a TLR4 ligand (see section 6.4). Blockade of B7-H1 enhances DC-mediated T cell activation and boosts the efficacy of adoptive T cell immunotherapy against autologous ovarian tumors engrafted in xenogeneic mice [40]. Of particular clinical interest, a retrospective analysis of human ovarian cancers revealed that patients with higher B7-H1 expression had a significantly poorer prognosis than those for whom the tumors had lower B7-H1 expression [42]. From these observations, blockade of B7-H1/PD-1 interaction may augment the efficacy of cellular immunotherapy for ovarian cancer. Alternatively, generation of antitumor effector T cells with diminished expression of PD-1 may allow the circumvention of B-H1-induced anergy or apoptosis upon tumor T cell infiltration.

Several studies have described high expression of B7-H4 in ovarian tumors and tumor-associated macrophages [43-45]. Kryczek and colleagues further showed that blockade of B7-H4 restored the T cell-stimulatory capacity of ovarian tumor-associated macrophages and contributed to tumor regression in vivo [43]. Related studies by these authors showed that Treg could stimulate antigen-presenting cell expression of B7-H4 [46], and further demonstrated that the intensity of B7-H4 expression by ovarian tumor-associated macrophages correlated with Treg numbers in the tumor [47]. Of particular clinical significance, B7-H4 expression by macrophages (but not by tumor cells) correlated inversely with patient outcome [47]. The mechanism of action of B7-H4 in inhibition of antitumor immunity is obscure, largely because a ligand for B7-H4 has not yet been identified. Nevertheless, the weight of experimental and clinical evidence indicates that blockade of B7-H4 may be a valuable adjunct to immunotherapy of ovarian cancer.

4.3 Tumor-associated DC

Ovarian tumor cells express significant levels of CXCL12 (stromal cell-derived factor 1), which drives infiltration of plasmacytoid precursor DC with immunosuppressive function [48]. Further investigation revealed that plasmacytoid DC recovered from ovarian tumor ascites induced tumor antigen-specific regulatory $CD8^+$ T cells that were capable of inhibiting myeloid DC-driven effector T cell responses by an IL-10-dependent mechanism [49]. Plasmacytoid DC are thus likely to make a significant contribution to the immunosuppressive infrastructure of ovarian tumors. Other recent studies in a mouse model of ovarian cancer have shown that DC expressing alpha-smooth muscle actin and VE-cadherin home to perivascular areas in ovarian tumors, and are important for maintenance of tumor vasculature. Selective depletion of DC bearing these markers led to vascular apoptosis and delayed growth of established tumors [50]. From these observations, selective depletion of tumor-associated DC may hold promise as an adjunct to adoptive T cell immunotherapy or tumor vaccination strategies.

4.4 Dendritic cell induction of regulatory T cells

While there is an increasing consensus that active immunotherapy or antitumor vaccination should be supported by selective and efficient depletion of Treg, there is also a new appreciation that vaccination or cellular immunotherapies may themselves induce or expand Treg, thus promoting tumor-specific tolerance. For example, vaccination with recombinant vaccinia virus in a mouse tumor model system resulted in expansion of both effector and regulatory T cells, with Treg function being dominant, blocking effector function *in vitro* and *in vivo* [51]. With respect to cellular immunotherapy, a notable clinical study showed that injection of DC matured with inflammatory cytokines (TNFα, IL-1, IL-6 and PGE_2) expanded $foxp3^+CD4^+$ Treg in 3 of 3 myeloma patients tested [52]. While it is well known that immature DC induce Treg and peripheral tolerance [53,54], the finding that mature DC can also expand Treg clearly raises a

barrier for current approaches to DC vaccination. From these observations, it is apparent that although depletion of Treg prior to vaccination may be necessary, Treg depletion alone will not be sufficient to lead to optimal effector function and antitumor immunity. Redirection of DC-driven maturation and function will also be required to prevent *de novo* induction of vaccine antigen-specific Treg.

