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Review

Cancer Immunotherapy by Dendritic Cells

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Cancerous lesions promote tumor growth, motility, invasion, and angiogenesis via oncogene-driven immunosuppressive leukocyte infiltrates, mainly myeloid-derived suppressor cells, tumor-associated macrophages, and immature dendritic cells (DCs). In addition, many tumors express or induce immunosuppressive cytokines such as TGF-β and IL-10. As a result, tumor-antigen crosspresentation by DCs induces T cell anergy or deletion and regulatory T cells instead of antitumor immunity. Tumoricidal effector cells can be generated after vigorous DC activation by Toll-like receptor ligands or CD40 agonists. However, no single immunotherapeutic modality is effective in established cancer. Rather, chemotherapies, causing DC activation, enhanced crosspresentation, lymphodepletion, and reduction of immunosuppressive leukocytes, act synergistically with vaccines or adoptive T cell transfer. Here, I discuss the considerations for generating promising therapeutic antitumor vaccines that use DCs.

Introduction

Dendritic cells (DCs) play a pivotal role in the initiation, programming, and regulation of tumor-specific immune responses (Steinman and Banchereau, 2007; Dhodapkar et al., 2008). Nevertheless, surprisingly little is known about the role of different DC subsets in cancer initiation and progression in patients. Rather, most of the efforts have been focused on utilization of the immunostimulatory power of fully activated DCs for immunotherapy of cancer (Figdor et al., 2004; Palucka et al., 2007; Lesterhuis et al., 2008). Despite this effort, a full understanding of the complex relations between tumors and the host, including DC-mediated regulation of host leukocyte responses, is likely to improve the precision and effectiveness of cancer immunotherapy. It is important to remember that passive immunotherapy with monoclonal antibodies is now a considerable success with great hopes for the future (Finn, 2008), and effective therapies promoting cell-mediated anticancer immune responses are likely to follow. In this review, I will discuss the evidence that cancer is associated with an environment disfavoring the DC activation required for proper effector $CD4^+$ and $CD8^+$ T cell activation, including evidence that DC phenotypes in cancer tissue and draining lymph nodes usually carry an ''immature'' phenotype and that myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) adversely affect DC function. I will then discuss how DC-based immunotherapy can be improved and emphasize the need for combining antigen-specific DC-based therapy with other therapies for effective treatment.

Immunosurveillance of Virus-Induced Tumors

Patients with T cell deficiencies or under immunosuppressive therapy are at an increased risk of virus-induced malignancies, induced by viruses such as Epstein-Barr Virus (EBV) or high-risk human papilloma virus (HPV) (Grulich et al., 2007; Angeletti et al., 2008). Nevertheless up to 15% of cancers in healthy immunocompetent people are also caused by viruses. Interestingly, those immunocompetent women that do not spontaneously clear such HPV infections, and are therefore at risk of developing cervical cancer, fail to generate interferon-γ (IFN-γ)-producing T cells to the early viral proteins E2 and E6 (de Jong et al., 2004; Welters et al., 2006) . This failure of IFN-γ production is HPV specific. Most infected women succeed, however, in clearing high-risk HPV infections by effective IFN-γ T cell responses (de Jong et al., 2004; Welters et al., 2006). Both high-risk HPV DNA, through CpG motifs triggering TLR9 (Hasan et al., 2007), as well as the viral capsid protein L1 (Lenz et al., 2001; Da Silva et al., 2007) possess DC-activating ability, thereby explaining the induction of robust T cell responses and viral clearance in the majority of people. Nevertheless, in a minority of women the virus, presumably aided by its capacity to downregulate type I IFN responses, persists and transforms keratinocytes (Nees et al., 2001), thereby avoiding DC activation by type I IFN. Further, keratinocytes transformed by high-risk HPV do not express TLR9 (Hasan et al., 2007), in contrast to normal differentiated keratinocytes; such a result ensures the lack of production of DC activating signals by the transformed cells through this pathway. This example illustrates the subtlety of the balance between virus and host: Virus control is the rule and oncogenesis the exception, at least in immunocompetent individuals. A similar subtlety seems to apply to nonviral cancers as exemplified by chemical carcinogenesis. Clearly, the adaptive T cell response cannot prevent chemical carcinogenesis, but tumor dormancy in a chemical carcinogenesis model is clearly kept in check by IFN-γ-dependent T cell immunity (Koebel et al., 2007), indicating that sufficient DC activation occurred spontaneously in this model to induce effector T cells with antitumor activity.

