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Review Article

Cancer immunotherapy: from promise to practice

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CONTENTS

Abstract

Tumor immunotherapy has advanced over the past decade due to the achievement of a better understanding of the immune system and the adoption of new approaches that attempt to take advantage of the interactions of the cells of the immune system while avoiding immunosuppressive consequences. The duality of the cells of the immune system translates into the promotion of either an immunostimulatory or an immunosuppressive response, depending on the situation. The central control of immunity lies with the dendritic cells, and the ability to steer these cells away from tolerance induction and towards immunity remains the major challenge for successful immunotherapy. As our knowledge of the molecular control systems improves, we will be able to provide more effective treatments that mediate a better end result with a higher degree of specificity and less toxicity.

Introduction

Amelioration or intervention in the progression of liquid and solid tumors has been a relatively unattainable goal of immunotherapy for decades. However, in the past few years, advances in immunology and molecular biology have allowed the development of therapeutic approaches that are beginning to pay dividends in a spectrum of cancers. With appropriate treatment, many cancers are evolving from a death sentence to a chronic condition where patient survival has been extended for years. While cures remain elusive, improvements in the management and diagnosis of malignancies have been significant. This review will examine some of the new therapies that are proposed or are currently in trials for cancer immunotherapy. We will focus on how the immune system is stimulated by these treatments and how the tumor fights back and attempts to interfere with induced antitumor immune responses.

Is there an immune response to cancer and why doesn't it protect us?

Cancers can occur in individuals with an intact immune system and progress despite the presence of antigens that can be recognized by immune defenders and provoke antitumor responses (1). Most tumor antigens are self-proteins that are expressed on normal tissue as well as malignant cells. The immune system is educated in the thymus to ignore self-antigens and mount immunity to foreign antigens. However, it is now understood that central tolerance to self-antigens caused by deletion in the thymus of autoreactive cells is also maintained in the periphery by T-regulatory cells, or Treg cells (2). Therefore, the development of an immune response to tumor-associated antigens (TAAs) requires breaking tolerance through inactivation of the inhibitory influence mediated by Treg cells or reversion of tumor escape mechanisms. Tumor escape strategies include loss of immunogenic molecules and secretion of inhibitory cytokines that limit immune responses, create an immunosuppressive microenvironment and promote tumor cell growth.

Evidence supporting the ability of the immune system to respond to malignancy includes spontaneous remissions and the presence of tumor-infiltrating lymphocytes in some regressing tumors (3). However, chronic stimulation of the immune system results in inflammation that contributes to cancer initiation and progression. The players that promote or inhibit antitumor immunity include T- and B-lymphocytes, natural killer (NK) cells and antigen-presenting cells (APCs). The interplay between these cell types can be modulated by effective immunotherapies to promote antitumor immunity. However, the successful invocation of an immune response to tumors requires an understanding of the cells involved and the double-edged nature of their interactions. The cells involved in immune interactions are presented in Table I.

Effector cells of adaptive immunity: CD4 and CD8 T-cells

Two major T-cell subsets comprise the effector cells of adaptive immunity and are defined by their expression of either CD4 or CD8 accessory molecules. CD4 T-cells carry a T-cell receptor (TCR) that recognizes peptides displayed on an APC in class II major histocompatibility (MHC) molecules. Two major subtypes of CD4 T-cells, the Th1 and Th2 cells, are recognized based on cytokine profiles. Th1 cells are helper cells that promote cell-mediated immunity and secrete interferon gamma (IFN-γ) and tumor necrosis factor α (TNF-α). Th2 cells secrete the interleukins IL-4, -5, -10 and -13, are responsible for humoral immunity and can suppress immune responses through IL-10 production (4). CD4 T-cells that function as helper cells promote B-cell immunoglobulin production (4) and support CD8 T-cell effector functions (5). CD4 T-cell help is mediated by CD40 ligand binding to CD40 on the APC, causing crosslinking of the CD40 molecule and APC activation (6).

CD8 T-cells respond to peptides displayed in class I MHC molecules on an APC. Stimulation of the CD8 T-cell response is the major goal of immunotherapy protocols since CD8 cells induce cytolysis through recognition of the class I-held peptide on the tumor cell surface and produce cytokines such as TNF-α, activate the Fas-Fas ligand pathway and secrete the cytolytic granules perforin and granzyme (7). Recently, it has become clear that helper CD4 T-cells are required for the development of effective CD8 T-cells, and protocols that enhance the CD4 response may improve the efficacy of CD8 T-cells. An additional type of CD4 T-cells, the immunosuppressive Treg cells, may inhibit anticancer immunity and the suppressive factors produced by Treg cells may have to be eliminated before effective immunotherapy can be achieved.

Treg cells

Treg cells are $CD4^+/CD25^+$ T-cells that can inhibit immune responses, avert autoimmune disease (8) and mediate tolerance to transplants (9). At least two types of Treg cells exist, thymus-derived and circulating inducible Treg precursors that make up 5-10% of the peripheral blood CD4 T-cell pool (8). Cell-surface antigens expressed on the Treg population include the IL-2 receptor (CD25), the inhibitory member of the co-stimulatory family cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR) and the hallmark of Treg —the forkhead transcription factor Foxp3. The disease immune dysregulation polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) has been associated with disruption in the *FOXP3* gene product in humans. IPEX in males is marked by lymphocyte activation and autoimmune disorders, with death resulting from overproduction of inflammatory cytokines. In the scrufy mouse, a similar disease is related to a mutation in the *Foxp3* gene (10). Murine Foxp3 expression is confined to Treg cells and has been proposed as a specific marker restricted to the Treg lineage (11).