5. Strategies to overcome tumor-associated immune suppression

Cellular suppression of immunosurveillance and tumor immunotherapy was clearly demonstrated several decades ago by Berendt & North [55]. Importantly, the North laboratory also played a pioneering role in demonstrating the value of combining cyclophosphamide with cellular tumor immunotherapy, showing that cyclophosphamide eliminated tumor-induced suppressor L3T4$^+$ (CD4$^+$) T cells [56,57]. Many other studies have since confirmed the value of low dose cyclophosphamide as an adjuvant for tumor vaccines and adoptive immunotherapy [58-61]. Although low dose cyclophosphamide has long been known for its ability to boost cellular immunity, its mechanism of action in alleviating T cell-mediated suppression (Treg function) has been obscure until recent studies revealed that inhibition of inducible nitric oxide synthase (iNOS) was directly linked to its immunostimulatory effects [59]. Cyclophosphamide also possesses anti-angiogenic properties, mediated by upregulation of thrombospondin-1 (Tsp-1), which induces apoptosis of endothelial cells [62,63] and sequesters vascular endothelial growth factor [64]. It is tempting to view the anti-angiogenic activity of cyclophosphamide as an added bonus for cancer treatment, but this may be offset by the recent finding that Tsp-1 promotes the generation of Treg from naïve or memory CD4$^+$CD25$^-$ T cells, through interaction with CD47 [65].

In vivo depletion of Treg can also be achieved by targeting CD25, which is preferentially expressed by CD4$^+$ Treg. Antibodies against CD25 can deplete Treg and enhance antitumor immunity in animal models [66-68], and an IL-2 immunotoxin fusion protein (denileukin diftitox, also known as ONTAK) depletes Treg and contributes to durable immune control of breast tumors in *neu*-transgenic mice [69]. ONTAK is an FDA-approved drug (for treatment of cutaneous T cell lymphoma), and thus has broad appeal for clinical applications. A clinical trial of ONTAK treatment in combination with tumor RNA-transfected DC vaccination for patients with renal cell carcinoma resulted in reduced numbers of Treg in the peripheral blood and enhanced the generation of tumor-specific T cell responses [70]. However, these encouraging results were counterbalanced by studies in melanoma patients, in which ONTAK failed to deplete Treg numbers, as measured by foxp3 expression, and did not diminish Treg activity of CD4$^+$CD25$^+$ T cells in co-culture suppression assays [71]. In addition, a practical limitation is that although CD25 may be a marker for Treg among resting T cell populations, it is also broadly expressed by activated T cells, and thus treatments that target CD25 run the risk of depleting activated, tumor-reactive effector T cells in addition to removal of Treg. Notwithstanding these concerns, ONTAK is currently being tested in a clinical trial enrolling patients with epithelial carcinoma, including ovarian cancer [72].

Other drug treatments may also have clinical utility for depletion of Treg, or inhibition of Treg function. The application of fludarabine combined with cyclophosphamide as a conditioning regimen for Treg depletion prior to adoptive immunotherapy is well established [73,74], and other studies have indicated that fludarabine may have clinical value as a stand-alone agent for in vivo inhibition of Treg [75], or as an adjuvant for DC-driven generation of tumor-specific T cells for adoptive transfer [76]. A notable recent study showed that imatinib mesylate (Gleevec) administered at clinical concentrations in mice inhibited foxp3 expression and Treg suppression, without altering expression of TGFβ and IL-10 [77]. Of particular interest, Gleevec enhanced antitumor responses following DC vaccination against a Gleevec-resistant

lymphoma, suggesting that Gleevec may have direct applicability as an adjuvant for cellular immunotherapy.

6. Prevention of Treg recruitment by redirection of DC maturation

Mature DC activate and expand $CD4^+foxp3^+$ Treg *in vitro* [78], supporting the clinical observation that vaccination with cytokine-matured DC expands $CD4^+foxp3^+$ Treg in myeloma patients [52]. It is thus probable that DC activation of anti-tumor effector T cell responses would be seriously compromised by concomitant activation of Treg. For DC vaccination to be clinically effective, the new challenge is to identify alternative pathways of DC differentiation that bias T cell responses away from Treg homeostatis and in favor of active anti-tumor immunity.

6.1 Inhibition of p38 mitogen-activated protein kinase (MAPK) signaling as a mechanism for attenuation of Treg activation

Recent studies have pointed to a role for p38 MAPK signaling in DC-driven induction of Treg responses. Jarnicki and colleagues have shown that TLR agonists promote the induction of Treg through DC production of IL-10 consequent upon p38 MAPK signaling [79]. These investigators further demonstrated that inhibition of p38 MAPK signaling attenuated Treg induction and enhanced the efficacy of DC pulsed with antigen and CpG (a TLR9 agonist) as an antitumor cellular therapy, demonstrating an increased frequency of IFNγ-expressing T cells and diminished $Foxp3^+$ Treg infiltration of tumors [79]. In addition, clinical studies in myeloma patients have shown that inhibition of p38 MAPK signaling could correct defects in DC function, restoring their ability to activate alloreactive and tumor antigen-specific T cells [80]. Activation of p38 MAPK may also play a direct role in Treg function. Adler and colleagues have shown that p38 activation in inducible Treg is critical for maintenance of anergy dependent on elevated expression of the cell cycle inhibitor $p27^{Kip1}$, and have further shown that p38 inhibition results in complete loss of regulatory function [81]. These findings suggest that the use of p38 MAPK inhibitors may be a valuable addition to our armory in overcoming Treg suppression of cellular immunotherapy and other antitumor vaccination strategies.