Cancer Associated with Chronic Inflammation

Chronic inflammation is considered to contribute to the development of ~15%–20% of malignancies worldwide, including esophageal, gastric, hepatic, pancreatic, and colorectal cancer (Mantovani and Pierotti, 2008). Inflammatory bowel disease is a known condition predisposing to colorectal cancer (Konda and Duffy, 2008; Atreya and Neurath, 2008). In addition, long-term use of nonsteroidal anti-inflammatory drugs such as aspirin is known to reduce the risk of colorectal cancer development (Wang and Dubois, 2008). Inflammatory mediators can also be

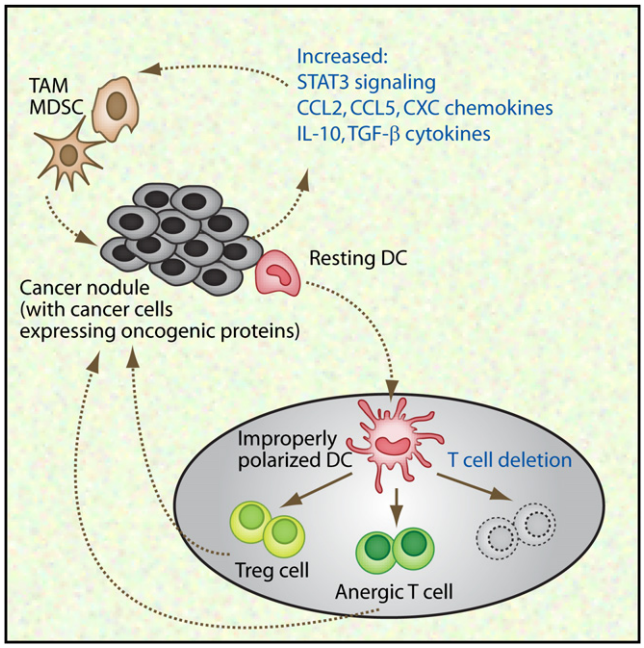

Figure 1. Subversion of Effector T Cell Responses by Cancerous Growth

Oncogenic protein expression in cancer cells causes overexpression of STAT3, production of CCL2, CCL5, and CXC chemokines, and production of IL-10 or TGF-β cytokines. Together, these factors attract tumor-associated macrophages (TAMs) and myeloid derived suppressor sells (MDSCs) into the tumor stroma. As a result, resting tumor-associated and tumor-draining lymph-node-resident DCs are insufficiently activated and improperly polarized. This leads to the generation in the lymph node of anergic T cells and Treg cells. An unknown proportion of tumor-responding T cells may also be deleted. The abnormal tumor vasculature hinders the smooth entry of tumor-specific effector cells into vascularized cancer nodules.

produced by the cancer cells, once cancer has been initiated, and by cells in the (pre-)malignant stroma, including leukocyte populations, in particular the MDSCs (Marigo et al., 2008) and TAMs (Sica and Bronte, 2007) (Figure 1), mainly under the influence of activated cellular oncogenes such as *MYC*, *RAS*, *RET*, and *BRAF* (Borrello et al., 2008).These mediators can cause leukocyte recruitment (Sica and Bronte, 2007; Marigo et al., 2008) and angiogenesis and can influence tumor cell survival, motility, and chemotaxis.(Borrello et al., 2008).

The well-known transcription factor NF-κB plays a particularly important role in liver cell homeostasis, cell survival, and carcinogenesis (Vainer et al., 2008) and is pivotal in the activation of DCs by a variety of stimuli. In some liver cancer models in mice, NF-κB expression in liver cells promotes cancer, whereas in other models, it impedes carcinogenesis (Vainer et al., 2008). Furthermore, breast cancer cells are known to produce the inflammatory chemokines CCL2 and CCL5, which are poorly expressed by normal breast cells (Soria and Ben-Baruch, 2008). These chemokines mediate a shift in the balance between different stromal leukocyte populations in favor of deleterious TAMs and inhibit potential antitumor T cells (Soria and Ben-Baruch, 2008). CCL2 also promotes angiogenesis. In addition, both chemokines act directly on the tumor cells and increase their migratory and invasion-related phenotype (Soria and Ben-Baruch, 2008).

Last but not least, a prominent feature of many cancer cells is constitutive oncogene- and cytokine-driven overexpression of the STAT3 protein (Wang et al., 2004; Kortylewski et al., 2005; Kortylewski and Yu, 2008) (Figure 1). STAT3 upregulates the expression of several immunosuppressive cytokines, including IL-10 and TGF-β, and suppresses T helper 1 (Th1) cell immune responses (Kortylewski et al., 2005; Kortylewski and Yu, 2008). STAT3 expression by tumor cells begets STAT3 production by a variety of leukocytes, including DCs, interacting with the tumor cells, with wide-ranging deleterious effects on antitumor effector leukocytes (Kortylewski and Yu, 2008). In particular, STAT3 expression in tumor-associated DCs causes reduced expression of costimulatory molecules and MHC class II (Kortylewski and Yu, 2008), as well as production of TGF-β (Kortylewski and Yu, 2008). Tumor progression indeed correlates with an accumulation of immature DCs that induce the expansion of regulatory T (Treg) cells in tumor-draining lymph nodes (Ghiringhelli et al., 2005).The STAT3 inhibitor Cucurbitacin I specifically decreases the amounts of phosphorylated STAT3 (P-STAT3) in many mouse and human cancer cell lines (Blaskovich et al., 2003; Shi et al., 2006b; van Kester et al., 2008) and inhibits the growth of tumors in mice (Blaskovich et al., 2003). In cutaneous T cell lymphomas, including Sezary syndrome, Cucurbitacin I also inhibited P-STAT3 and caused tumor cell apoptosis in vitro (van Kester et al., 2008). This inhibitor thus could help to not only directly kill tumor cells but also redress the immunosuppression associated with P-STAT3 signaling.