Treg cells are generally nonresponsive and do not proliferate after TCR stimulation (12, 13). However, Treg cells proliferate *in vitro* in the presence of mature antigen-

Table I: Effector cells of adaptive and innate immunity.

Cell type	Function	Ref.
CD4 T-cells	Th1 cells secrete IFN-γ and TNF-α Th2 cells secrete IL-4, -5, -10 and -13 Promote cellular and humoral immunity	4, 5
CD8 T-cells	Cytolysis of target cells	7
Treg cells	Inhibit immune responses Control transplant rejection and autoimmune responses	8, 9
NK, NK T-, $\gamma\delta$ T-cells	Detect infectious pathogens or tumor cells Mediate cytolysis Induce DC maturation	18, 19
DCs	Immature DCs are phagocytic and pinocytic Mature DCs are potent APCs Mediate immunity and tolerance	22, 23

IFN-γ, interferon gamma; TNF-α, tumor necrosis factor α; Treg, T regulatory; NK, natural killer; DC, dendritic cells; APCs, antigen-presenting cells.

loaded dendritic cells (DCs) without the addition of exogenous cytokines (14). Mature DCs can present self-antigen to CD4 T-cells and drive their conversion to Treg, thereby regulating autoimmunity and effector T-cell expansion. The mechanism of Treg cell suppression remains to be fully elucidated and may vary with culture conditions and the assay system used to monitor the immunosuppression (13). Remote inhibition mediated through secretion of inhibitory cytokines such as IL-10 and transforming growth factor β (TGF-β), and direct cell contact through CTLA-4 blockade of effector T-cell access to B7-1 and -2 on the APCs, have been described in several models. In addition, Treg expression of high levels of membrane CD25 can deplete IL-2, which is a required growth factor for antigen-responsive CD4 T-cells (12).

Treg cells have been found in some tumors, including melanoma (15), lung, ovarian (16), pancreatic and breast cancer (17), indicating that Treg could suppress antitumor immune responses and thereby perpetuate the malignancy. The presence of Treg-infiltrating tumors may explain the limited efficacy of many antitumor immunotherapies, where induction of an immune response is short-lived and abrogated by the tumor milieu. Improved immunotherapy will require the depletion or inactivation of the Treg response.

Innate immunity: NK, NK T- and γδ T-cells

In addition to adaptive immune responses, innate effector cells play an important role in immune stimulation and also serve to mature DCs into effective APCs (18). The innate immune system includes NK, NK T-cells and γδ T-cells, which detect infectious pathogens or malignancy and react by secreting IFN-γ and mediating cytolytic activity. NK cells lyse malignantly transformed cells without prior activation and recognize tumor cells through activating or inhibitory receptors. Class I MHC molecules are inhibitory for NK cells, and the loss of class I expression on tumor cells, which serves as a mechanism of immune evasion, can activate NK cells to promote tumor cytolysis (19). NK T-cells express both NK markers and an invariant TCR. NK T-cells recognize foreign and self-glycolipids when they are presented by the nonclassical MHC molecule CD1d. NK T-cells also detect altered self-ligands that may be displayed on tumor cells and initiate a cytolytic response. γδ T-cells react to microbial and tumor-related antigens and stress-induced self-proteins (20). Activated NK, NK T- and γδ T-cells can induce DC maturation and the mature DCs can feed back to stimulate innate as well as adaptive immunity. Therefore, immunotherapy protocols that activate innate immunity through pulsing DCs with molecules such as α-galactosylceramide (α-GalCer) that stimulate NK T-cells may enhance immune responses (21).

DC biology

DCs are the central promoters of both immunostimulatory and -inhibitory immunity (22). At least two major subtypes of DCs are recognized based on their differentiation from a myeloid or lymphoid bone marrow precursor (23). $CD34^+$ bone marrow-derived cells are found in the peripheral blood and have potential to develop into monocytes. In the skin, Langerhans cells (LCs), the major APCs of the epidermis (24), or DCs that populate tissues (25), are derived from circulating CD34 precursors. Myeloid DCs are the main cell type that has been employed for immunotherapy protocols since they are easy to differentiate from peripheral blood monocytes. DCs of lymphoid origin and termed “plasmacytoid” DCs are also present in the bood and produce interferons alfa and beta in response to viral infections (25). When DCs are immature, they are aggressively phagocytic and pinocytic. As DCs mature, they become potent APCs and promote immune responses (26). Since immature DCs have been implicated as mediaters of tolerance (27), the status of DC maturation has become a focus in optimizing immunotherapy protocols for cancer. The salient features of DC biology are presented in Table II.

DC control of immunity

DC maturation has been defined by the phenotypic markers on the DC membrane and in the cytoplasm. DC interaction with adaptive and innate immune responses is presented in Figure 1. Immature DCs express lower levels of the co-stimulatory molecules B7-1 and -2 (CD80 and CD86), CD40 and class I and II MHC molecules, are poor stimulators of immunity and may promote tolerance. Mature DCs lose expression of markers commonly asso-

Table II: Dendritic cells and the control of immunity.