6.2 Polarization of DC with type I and type 2 interferons

Several studies have shown that maturation of DC in the presence of IFNγ induces high expression of IL-12p70 and favors activation of strong $CD4^+$ Th1 responses and $CD8^+$ CTL responses [82-84]. More recent observations from Kalinski and colleagues have also pointed to a valuable role for IFNα in modulating DC interactions with Treg. These authors found that DC matured in the presence of PGE_2 attract Treg through secretion of CCL22, and further showed that DC maturation in the presence of IFNα inhibited CCL22 production and Treg recruitment, while promoting expression of Th1-attracting chemokines [85].

6.3 Inhibition of A20 expression in DC

A20 is a zinc-finger protein that negatively regulates the Toll-like receptor and TNF receptor signaling pathways through dual ubiquitinase and de-ubiquitinase activity. A20-silenced mouse DC inhibit Treg activation and strongly enhance T helper cell and CTL responses that are refractory to Treg suppression [86]. The proposal that A20 silencing in DC could be a powerful approach to boosting antitumor immunity is supported by studies with human DC, showing that A20 is a negative regulator of NF-κB and AP-1 activation, and that its downregulation enhances the ability of DC to stimulate tumor antigen-specific CTL responses [87].

6.4 Maturation of DC with anti-CD40 and TLR4 agonists

Recent studies in mice have shown that $CD8\alpha^+$ DC induce conversion of naïve $CD4^+$ T cells into adaptive $foxp3^+$ Treg in the presence of TGFβ, and have further shown that multiple coinhibitory pathways regulate foxp3 induction [88]. Blockade of PD-L1 (B7-H1), CTLA4 or GITR were all capable of inhibiting DC-driven conversion of naïve $CD4^+$ T cells to $Foxp3^+$ Treg. Notably, PD-$L1^{-/-}$ DC were severely impaired in their ability to induce foxp3 expression in the presence of TGFβ, suggesting that PD-L1 signaling is critical for the induction of adaptive $foxp3^+$ Treg. Maturation of DC with agonist anti-CD40 antibody or the TLR4 ligand LPS, and particularly a combination of both markedly diminished DC-driven foxp3 expression, suggesting that adaptive Treg induction could be regulated by manipulation of DC maturation [88]. Further studies will be needed to determine whether similar mechanisms are operative with human DC activation and expansion of $foxp3^+$ Treg.

6.5 Opposing roles for IL-2 and IL-15 in regulation of tumor immunity

Under normal conditions, innate thymus-derived $CD4^+$ Treg play an important role in controlling autoimmune pathology, chiefly through maintenance of peripheral tolerance to self antigens. Conversely, the pathology of cellular autoimmune diseases involves loss of tolerance and abrogation of Treg suppression of autoreactive T cells. Dysregulation of cytokine expression plays a major role in autoimmune pathogenesis, and increasing evidence points to overexpression of IL-15 as a key factor in a variety of autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, ulcerative colitis, celiac syndrome, psoriasis and sarcoidosis [89]. It is thus possible that IL-15 contributes to reduced Treg activity.

IL-15 shares some functions with IL-2, but also has some important opposing actions. Whereas IL-2 is critical for maintenance of $CD4^+$ Treg and is involved in activation-induced cell death (AICD) of autoreactive T cells, IL-15 inhibits AICD and promotes survival of memory $CD8^+$ T cells [89]. Recent studies have shown that treatment with IL-2 results in expansion of Treg in patients with melanoma [90] and ovarian cancer [91]. IL-2 also promotes Treg trafficking to tumors by inducing expression of CCR4 and CXCR4, which bind CCL22 and CXCL12 (stromal cell-derived factor-1), respectively [37,91], both of which are abundantly produced in ovarian tumors [37,48]. In contrast with the Treg-promoting properties of IL-2, IL-15 enhances T cell adoptive immunotherapy of cancer [92], in vivo reactivity of antitumor $CD8^+$ T cells [93] and boosts antigen-specific primary $CD8^+$ T cell responses to DC vaccination [94]. An important recent study has shown that IL-15 can abrogate tolerance in tumor-antigen-specific $CD8^+$ T cells, rendering them effective for immunotherapy of established tumors [95]. These latter observations provide a direct link between the known activity of IL-15 in cellular autoimmune disease and the potential for IL-15 to boost anti-tumor immunity through breakdown of peripheral tolerance to self tumor antigens. IL-15 can also act directly on DC, conferring a survival benefit [96] and skewing differentiation towards a Langerhans cell phenotype [97]. DC generated in the presence of IL-15 induce more potent $CD8^+$ T cell responses, characterized by higher expression of Th1 cytokines, than DC generated with GM-CSF alone or GM-CSF plus IL-4 [98], suggesting that IL-15-treated DC may be more effective than standard DC for tumor immunotherapy. Collectively, these observations discourage the use of IL-2 as an adjuvant for cellular immunotherapy, and suggest that IL-15 treated DC would diminish activation and expansion of Treg and favor stimulation of effector T cell responses.