DC Numbers and Phenotypes in Cancer

DC phenotypes in cancer tissue and cancer-draining lymph nodes are often those corresponding to resting, nonactivated, "immature" DCs, both in tumor-bearing animals and in patients with cancer (reviewed in Gabrilovich, 2004; Schuurhuis et al., 2006a; Dhodapkar et al., 2008) (van Mierlo et al., 2004; Boonman et al., 2005) Early studies have suggested that patients with high numbers of DCs present in tumors survived longer than patients with few or no DCs in the cancer tissue (Becker, 1993). However, in a more recent study, colorectal cancer patients with relatively high numbers of "mature" $CD208^+$-infiltrating DCs in the tumor epithelium had a markedly shorter survival rate (Sandel et al., 2005). In addition, patients with relatively high numbers of $CD1a^+$ DCs in the advancing margin of the tumor had a markedly shorter disease-free survival rate (Sandel et al., 2005). In the case of human breast cancer, infiltration of cancer tissue with mature DCs is frequently seen, but these DCs drive a $CD4^+$ T cell response associated with secretion of IL-13, thereby facilitating tumor growth rather than inhibition, which explains the eosinophil granulocyte infiltrations often seen in breast cancer tissue (Aspord et al., 2007). In contrast, in a cohort of 39 patients with advanced melanoma (stages III B or C and IV), treated with the cytokine GM-CSF, a greater increase in mature DCs after treatment was associated with disease remission or delayed recurrence (Daud et al., 2008). The immunosuppressive milieu created by cancers generally causes a decrease in the numbers of conventional "myeloid" DCs, with little or no effects on the numbers of plasmacytoid DCs (Gabrilovich, 2004). Thus, immature myeloid DCs promote the expansion of Treg cells in tumor-draining lymph nodes, associated with tumor progression in a TGF-β-dependent fashion (Ghiringhelli et al., 2005). However, these

findings were made in rodent models. In general, it is safe to assume that neither the decreased numbers of immature myeloid DCs found in human cancers nor the immature pDCs contribute to immune defenses against these cancers, but more work is required to chart the clinical significance of the different human cancer-associated DC populations. Even if these improperly activated or even dysfunctional tumor-associated DCs support immune responses compatible with oncogenesis or even promote oncogenesis, therapeutic activity can be imprinted in these DCs by molecularly defined triggers of DC maturation causing induction of robust tumor-specific effector T cells, as discussed below.

Suppression of DC Function in the Tumor Environment

The inflammatory nature of many cancers and the resulting tumor infiltration with assorted leukocytes, in particular myeloid MDSCs and TAMs (Sica and Bronte, 2007; Marigo et al., 2008), creates an immunosuppressive environment that leads to suppression of DC-instructed effector $CD4^+$ and $CD8^+$ T cell responses and the induction of Treg cells. Much of this immunosuppression is mediated by cytokines secreted by the tumor or the tumor-infiltrating MDSCs and/or TAMs acting on the DCs in the draining lymph nodes presenting tumor-associated antigens (Gabrilovich, 2004). In addition, direct chemical and enzymatic interactions between leukocyte products and tumor-responding T cells, such as nitrotyrosination of the T cell receptor (TCR) and CD8 molecules, have been described (Nagaraj and Gabrilovich, 2008). The relative importance of direct deleterious effects exerted by tumor-associated MDSCs and TAMs on T cells versus effects exerted on migratory DCs from tumors and DCs in tumor-draining lymph nodes is not known. Nevertheless, the end result is immunosuppression of tumor-directed T cell responses. It is likely that both mechanisms operate in a tumor setting. Further dissection of these complicated mechanisms is likely to yield new therapeutic possibilities. In a study of sporadic tumorigenesis by an SV 40 large T oncogene in mice, the prominent induction of MDSCs with deviation and suppression of the anti-SV 40 T cell responses was shown to be associated with tumor outgrowth (Willimsky et al., 2008). However, the interpretation that this model is a reflection of the general absence of immunoediting and immunosurveillance against sporadic nonviral tumors is probably premature. More likely, the balance between immunosuppression and immunosurveillance exerted by spontaneously arising tumors varies depending on the inflammatory signature installed by the distinctive oncogenic events in each tumor or premalignant lesion. In the case of SV 40 large T, powerful inflammatory abnormalities could have been caused in the premalignant and malignant stages, which are not generally representative of other tumor models or human cancer. Also, the evidence from different tumor models is overwhelming that the lack of T cell effector function, apart from the suppressive effects of MDSCs, TAMs, and $Foxp3^+$ Treg cells, is caused by lack of expression of costimulatory molecules on tumor-associated DCs or by dysregulation of costimulatory pathways (Kortylewski and Yu, 2008; Keir et al., 2008). In the case of SV40 oncogenesis, this situation can be addressed therapeutically as illustrated in a nonsporadic model of tumorigenesis by SV40 large T (expressed under the rat insulin promoter), in which tumor-specific $CD8^+$ T cells were not ablated by the insufficiently activated DC, but could be aroused to full effector function, associated with a greatly enhanced lifespan of the tumor-bearing mice by intravenous vaccination with tumor-derived peptide and agonistic DC-activating CD40 antibody or DC-activating viral immunization and boosting (Nguyen et al., 2002).