Feature	Impact on immune response	Ref.
Immature DCs	Phagocytosis and pinocytosis of exogenous antigens	22
Mature DCs	Potent antigen presentation to stimulate antipathogen and antitumor immunity Presentation of self-peptides to mediate tolerance	14, 28
Cytokine secretion	IL-12 promotes immunity IL-10 mediates immunosuppression	22
Control of DC plasticity	Pathogen recognition through TLRs Danger signals	30-32

DCs, dendritic cells; TLRs, Toll-like receptors.

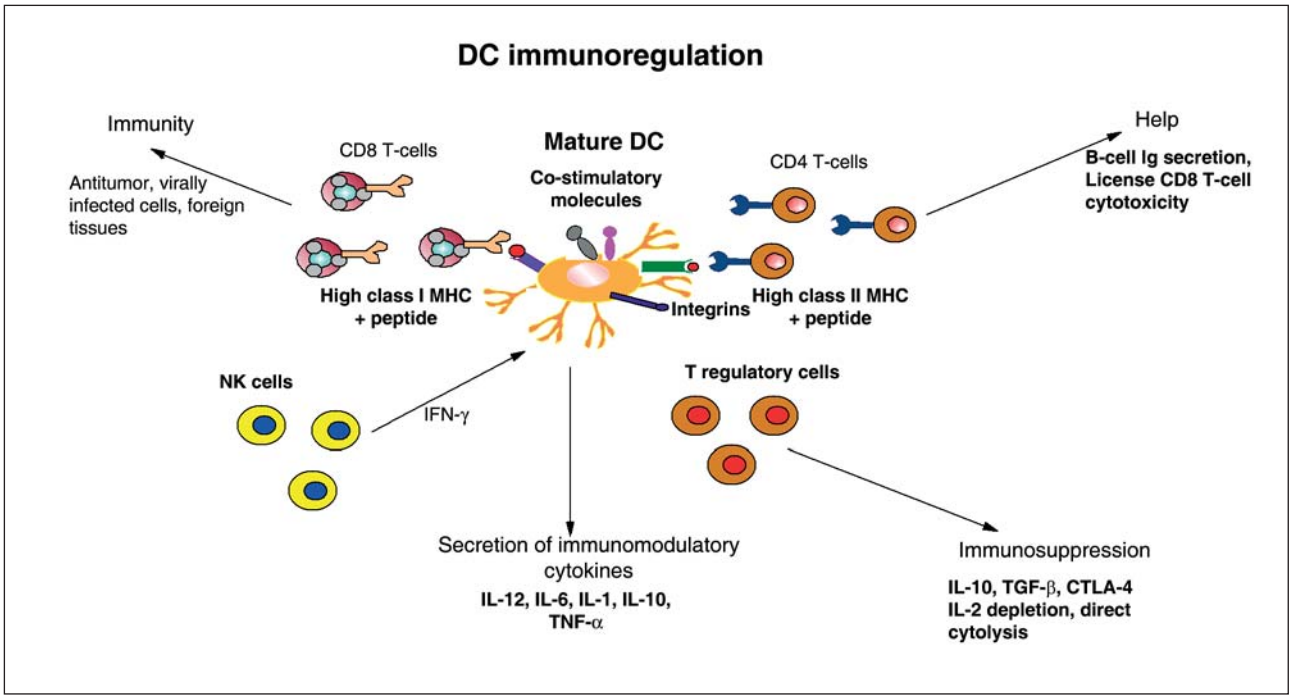

Fig. 1. The role of dendritic cells (DCs) in controlling immune responses. Mature DCs express high levels of class I and II major histocompatibility (MHC) molecules, co-stimulatory and adhesion molecules (integrins). CD8 T-cells stimulated by peptides presented in class I MHC become cytotoxic effectors that recognize the same peptide displayed on the surface of the target cell. CD4 T-cells recognize peptides displayed in class II MHC on the DC surface and help B-cell production of immunoglobulin and promote CD8 T-cell cytotoxicity. Activated natural killer (NK) cells secrete interferon gamma (IFN-γ), which induces DC maturation. T regulatory cells are stimulated by DC presentation of self-peptides to suppress immune responses. DCs secrete cytokines which are either immunostimulatory or inhibitory.

ciated with monocytes and increase the expression of co-stimulatory molecules (CD80, CD86, CD40) and class I and II MHC molecules. Lysosomal markers linked to antigen processing (DC-Lamp, marker of mature DCs) and the lymph node-homing molecule CCR7 are also upregulated. Mature DCs are potent immunostimulators of even naïve T-cells due to their display of high levels of MHC, co-stimulatory and adhesion molecules (28). Recent observations suggest that the dogma of DC maturation as the hallmark of immunity may require revision, since even immature DCs express sufficient class I MHC molecules for stimulation of a CD8 T-cell response (29), and mature DCs may induce tolerance (14). A central question for the development of cancer vaccines using DCs is: how can the plasticity of DCs be controlled so that an immunogenic or a tolerogenic response is achieved? While the answer to this question remains enigmatic, clues from immunology and molecular biology have shed some light on the matter.