7. Subversion of regulatory T cell responses to self antigens as a novel stratagem for cellular immunotherapy

Accumulating evidence has clearly shown that the distribution of self-reactive T cells with high affinity is heavily skewed in favor of the $CD4^+$ Treg compartment, as a result of thymic selection that is biased toward direction of autoreactive T cells into that compartment

[99-102]. In addition, self-antigens drive peripheral expansion of Treg [103], and direct their accumulation in sites where the antigen is processed and presented [104,105]. Furthermore, remarkably few Treg appear to be required to suppress effector T cell responses in vivo [106]. In the face of this reality, it is probable that most tumor vaccination strategies will face defeat, because the frequency of self-reactive effector T cells will be markedly lower than the frequency of antigen-specific Treg, and because the affinity of self-reactive effector T cells will also likely be lower than the affinity of their Treg counterparts. How can this seemingly intractable problem be resolved? Novel approaches to Treg depletion have gained some currency, but these strategies do nothing to improve the repertoire or affinity of self-reactive effector T cells. Perhaps a better option is not to deplete Treg, but to tap into the Treg compartment as a source of high affinity self-reactive (and tumor-reactive) T cells, and to reprogram their function such that they act as antitumor effector T cells. The developing consensus that Treg and Th17 differentiation and expansion are reciprocally regulated [107, 108] affords such an opportunity. The idea that subversion of Treg responses in favor of Th17 responses may have therapeutic benefit for cellular immunotherapy of ovarian cancer is strongly supported by clinical evidence that the presence of infiltrating Th17 cells in ovarian tumors has a pronounced positive correlation with prolonged overall survival (Kryczek et al, *Th17 cells in ovarian cancer patients*, 23rd Annual meeting of the International Society for Biological Therapy of Cancer, San Diego, October 31-November 2, 2008).

Redirection of Treg responses to self antigen-reactive Th17 responses may be achieved by activation of T cells in the presence of cytokines that drive Th17 differentiation. The exact nature of the requirements for Th17 differentiation has been blurred by some apparently conflicting reports [109], but recent work has pointed to a central role for IL-1β [110-112]. In contrast, IL-2 signaling inhibits Th17 T cell responses [113], and important studies from Kryczek and colleagues have shown that IL-2 and IL-1 play opposing roles in regulating Th17 differentiation, with IL-2 blocking Th17 differentiation and IL-1 playing a dominant role in Th17 induction [114]. These authors have also shown that IL-2 reduces Th17 differentiation in the tumor microenvironment and promotes Treg expansion [115], pointing to dynamic regulation of Treg and Th17 responses, and lending further credence to the position that adjuvant IL-2 administration may be counterproductive for tumor vaccination or immunotherapy (see above).

The ability to manipulate Treg versus Th17 responses with cytokines may be advantageous for adoptive T cell immunotherapy, in which tumor-specific T cells are expanded in vitro, but is less helpful for DC vaccination, since the adjuvant use of inflammatory cytokines such as IL-1β or IL-15 (see above) is likely to incur unacceptable off-target toxicity. The challenge, then, is to drive Th17 differentiation at the expense of autoreactive Treg responses by manipulation of DC alone. Evidence that this may be achievable has been provided by the observation that DC treated with an antibody that cross-links the B7-DC receptor are capable of reprogramming $Foxp3^+$ Treg to become Th17 autoimmune effectors [116].

Apart from inhibition of Treg activation and induction of high affinity tumor antigen-specific effector T cell responses, other benefits may accrue from Th17 induction. Perhaps most important, a considerable weight of evidence suggests that Th17 responses are markedly less sensitive than Th1 responses to suppression by Treg [117-120], which may remain a barrier to cellular immunotherapy in ovarian cancer patients, even in those pre-treated with cyclophosphamide or ONTAK to deplete tumor-associated Treg.