Mechanisms of Crosspresentation for $CD8^+$ T Cell Recognition of Tumor Antigens

DCs can present antigens to $CD8^+$ T cells either through endogenous processing after, e.g., microbial infection or by uptake and processing of exogenous antigens, a process known as cross-presentation. Both modes of antigen presentation probably play a role in infectious disease (Melief, 2003; van der Bruggen and Van den Eynde, 2006; Lin et al., 2008b), but in the case of cancer, crosspresentation after uptake and processing of soluble or particulate matter from apoptotic, necrotic cancer or even live cancer cells is the only important natural mode of presentation (Melief, 2003; Gabrilovich, 2004; van der Bruggen and Van den Eynde, 2006; Lin et al., 2008b). Our knowledge of mechanisms operating in the ingestion, processing, and, ultimately, crosspresentation of tumor-associated antigens to $CD8^+$ T cells by DCs is mainly derived from in vitro experiments. Clearly, a variety of mechanisms can transfer antigens from tumors to DCs for MHC class I presentation, including: (1) antigens from dead cells (apoptotic or necrotic tumor cells) (Albert et al., 1998; Blachere et al., 2005; Berg et al., 2006; Fonseca and Dranoff, 2008), (2) soluble antigens bound to chaperonins such as heat shock proteins, docking onto scavenger receptors on DCs (Binder et al., 2007; Giodini and Cresswell, 2008), (3) soluble proteins (Norbury et al., 2004), (4) antigen-carrying vesicles secreted by some tumor cells, called exosomes (Zeelenberg et al., 2008), (5) transfer of small antigenic protein fragments through so-called gap junctions (Neijssen et al., 2005), (6) plasma-membrane fragments "nibbled" from live tumor cells by DCs (Harshyne et al., 2001), and (7) an apparently unrelated mechanism called "crossdressing": Peptide-MHC class I complexes are acquired by direct contact of DCs with dead, but not live, tumor cells (Dolan et al., 2006). Mechanisms 6 and 7, in contrast to the others listed, operate in the absence of antigen processing by the DC and involve direct transfer of peptide-MHC complexes to the DC. This last mechanism also seems to operate in vivo in influenza virus infection, in that TAP-deficient DCs acquired influenza peptide-MHC class I complexes from apoptotic influenza-infected cells (Blachere et al., 2005).

The rules and mechanisms are much less known for crosspresentation of tumor-associated antigens in vivo. It seems reasonable, however, to rely in large part on the general rules for in vivo crosspresentation delineated from work outside the cancer field. In mouse experiments with the model antigen ovalbumin (Ova), low expression of Ova is associated with the lack of crosspresentation, whereas high expression of Ova is associated with crosspresentation and, in the absence of DC activation, with peripheral deletion of Ova-specific T cells (Kurts et al., 1999). The amount of antigen expression is therefore very important for crosspresentation to $CD8^+$ T cells.

In another study with DNA vaccines, the stability of the protein expressed from the DNA used for vaccination is also important for immunogenicity, in the sense that high protein stability corresponds to high immunogenicity (Bins et al., 2007). High protein

expression was achieved in the skin by short-interval intradermal delivery of DNA encoding the tumor-associated antigen HPV 16 E7, a vaccine delivery method inducing robust $CD8^+$ cytotoxic T lymphocyte (CTL) responses, again consistent with the notion that high protein expression promotes crosspresentation to $CD8^+$ T cells (Bins et al., 2005). In mice deficient for the GTPase Rac1, crosspresentation is severely disturbed, causing lack of T cell tolerance for self tissues, indicating that self-tolerance depends on crosspresentation to $CD8^+$ CTL precursors of normal tissue antigens, probably from natural tissue apoptosis (Luckashenak et al., 2008). Thus, high amounts and stable protein expression by tumors, as well as a high degree of spontaneous tumor apoptosis or necrosis that releases high amounts of proteins, favor crosspresentation.