Control of DC immunogenicity by pathogen recognition and danger signals

Two hypotheses have been advanced to explain how DCs modulate immune responses. It was suggested by Janeway that APCs express pattern recognition receptors that bind molecules associated with pathogenic organisms (30). This hypothesis was supported by the discovery of Toll-like receptors (TLRs) on DCs, which bind molecules on pathogens and thus activate tolerogenic DCs, turning them into immunostimulatory DCs that can cross-prime immune responses (31). This model explains how DCs are stimulated by pathogens, but it does not elucidate the generation of immune responses to tumors.

To explain the immune response to tumors, Matzinger has proposed the “danger model” that hypothesizes that signals released from damaged, dying cells can activate DCs (32). Molecules that can signal danger include heat shock proteins (HSPs) and mitochondrial products, the presence of which indicates that tissue components have been released from damaged tumor cells. An immune response invoked through cytotoxic T-cells targeted by peptides presented on the class I MHC molecules of the cross-presenting DCs could then destroy the malignant cells.

A central tenet of both of these models is that the basal state of DCs, even when loaded with antigen, is tolerogenic or resting, and that inflammatory conditions associated with infection or neoplasia may be required to convert the DCs to an immunostimulatory role. This hypothesis has been supported by experiments indicating that, despite a phenotype associated with DC maturity, in the absence of inflammatory stimulation, T-cell activation is limited and tolerance ensues (33). If DCs loaded or not

with antigen and mature or immature are functionally tolerogenic, the consequences for immunotherapy protocols using DCs to induce antitumor immunity are substantial. Into this arena of immunosuppression generated by the tumor cells themselves or mediated by Treg cells and tolerogenic DCs steps the immunotherapist, whose protocols appear to be doomed to failure by the inhibitory nature of the immune system they hope to stimulate. It is only through an improved understanding of the immune response that more effective immunotherapies can be devised to circumvent these difficulties. Despite these substantial obstacles, new immunotherapies have been devised that show promise for the therapy of some malignancies (Table III).

Monoclonal antibody immunotherapy

Monoclonal antibodies (mAbs) have shown efficacy as adjuvants for immunotherapy. MAbs were first described in the 1970s (34) and have since become useful therapy for some malignancies, demonstrating an ability to enhance the antitumor response, a relatively safe toxicity profile and high levels of specificity. MAbs can be divided into specific classes based on the mechanism through which they mediate an antitumor response. The initial mAbs (35) used for antitumor therapy operated by antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). In recent years, antibodies have been developed that are directed against a variety of tumor targets, including growth factors such as vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), and a number of specific cell-signaling molecules and receptors on the cells of the immune system (36-38).

Rituximab (Rituxan®) was the first antitumor mAb therapy approved by the FDA for the treatment of refractory non-Hodgkin's lymphoma (NHL). Subsequently, trastuzumab was approved for HER2/Neu$^+$ breast cancers. Six additional mAbs have been approved for clinical use, including the anti-CD52 alemtuzumab for refractory B-cell chronic lymphocytic leukemia (B-CLL), the anti-CD33 gemtuzumab ozogamicin conjugated to calicheamicin for refractory acute myeloid leukemia (AML), the anti-CD20 radioisotope conjugates ibritumomab and tositumomab for refractory NHL, bevacizumab in combination with chemotherapy to target VEGF in metastatic colon cancer and the anti-EGFR cetuximab for metastatic colon cancer (39). These antibodies mediate their antitumor effects through stimulation of ADCC and CDC or inhibition of specific growth factors or their receptors that are essential for tumor proliferation. Clinical trials using mAbs have been reviewed (40) and other trials have been initiated to determine the efficacy of a large variety of other anticancer mAbs (41). MAbs remain limited by the tumor cell's propensity to alter TAAs or the expression of these antigens on normal tissues. Despite these problems, mAbs have made a valuable contribution to the anticancer arsenal.

Table III: Options for immunotherapy..

Immunotherapy	Features	Limitations	Ref.
Monoclonal antibodies	Specificity	Antigen loss Antigen expression on normal tissues	36-41
DC vaccines	Availability in blood Loading with a variety of antigens Potent immunogenicity	Tolerance induction Unresolved optimal dose, antigen type, route, immunization schedule	43-46
Reversal of Treg immunosuppression	Anti-CTLA-4 Anti-GITR Anti-TGF-β Anti-IL-10	Induction of autoimmune sequelae	56-59
Cytokines	IL-2 IL-12 IL-15	Toxicity Potential for Treg expansion	12, 68, 69
HSPs	Specificity Carriers of a large variety of peptides Induce CD8 T-cell response	Variable clinical response Induction of Treg	74, 91
ECP and TI	Clinically practical, safe, broadly applicable, amenable to extension to other diseases Potential for steering between immunity and tolerance	Availability Cost	93-103
Molecular medicine	DNA arrays Diagnosis Identification of target molecules siRNA	Cost Technical complexity In clinical development	105, 107, 108

DC, dendritic cells; HSPs, heat shock proteins; Treg, T regulatory; ECP, extracorporeal photopheresis; TI, transimmunization.