8. Expert Opinion

Although various studies have suggested that ovarian tissues are intrinsically immunogenic, and that cellular immune responses can develop against ovarian tumors, it has become

increasingly apparent that ovarian tumors have recruited diverse and powerful defensive strategies to protect against immunological attack. The critical role played by tumor-infiltrating Treg in protection against antitumor T cell immunity is now widely recognized for many types of cancer, and Treg represent a particularly important defense mechanism in ovarian cancer. The clinical significance of tumor-associated Treg has been highlighted by Curiel and colleagues, who showed that Treg are recruited to ovarian tumors by the chemokine CCL22, and that the presence of Treg confers immune privilege and is associated with a poor prognosis and increased mortality [37]. Diligent investigation has revealed that other defense mechanisms are also operative in the ovarian tumor microenvironment, notably the expression of chemokines that recruit suppressor cell populations, including Treg and plasmacytoid DC, and the expression of inhibitory members of the B7 family of receptors (B7-H1 and B7-H4). Collectively, these immunosuppressive mechanisms present a truly formidable barrier to cellular immunotherapy.

The identification of drugs that may selectively deplete tumor-associated Treg has raised the prospect of their use in cancer treatment, either as free-standing agents or as adjuvants in support of tumor vaccination or cellular immunotherapy. Cyclophosphamide has long been recognized for its immunopotentiating qualities, and more recently the use of denileukin diftitox, which binds CD25 on Treg, has also come into vogue. Both these agents, and other drugs discussed in this review, may help to break down a major barrier to successful immunotherapy. Blockade of tumor-associated chemokines that recruit suppressor cells, or blockade of inhibitory coreceptors may also facilitate a counterattack by vaccine-induced antitumor immunity or cellular immunotherapy.

From the above discussion, one can conclude that considerable progress has been made in identifying mechanisms of tumor-associated immunosuppression, and in developing strategies that may surmount these barriers. Unfortunately, the recent recognition that antitumor vaccination or immunotherapy itself may contribute to activation and expansion of tumor antigen-specific Treg responses poses a further challenge that demands a highly creative solution. The root of the problem lies with the mechanisms by which the thymus shapes the peripheral T cell repertoire. Self-reactive precursor T cells may suffer one of two fates, either negative selection and apoptosis, or differentiation into peripheral Treg with high affinity for self antigen. As a result, a majority of self-reactive T cells are suppressor cells, with the primary role of preventing autoimmune disease. Antitumor vaccination or cellular immunotherapy may be capable of activating a relatively low affinity effector T cell response, but is also likely to activate and expand high affinity self-reactive Treg that will clearly impose major constraints on the efficacy of the treatment. In the light of this reality, it is hardly surprising that most attempts at tumor vaccination or immunotherapy have failed to live up to expectations in clinical trials.

A possible solution to the challenge posed by thymic selection of self-reactive Treg lies in the increasing body of evidence showing that Treg and Th17 T cell responses are reciprocally regulated, and that it is possible to reprogram Treg and generate effector Th17 responses that may support strong antitumor immunity. Important recent studies have shown that IL-2 and IL-1β may play key roles in direction of Treg and Th17 responses, with IL-2 favoring Treg activation and expansion and IL-1β playing a dominant role in Th17 differentiation. Apart from suggesting that the longstanding clinical use of IL-2 as a freestanding or adjuvant antitumor agent may in fact be counterproductive, these observations point to the use of IL-1β for generation of antitumor T cells from a high affinity pool of self–reactive T cells for adoptive immunotherapy. The use of IL-1β (or other proinflammatory cytokines such as IL-15) may have utility for ex vivo applications, but would likely incur unacceptable toxicities in the adjuvant setting. An alternative and more attractive approach may be to endow DC with the

capacity to reprogram Treg and induce Th-17 driven antitumor immunity, as described recently by Radhakrishnan and colleagues [116].

In closing, there are two further points in favor of the generation of antitumor Th17 responses from the high affinity self-reactive Treg repertoire. First, Th17 responses are broadly refractile to Treg suppression, suggesting that antitumor effector T cells would be less prone to inhibition in the tumor microenvironment. This is a quality that is conspicuously not shared by $CD4^+$ Th1 responses, which for many years have been regarded as desirable for antitumor immunity. Second, the recent clinical evidence that IL-17 expression in ovarian tumors has a strong positive correlation with improved overall survival provides a convincing rationale for Th17-driven cellular immunotherapy for ovarian cancer.

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