Crosspresentation of Tumor-Associated Antigens by DCs in Tumor-Draining Lymph Nodes

The outcome of crosspresentation is determined by the activation status of the DCs (Melief, 2003; Steinman et al., 2003). During microbial infection, ''danger signals,'' including TLR ligands (Medzhitov, 2007) encoded by the microbe, induce DC maturation, which in turn induces robust IFN-γ-producing Th1 cell responses and cytotoxic T lymphocyte (CTL) responses. In the case of cancer cells, the extent of DC maturation that leads to tumor-antigen crosspresentation is usually very much weaker than that induced by virulent microorganisms, despite the fact that many cancer cells and/or the cancer-associated stroma possess an inflammatory signature (see above) and despite the fact that so-called endogenous danger signals (Matzinger, 2002) such as uric acid, heat shock proteins, and high-mobility group box 1 (HMGB1) protein can cause DC activation from dying cancer cells (reviewed in van der Most et al., 2008). HMGB1 protein reportedly binds to TLR2 and TLR4 (Park et al., 2006), consistent with immune activation by dying tumor cells involving both HMGB1 and TLR4 (Apetoh et al., 2007). A dampening influence on $CD8^+$ T cell activation by DCs crosspresenting cancer-associated antigens may be exerted by the selective recruitment of the pattern recognition receptor pentraxin 3 (PTX3) to the synapse between crosspresenting DCs and dying (apoptotic) cells (Baruah et al., 2006). PTX3 was found to suppress the $CD8^+$ T cell response to tumor-associated self-antigens, but not to microbial antigens, indicating an important role of PTX3 in censorship of autoimmunity (Baruah et al., 2006). Apart from molecules with modulating properties such as pentraxins, another factor is the amount of apoptosis in growing tumors, which is apparently too low in many instances to generate enough uric acid and heat-shock-bound tumor-associated proteins to cause DC activation. This finding is in line with the immature phenotype of DCs found in many cancers, and is also consistent with the fact that optimal crosspresentation of tumor-associated antigens usually needs tumor apoptosis-enhancing treatments.

The result of antigen presentation to either $CD4^+$ or $CD8^+$ T cells by immature or partially mature DC can be deletional tolerance (Steinman and Nussenzweig, 2002; Steinman et al., 2003) or induction of regulatory $CD4^+$ T cells (Zou, 2006), regulatory $CD8^+$ T cells (Zou, 2006) or of ''poised'' T cells (van Mierlo et al., 2002; van Mierlo et al., 2004). The latter population is interesting for therapeutic purposes because these central memory-like $CD8^+$ T cells that reside in tumor-draining lymph nodes in a mouse model could be rescued for therapy by vigorous DC stimulation in situ by the injection of TLR ligands (CpG and LPS) or CD40 agonist monoclonal antibody (van Mierlo et al., 2002; van Mierlo et al., 2004). Such stimulation was associated with expansion and systemic spread of $CD8^+$ T cells, presumably by proper CTL effector programming by DCs in the tumor-draining lymph nodes that were shown to crosspresent tumor antigen (van Mierlo et al., 2004). Consistent with that, immature DCs loaded ex vivo with an MHC class I-binding peptide induced poised T cells that could be rescued by restimulating the response in vivo with infectious virus expressing this CTL epitope (Dumortier et al., 2005). A potential clinical application of this finding would be to cause expansion and systemic migration of T cells from local tumor draining lymph nodes by CD40 agonist therapy or local TLR ligand administration before surgical removal of tumor-draining lymph nodes. Similar results were obtained in a different mouse tumor model, in which natural presentation of a CTL epitope from the melanocyte antigen tyrosinase crosspresented from a transplanted melanoma cell line caused incomplete CTL activation in tumor-draining lymph nodes associated with tumor outgrowth (Hargadon et al., 2006). Once the tumor had metastasized to these nodes, direct presentation contributed to this incomplete activation. Ex vivo-activated DCs loaded with tumor-associated antigen were able to cause full development of effector CTLs in this situation. A separate mechanism of activation of resident dormant CTLs was achieved by intratumoral administration of the TLR ligand poly (I:C). This resulted in complete tumor resolution caused by type I IFN-mediated activation of the inert intratumoral $CD8^+$ T cells (Currie et al., 2008). In this case, no systemic migration of the tumor-specific T cells was observed. Usually, crosspresentation of tumor-associated antigens by DCs is only effective in the case of high expression and stability of the tumor-associated protein or in the case of a high spontaneous cancer apoptosis or necrosis rate, consistent with the results from model antigen systems and from DNA-vaccination experiments. If spontaneous crosspresentation of tumor antigens is insufficient, processing and presentation can be driven by a variety of tumor cell-death-promoting therapies, including chemotherapy, cryoablation, hyperthermia, and irradiation (Figure 2).