DC-based vaccines in cancer immunotherapy

The first peptide-pulsed DC cancer vaccine clinical trial was described in 1995 (42), and numerous clinical trials have followed using a variety of DC preparations, injection routes and loading strategies. DC anticancer vaccines are designed to treat tumors by inducing tumor-specific effector T-cells that reduce the tumor mass and induce memory T-cells that control tumor relapse (43). Thus, when properly prompted, the immune system's innate, antigen-non-specific immunity and adaptive, antigen-specific immunity synergize to eradicate the tumor. The induction, coordination and regulation of the adaptive and innate immune system are ultimately controlled by DCs (43, 44).

A large variety of antigens for loading of DC vaccines are being tested, including peptides, viral vectors and modified tumor cells. DC vaccines have demonstrated good response rates (45), although completed trials have shown varied rates of success (46). Many clinical trials have been published describing the results of DC vaccination in more than 1,000 patients with different cancers, including adenocarcinoma, bladder, breast, lung and colorectal cancer, chronic myelogenous leukemia (CML), cancers of the gastrointestinal system, tumors of the neurological system, gynecological cancers, tumors of the head and neck, hepatocellular cancer, multiple myeloma, lymphoma, prostate cancer, sarcomas, thyroid cancer and others (46).

Characteristics of the optimal DC vaccine preparation remain a matter of continuing investigation. The source of DCs, preparation of tumor antigen, degree of DC maturation, route of vaccination, number of administrations, vaccine preservation, number of DCs required and the timing of vaccinations remain to be determined. The most effective antigen source for use in loading DCs is not clear, with peptides, whole tumors, nucleic acids or viral vectors being tested. Adverse effects that have been reported to be associated with DC vaccinations are minimal, consisting mostly of fever, injection-site reactions and adenopathy. Autoimmunity was uncommon, and only a few patients experienced conversion to positive antinuclear antibody (ANA), rheumatoid factor (RF), anti-dsDNA and antithyroid titers that were generally not clinically significant (46).

Some indications as to the best methodology for DC vaccine construction have been reported. Vaccine treatments are most effective in patients with predominantly lymphatic or cutaneously restricted disease (45, 47). One explanation for this observation is that, in contrast to solid tumors, lymphoid tumors and possibly cutaneous tumors allow direct circulatory access, with the best results seen when the vaccines are injected subcutaneously or intradermally rather than intravenously (48). In almost all vaccines, the DCs were obtained from the patient's peripheral blood. Some investigators are exploring the possibility of using bone marrow-derived DCs in vaccines, based on promising results obtained in animal models (49). However, DCs from whole blood are readily accessible and can be subsequently expanded in culture (50).

Adjuvants have been used to boost the activity of DC vaccines. One common adjuvant is keyhole limpet hemocyanin (KLH), an inducer of strong $CD4^+$ T-cell helper responses as a neoantigen not previously encountered by the immune system (51). When KLH is given in conjunction with a source of tumor antigen, it amplifies the immune response through the production of cytokines in the lymph node microenvironement, stimulating an enhanced $CD8^+$ T-cell response. Additional adjuvants commonly added to DC vaccines in an attempt to amplify antigenicity and cause the maturation of monocytes to immature DCs include IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), used to differentiate monocytes to immature DCs that can take up tumor antigen and become mature DCs.

DC vaccines are frequently pulsed with an individual peptide, creating cytotoxic T-lymphocytes (CTLs) that are restricted to recognition of a single tumor epitope. However, as tumors accumulate gene mutations in antigenic genes, the antigens change their structure or may not be expressed at all (52). Thus, limiting vaccines to one antigen may not be as effective as a whole-tumor lysate that could provide multiple antigens for the induction of many CTL clones.

Future vaccines will need to overcome the immune system's regulatory balances. The immune system has naturally evolved robust suppressor systems, predominantly mediated by Treg, to prevent detrimental antigen-specific responses to self, thereby averting excessive host tissue damage (53). Knowledge regarding the biology of these counterregulatory mechanisms will become crucial for the successful application of cancer immunotherapy.

Modulating Treg cell immunosuppression

The presence of Treg cells in tumors has supported the role of these cells in inhibiting antitumor immunity and maintaining an immunosuppressive milieu that allows the persistence and expansion of the malignancy (54). Depletion or inactivation of Treg cells may be a requirement for optimal immunotherapy. The expression of CTLA-4 on the surface and in the cytoplasm of Treg cells may provide a target for removal of Treg cells and reversal of immunosuppression. The first clinical trial using anti-CTLA-4 therapy was conducted in 9 previously immunized patients with ovarian cancer or melanoma. Each patient received a single intravenous injection of an anti-CTLA-4 MAb, and some tumor cell death was achieved, as shown by histopathological evaluation or by stabilization of biochemical tumor markers (55). In a second trial, patients with metastatic melanoma were given a peptide-pulsed melanoma vaccine along with anti-CTLA-4 mAb. All patients developed T-cell reactivity against the immunizing peptides and some objective cancer regression was obtained. However, many of the patients experienced grade III/IV autoimmune disease, including dermatitis, colitis and hepatitis. When the patients were treated with supportive care and/or steroid therapy, they

recovered from the acute toxicity (56). A number of other studies have been conducted using co-administration of anti-CTLA-4 antibody and peptide vaccines, and these studies provided encouraging results in terms of both objective tumor regression and clinical response (57). These trials provide proof of principle that substantiates the proposition that depletion of the Treg cell population can result in an enhanced tumor vaccine effect and that Treg cells control autoimmunity.