DC Activation with Cancer Chemotherapeutics and Other Tumor Cell-Death-Promoting Therapies

Many tumors do not express protein antigens at a sufficiently high amount to cause ample crosspresentation (Nowak et al., 2003a; Heath et al., 2004; van der Bruggen and Van den Eynde, 2006; van der Most et al., 2008; Zitvogel et al., 2008). Moreover, most growing tumors, despite their often inflammatory nature, do not cause robust DC maturation (Melief, 2003; Zitvogel et al., 2008). Both of these obstacles can be overcome by certain forms of cancer chemotherapy. The massive tumor apoptosis and/or necrosis induced by chemotherapy releases large amounts of tumor-associated proteins, strongly driving efficient crosspresentation by tumor-associated DCs in the tumor-draining lymph nodes (van der Most et al., 2008; Zitvogel et al., 2008) (Figure 2). In its most extreme form, this even leads to the antigen loading of not only DCs but also of stroma cells, including vascular cells, allowing the destruction by CTL-mediated therapy of even MHC class I-low or -negative tumors (Zhang et al., 2007).

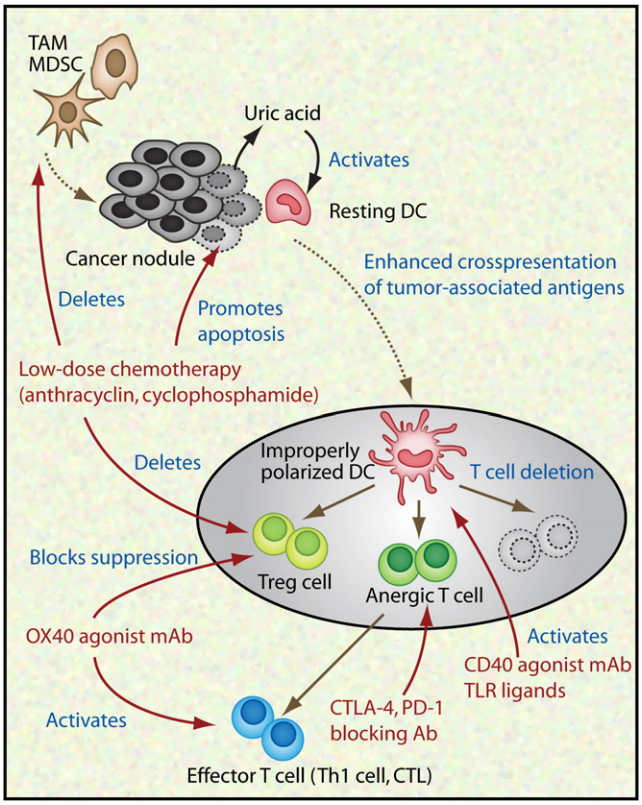

Figure 2. Sites of Action of Immunotherapy of Cancer
Different treatment modalities act in concert to thwart the immunosuppression associated with cancer and promote induction and expansion of effector T cells by a variety of mechanisms. STAT3 inhibitor reverts immunosuppression associated with enhanced STAT3 signaling. Low-dose chemotherapy causes enhanced tumor cell apoptosis, associated with release of uric acid. This has two effects: enhanced crosspresentation of tumor-associated antigens by DCs and DC activation via crystalline uric acid. Low-dose chemotherapy also deletes Treg cells, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) and allows homeostatic expansion of vaccine-driven effector T cells. CD40 agonist antibody and TLR ligands directly activate DC in tumor-draining lymph nodes, like therapeutic vaccines driving robust effector T cell responses (Th1 cell and CD8 CTL responses). CTLA-4 and PD-1 blocking antibodies release the brakes of costimulation and thereby converts anergic T cells into effector T cells. OX40 agonist antibody blocks suppression by regulatory T cells and activates effector T cells.

In most instances, the enhanced crosspresentation of tumor antigens by DCs after chemotherapy is not enough to induce a sufficiently robust T cell response for tumor eradication (van der Most et al., 2008; Zitvogel et al., 2008). However, after additional activation of the DCs by molecularly defined DC-activating compounds such as CD40 agonistic antibody, a strongly synergistic antitumor effect mediated by tumor-specific $CD8^+$ T cells has been observed (Nowak et al., 2003a; Nowak et al., 2003b; Nowak et al., 2006). The benefit of chemotherapy is to allow the use of the tumor as its own vaccine for appropriate DC loading, thereby necessitating only the use of a strong DC activator to achieve meaningful therapeutic effects. This has the added advantage that cancer epitopes based on tumor-unique mutational sequences will have a chance to induce powerful tumoricidal T cell responses, without the need to identify and sequence these mutations. In a study with a murine tumor transduced with the model antigen influenza hemagglutinin (HA), treatment with the chemotherapeutic drug gemcitabine markedly enhanced crosspresentation of HA to $CD8^+$ T cells, without causing deletion of these T cells. Drug-induced cancer apoptosis primed the host for a strong antitumor response to vaccination with a second, influenza virus-mediated HA presentation in a more costimulatory context (Nowak et al., 2003a).

Certain ''immunogenic'' forms of cancer chemotherapy that cause substantial DC activation by molecularly defined pathways even in the absence of added DC-activating compounds have been described. One of these chemotherapeutic drug classes are the anthracyclins, DNA-damaging compounds. Anthracyclins induce the rapid translocation of calreticulin, an ER-based molecule in the class I processing pathway, to the cell surface. This process promoted phagocytosis of cancer cells by DCs and is associated with DC activation and immunogenicity in vivo (Obeid et al., 2007). It was subsequently found that the ectocalreticulin display is by itself not sufficient to cause DC activation and tumor eradication but that the HMGB 1 alarmin protein secreted by dying tumor cells interacts with TLR4 on DCs to cause immunogenic crosspresentation (Apetoh et al., 2007). Another compound, Cyclophosphamide (Cy), can enhance tumor-specific immunity in a variety of ways. Reportedly, a low dose of Cy selectively depletes $CD4^+25^+$ Treg cells (reviewed in Zou, 2006; van der Most et al., 2008; Zitvogel et al., 2008; and Brode and Cooke, 2008). Also, Cy and other chemotherapeutic agents induce production of uric acid caused by tumor cell death. Uric acid, at least supposedly in crystal form, activates DCs, promoting tumor rejection (Hu et al., 2004) (Shi et al., 2003; Shi et al., 2006c). The considerable DC-activating potency of uric acid was recently demonstrated by the finding that alum, the most widely used adjuvant in vaccines, has no intrinsic DC-activating ability in vivo, but acts through uric-acid-induced DC activation in vivo (Kool et al., 2008). Cy also induces a profound and systemic type I IFN release (Schiavoni et al., 2000) that results in DC and T cell activation and appears to be partially responsible for its antitumor effect (Mokyr et al., 2006). Finally, many forms of chemotherapy cause some degree of lymphopenia, which is likely to create an excellent stage for homeostatic expansion of tumor-specific $CD8^+$ T cells, in particular in combination with robust therapeutic vaccination or adoptive transfer of tumor-specific T cells (Muranski et al., 2006; Rosenberg et al., 2008).

In a murine tumor model system with OVA-transduced B16 melanoma cells, cryoablation with liquid nitrogen of tumors alone enhanced crosspresentation by DCs in vivo. However, this process can only lead to highly effective in vivo antigen presentation by DCs when performed in combination with additional DC stimulation by TLR9 ligand CpG vaccine (den Brok et al., 2006b). In an in vitro study, it was shown that human DCs loaded with melanoma cells that were heated to 42°C and subsequently killed are more efficient at crosspriming naive human CTL in 3 week cultures than DCs loaded with unheated killed melanoma cells (Shi et al., 2006a). The heat-treated melanoma cells expressed enhanced amounts of HSP70, and the enhanced crosspriming could be reproduced by overexpression of HSP70 in melanoma cells. The hyperthermia also caused increased transcription of several tumor-associated antigens, including MAGE-B3, MAGE-B4, MAGE-A8, and MAGE-A10 (Shi et al., 2006a). Not unexpectedly, tumor cell death caused by radiotherapy also promotes crosspresentation (Chen et al., 2005; den

Brok et al., 2006a; Apetoh et al., 2007). Thus, the accelerated cancer cell death associated with conventional cancer therapies not only leads to enhanced crosspresentation of tumor-associated antigens to DCs but also to DC activation by the enhanced release of uric acid from dead cells. Certain forms of cancer chemotherapy further enhance DC activation as exemplified by release of type I IFN after therapy with Cy and ectocalreticulin display after anthracyclin therapy. Because optimal DC activation depends on synergistic triggering of several molecules (Napolitani et al., 2005), additional clinical benefit can be achieved by administration of exogenous TLR ligands and CD40 agonists.

Subsets of DCs that Crosspresent Tumor Antigens

Most data on subsets of DCs involved in crosspresentation to CTL precursors have been generated in mice (reviewed in Heath et al., 2004). In this species, the DC subset expressing CD8α is particularly effective in crosspresentation to CD8 CTL precursors (Heath et al., 2004). The CD8α subset of DC appears to originate from the CD8α negative subset by a maturation process involving upregulation of not only CD8α but also the C-type lectin DEC-205 and CD24 (Martinez del Hoyo et al., 2002). The dominant role of this subset in crosspresentation is not due to differences in antigen capture but rather to a greater processing efficiency (Schnorrer et al., 2006). Not all $CD8\alpha^+$ DC appear to possess this specialized function because intravenous injection of cytochrome C caused apoptosis of approximately half the $CD8\alpha^+$ DC population, but virtually completely ablated crosspresenting ability and thereby CTL induction, reducing subsequent immunity to tumor challenge (Lin et al., 2008a). Particularly relevant for tumor-antigen processing is the finding that $CD8^+$ DCs, residing in the T-dependent areas in lymph nodes and spleen, are specialized in the uptake and crosspresentation of apoptotic cells (Iyoda et al., 2002; Schulz and Reis e Sousa, 2002). In the crosspresentation of skin-derived antigens, exemplified by herpes simplex virus skin infection in mice, collaboration was observed between migratory DCs transporting antigen to sessile lymphoid-resident DCs (Allan et al., 2006). Similar collaboration between migratory $langerin^+CD11b^-$ DCs and sessile CD8α DCs was observed in the clearance of lung infections from acute influenza virus infection (GeurtsvanKessel et al., 2008). In contrast, plasmacytoid DCs (pDCs), which are strong type I IFN producers, do not crosspresent to CD4 or CD8 virus-specific T cells and are not important for a protective T cell response to this virus, possibly because in influenza infection other cell types produce enough type I IFN (GeurtsvanKessel et al., 2008). Type I IFN is indeed produced rapidly in response to infection by activated NK cells, pDCs, and other cell types, but not in cancer, except after cyclophosphamide treatment.