In addition to anti-CTLA-4 therapy, a number of other options exist for selective targeting of Treg cells to enhance the efficacy of cancer immunotherapy. Glucorticoid-induced TNFR-related protein (GITR), a member of the tumor necrosis factor/nerve growth factor receptor (TNFR/NGFR) family, has been described on the surface of $CD4^+/CD25^+$ T-cells and is thought to play a role in regulating suppression. Stimulation of this receptor via an activating antibody has been shown to reverse the induction of suppression, and removal of $GITR^+$ cells resulted in organ-specific autoimmunity in murine models, indicating depletion of Treg cells (57, 58). Other molecules that can be targeted to inactivate Treg cells include cell-signaling molecules such as TNF-related activation-induced cytokine (TRANCE) and receptor activator of NF-κB (RANK), which have been shown to be involved in activating the signaling pathways of $CD4^+/CD25^+$ T-cells (59). Depletion of these molecules resulted in a rapid onset of diabetes in murine models.

Another alternative for Treg cell inactivation lies in the close association of Treg cells with solid tumors. High levels of $CD4^+/CD25^+$ T-cells are present in lung, ovarian, breast and pancreatic tumor samples (17, 60), and there appears to be an inverse relationship between the number of Treg cells and survival (61). The chemokine CCL22 and the chemokine receptor CCR4 are required for the migration of Treg cells to the tumor site, and inhibition of CCL22 results in reduced migration of Treg cells (62). Selective depletion of Treg cells in tumor sites while sparing the circulating Treg cell population that controls autoimmunity would enable eradication of tumor immunosuppression while preserving regulation of autoimmunity.

Cytokine immunotherapy targeting Treg cells

Cytokines play a major role in the development and functional capacity of Treg cells. Treg cells produce a number of soluble, inhibitory cytokines, such as IL-10 and TGF-β. Selective inhibition of these cytokines has been shown to reverse generalized immunosuppression in a number of murine models and in humans (63, 64). T-cell-specific blockade of TGF-β allows the generation of an immune response capable of eliminating tumors in murine model systems (65). Phase I clinical trials are examining the efficacy of anti-TGF-β therapy in the setting of glioblastoma and non-small cell lung cancer (66). Blockade of IL-10 has demonstrated enhanced tumor destruction in murine models and may also offer some clinical utility (67).

One of the best-studied cytokines for tumor immunotherapy is IL-2. IL-2 has been found to enhance the potency of immunotherapy due to its role as an activator/expander of tumor-specific T-cells. When IL-2 was used as monotherapy, durable regressions were demonstrated in 20% and complete responses in 9% of renal cell carcinoma patients. Furthermore, in metastatic melanoma, a regression rate of 17% and a complete response rate of 7% were noted (68).

Studies are under way to determine whether IL-2 can be used synergistically with IL-12 to enhance the antitumor effects of a vaccine (68). *In vitro* studies have shown that a synergism between these two cytokines can augment the immune response in patients with cutaneous T-cell lymphoma (CTCL) (69, 70). Studies indicate that although IL-2 appears to be necessary for T-cell activation and growth, it also supports the growth and differentiation of Treg cells, and this may limit the beneficial aspects of this cytokine. Similarly, a number of newer studies have looked at the use of alternative cytokines, such as IL-15, and cytokine combinations with vaccines in hopes of more specifically activating the tumor-infiltrating lymphocytes without expanding the Treg lymphocyte population (12).

Members of the naturally occurring Treg cell subpopulation are $CD25^+$. Given the relative specificity of CD25 to Treg cells, antibody-dependent cell death via CD25 may serve as a means to eliminate these cells. The FDA has approved denileukin diftitox (Ontak®), a recombinant cytotoxic protein composed of diphtheria toxin conjugated to the IL-2-binding domain, for use in CTCL patients. Due to its specificity for the IL-2 receptor, this antibody should ideally be able to deplete the $CD4^+/CD25^+$ cell population *in vivo,* in hopes of enhancing the efficacy of adjunct immunotherapy. One caveat, however, is that CD25 is also expressed on newly activated $CD4^+/CD25^-$ T-cells, as well as effector $CD8^+$ T-cells (71). Thus, destruction of the naturally occurring Treg cells through a CD25-specific mechanism may potentially destroy some of the effector T-cells capable of mounting a clinically salient immune response.

An alternative approach to selectively removing the $CD25^+$ population prior to introduction of a tumor vaccine is total lymphocyte ablation *in vivo*, followed by introduction of the appropriate immunotherapeutic. Melanoma patients had their tumors harvested *ex vivo* for selection of tumor-infiltrating lymphocytes. Prior to reinfusion of the expanded tumor-specific lymphocyte population, cyclophosphamide and fludarabine were administered to deplete the lymphocyte population *in vivo*. Marked expansion of tumor-specific T-cells was observed and 51% of the melanoma patients achieved objective responses (72, 73).

HSPs as anticancer vaccines

Another option for producing an effective cancer vaccine is through the use of highly immunogenic HSPs as tumor-associated antigenic adjuvants. The dichomatous role of HSPs as both immunostimulants and immunosup-

pressors, perhaps through induction of a Treg response to counter inflammation, has been the subject of much critical debate in recent years. The ability of HSPs to elicit an immune response was first demonstrated in 1986 by Srivastava and colleagues, who showed that mice immunized with gp96 produced tumor-specific immunity against the tumors that were used to isolate the HSP, but not against other tumors (74). These results were validated in studies on a number of other HSPs, including HSP70, HSP90, calreticulin, HSP110 and GRP170 (75-78). In each of these experiments, it was shown that the HSPs isolated from cancer cells elicited immunity, while HSPs isolated from normal tissues did not.

This initial work led to subsequent discoveries of the immunostimulatory effects that HSPs have on nearly all cells of the immune system, including T-cells, B-cells, macrophages and DCs (79). HSPs also function as carriers of self- and non-self-peptides. As intracellular chaperones, HSPs bind a wide variety of peptides *in vivo* (80). In the case of a malignant cell, the HSP-peptide complexes presented on the surface of the cell are unique TAAs and thus can be attacked by the immune system of a vaccinated patient. This is supported by research that showed that the antitumor response generated by HSP-associated tumor cells was derived from peptides bound to the HSPs and not directed against the HSPs themselves (77, 81). Given singly, neither HSPs nor peptides were immunogenic; only the HSP-peptide complex was able to elicit a $CD8^+$ cytotoxic T-cell response (82). Thus, the specific immunogenicity of each HSP-peptide preparation is due to the inherent antigenic variety found in the malignancy (83).

This research has defined a new type of cancer vaccine that can be created using tumor-derived HSP-peptide complexes (HSPPCs) as effective immunological targets. HSPs could be loaded *in vitro* with synthetic peptides and injected back into the patient to achieve a desired immune response. In order to achieve immunity by HSP vaccination, both APCs and $CD8^+$ T-cells are required, as depletion of either cell type diminishes the protective effects of the vaccine (84). It has also been shown that HSPs can mediate efficient cross-presentation into the class I pathway after being endocytosed by APCs, through the CD91 receptor, rendering them more effective at generating the desired cytotoxic T-cell response (84, 85). In addition to the direct effects on antigen presentation, the interaction of HSPs with APCs leads to secretion of proinflammatory cytokines, such as TNF-α, IL-1β, IL-12 and GM-CSF, maturation and migration of DCs and translocation of NF-κB (75, 86, 87). A number of murine models have demonstrated that HSP immunization slows the progression of primary tumors and reduces the overall metastatic burden as well (76).

The initial autologous HSP vaccine to be tested in a clinical trial was HSPPC-96 (Oncophage®, vitespen; see monograph this issue), a gp96 HSP-peptide complex made from resected tumor tissue and given back to the patient via intradermal or subcutaneous injection. Several clinical trials have looked at the safety profile of this vaccine, as well as the optimal route of administration and dosing schedule (88). The most common side effects noted were transient and included injection-site inflammation and low-grade fever. The results demonstrated that HSPPC-96 treatment resulted in a better quality of life during treatment when compared to those patients undergoing chemotherapy or high-dose cytokine immunotherapy (89). In addition, no clinical signs of autoimmunity have been documented in the more than 500 patients treated.

The encouraging safety profile of the HSPPC-96 vaccine has led to a number of phase II and III clinical trials to determine whether or not the vaccine can have a beneficial effect in cancer patients. The frequency of measurable immune response correlated well with favorable clinical responses. In a melanoma trial, the majority of the clinical responders were also those who had measurable immune responses, and conversely, those without clinical responses were less likely to have measurable immune responses (90, 91).

Currently, there is sufficient evidence to support the idea that anticancer vaccination with HSPPCs can produce measurable antitumor responses in cancer patients. Why these measurable immune responses do not always translate into an observable clinical response may relate to the role of HSPs as autoantigens that generate a Treg cell response (92). If high levels of HSPs are present in apoptotic cells engulfed by DCs, peptides derived from the HSPs may be presented in class II MHC molecules and induce Treg cell stimulation. As in other forms of immunotherapy, the immune system may both potentiate and limit the success of the treatment, and a combination approach using anti-Treg modalities to supplement immunotherapy may allow us to circumvent the difficulties while preserving the benefits.

Extracorporeal photopheresis and transimmunization

We have developed a form of immunotherapy that may address many of the limitations imposed by current approaches to immunotherapy. This treatment is clinically practical, and obviates the need for extensive cell manipulation and potential breaches of sterility. The therapy is broadly applicable and does not need to be tailored to the individual patient. The recent modification of the standard therapy has defined a possible mechanism and provided a means to extend the therapy to a broader range of malignancies and the opportunity to potentially steer the immune response between tolerance and immunity.

In its initial incarnation, extracorporeal photopheresis (ECP) was approved by the FDA for the treatment of CTCL (93). ECP was developed from a combination of therapeutic leukapheresis, a cytoreductive treatment (94), and psoralen and ultraviolet light therapy (PUVA), used for the treatment of psoriasis and early-stage skin-limited CTCL (95). In ECP, the photoactivatable drug 8-methoxypsoralen (8-MOP) is added to a therapeutic leukapheresis, which is passed through a UVA exposure field (96). The drug is hydrophobic and enters the DNA helix, where after photoactivation it can form cross-links between adjacent pyrimidine bases (97). The cross-

linked DNA does not replicate and is poorly repaired, leading to gradual apoptotic cell death over a 6-day period (96, 98). ECP has become an important therapeutic option for CTCL (93) and has also shown activity for the reversal of transplant rejection (99) and the treatment of graft-versus-host disease (100).

We have recently modified this therapy by the introduction of a simple overnight incubation phase, which has revealed clues to the mechanism of the therapy (101). We demonstrated that the combination of the physical interactions caused when the leukapheresis cells pass through the large plastic UVA exposure plate, inducing adherence and release of monocytes, combined with the apoptotic death of the malignant T-cells has profound consequences for the reinfusate returned to the patient. The monocytes become activated and begin to transition into phagocytic, immature DCs, while the malignant T-cells are rendered apoptotic and are engulfed by the DCs. Therefore, after overnight incubation, DCs loaded with apoptotic T-cells are returned to the patient. During the overnight culture, the monocytes secrete cytokines that are similar to the constituents of monocyte-conditioned media (MCM), which have been shown to induce DC maturation (102). In addition, the phagocytosis of apoptotic material drives DC differentiation into maturing DCs demonstrating increased expression of CD83, class II MHC, the co-stimulatory molecule CD86 and loss of markers associated with the monocyte lineage, such as CD14 and CD36 (101). The maturing DCs are better stimulators in mixed leukocyte cultures than leukapheresis leukocytes, confirming their expression of high levels of class II MHC molecules.

Transimmunization (TI) therapy has been used clinically in a phase I trial that demonstrated an excellent safety profile and preliminary signs of efficacy, with partial responses found in 55% of CTCL patients who had failed all other forms of therapy (103).

An additional benefit of the TI procedure is access to all the participants in the immune response, permitting the addition of exogenous agents such as drugs, antibodies or cytokines. The level of apoptosis can be controlled, and since high numbers of apoptotic cells appear to correlate with induction of a Treg cell response (104), it may be possible to tailor the therapy for the treatment of other disorders due to a failure of immunoregulation. The high levels of apoptotic cells generated by ECP may also play a role in the efficacy of this therapy in autoimmune disorders and organ rejection. Other types of malignancies might also be amenable to treatment with TI, since the nature of the apoptotic cells added to the overnight culture can be simply modified through the use of other mediators of programmed cell death (such as irradiation of isolated cell suspensions from solid tumors) and co-incubation with TI-generated transitioning DCs overnight.

Molecular approaches for immunotherapy

The potential of molecular medicine as a diagnostic and prognostic tool is beginning to be realized. It is likely that new therapeutics will be identified through expression array analysis of a variety of cancers, which will identify genes that are upregulated in tumor cells but not highly expressed in normal tissue. In the case of CTCL, gene expression studies of patients with CTCL were compared with normal controls and 385 genes were identified that were differentially expressed (105). Genes that were found to be overexpressed included: the transcription factors GATA-3 and jun-B, the integrin β_1, proteoglycan 2, the oncogene *RHOB* and phosphatase 1. In addition, T-plastin, which is not normally expressed in lymphoid cells, was found to be restricted to CTCL cell samples and may be useful as a tumor marker for this disease. A 4-fold upregulation of expression of the chemokine receptor CX_3CR1 was found, as was increased expression of ICAM-2 (intercellular adhesion molecule 2) in comparison to normal controls. Some genes that were underexpressed in CTCL included those for STAT4 (signal transducer and activator of transcription 4), CD26 and IL-1 receptors. Identification of these genes may serve as new diagnostic tools for monitoring CTCL and may also provide targets for the development of new drugs.

One example of a molecular approach that is being tested for immunotherapy is the use of small interfering RNA (siRNA) to shut off the expression of genes and thereby modulate immune responses. In an attempt to enhance the immunostimulatory capacity of DCs, siRNA was used to inhibit the suppressor of cytokine signaling 1 (SOCS1). SOCS1 is a member of a family of proteins that regulate immune cell responses to cytokines (106). SOCS1 inhibits specific kinase function and targets interacting proteins into the degradation pathway via the ubiquitin-transferase complex. SOCS1 also binds to the p65 subunit of NF-κB and causes its degradation (107). When the *SOCS1* gene was silenced by siRNA, DCs became hyperactive, mediating enhanced antigen presentation, CTL stimulation and antitumor immunity (108). This approach may allow the immunotherapist to inactivate the tolerogenic profile of DCs and enhance their immunostimulatory capacity. Further development of other approaches elucidated through molecular characterization of immune responses will enable the development of more sophisticated immunotherapy protocols.

Conclusions

The future of immunotherapy for cancer appears brighter than it has in many years due to advances in immunology and molecular biology. Despite numerous trials with disappointing results, newer approaches are more targeted and less toxic. As our understanding of the obstacles which defeat our best efforts are understood, novel immunotherapeutics can be designed and tested. One of the most important observations is the duality of the immune system, with the same cell capable of mediating both suppressive and stimulatory functions. Once the factors that control the nature of the induced immune response are clarified, it may be possible to steer the

immune system towards a desired endpoint, and thereby not only treat malignancy but also prevent unwanted suppressive sequelae.

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