Type I IFN, in particular IFN-α, has a major effect on crosspresentation by two distinct mechanisms: promotion of crosspresentation by DC activation (Le Bon et al., 2003) and direct stimulation of CTL via type I IFN receptors on these T cells (Le Bon et al., 2006). Mobilizing type I IFN production by pDCs could therefore have a beneficial effect on crosspresentation by common $CD8\alpha^+$ DCs and reveal yet another level of collaboration between DC subsets to be exploited for cancer immunotherapy. Immature human cDCs, but not mature cDCs, were found to upregulate STAT1 in response to type I IFN. Conversely, exposure of mature cDCs to type I IFN leads to signaling via STAT4. STAT1 signaling resulted in inhibition of CD40L-induced IL-12 production, causing inhibition of $CD8^+$ T cell activation (Longman et al., 2007). Type I IFN signaling therefore has differential effects on crosspresentation by mature versus immature cDCs, leading to the conclusion that application of type I IFN in cancer treatment needs to be conducted with care and should be applied in conjunction with full DC activation. In a recent study, TLR9-activated pDCs were directly injected into subcutaneous transplanted B16 melanoma tumors in a mouse model. This treatment induced robust, spontaneous CTL crosspriming against multiple B16 tumor-associated antigens, causing regression of both the treated tumors as well as of distant contralateral tumors (Liu et al., 2008). The T cell crosspriming was mediated by cDCs and was completely dependent on the early recruitment of NK cells at the tumor site (Liu et al., 2008). This NK cell recruitment was mediated by CCR5 via chemokines secreted by the pDCs. The combined data suggest that activated pDCs can initiate effective and systemic antitumor immunity through a series of cellular events involving sequential activation of NK cells, cDCs and $CD8^+$ T cells (Liu et al., 2008).

Human pDCs of melanoma patients can process and present exogenous proteins to $CD4^+$ T cells after FcγRII-mediated protein ingestion (Benitez-Ribas et al., 2006). Both Langerhans cells and cDCs can crosspresent melanoma-associated antigens to CTL and activate them in vitro (Cao et al., 2007). The conclusion is that crosspresentation to CTL precursors in tumor systems involves an intricate interplay between different cell types of the innate and adaptive immune system. Migratory DCs (from organ-specific tissues) and sessile DCs (in lymphoid organs) are likely to collaborate in crosspresentation as in the viral systems discussed above. The prime crosspresenting DC is the cDC, but orchestration of crosspresentation by cDCs appears to be mediated by IFN-α-producing pDCs and by NK cells. A very special case of crosspresentation of tumor-associated antigens from apoptotic tumor cells is crosspresentation by liver sinusoidal endothelial cells, which causes $CD8^+$ T cell tolerance (Berg et al., 2006). This study reveals the existence of yet another professional APC subset that in an organ-specific way engages in crosspresentation of tumor antigens to $CD8^+$ T cells. $CD4^+$ T helper cells are also likely to play a major role through their well-established role in CD40L-mediated DC activation (Schoenberger et al., 1998; Bennett et al., 1998). Indeed, because of this, studies of crosspresentation by these DC subsets in tumor systems should be expanded to MHC class II processing and presentation. That this can be rewarding is illustrated by a recent study in mice in which it was shown that a major cause of defective effector CTL generation against tumors might be defective MHC class II processing of tumor-associated antigens, rather than defective crosspresentation to $CD8^+$ T cells (Gerner et al., 2008).

Therapy with Ex Vivo-Activated DCs versus Direct In Vivo Targeting of DCs

In the last decade, after the initial successful results with preventive and therapeutic DC vaccination in mice (Mayordomo et al., 1995), numerous investigators have initiated clinical trials of established cancer with tumor antigen-loaded DCs, mainly conventional DCs formerly called myeloid DCs as distinct from type I IFN-producing pDCs. These efforts can help to establish proof of

principle that properly activated DCs, loaded with the proper form and dose of antigen and properly activated, as well as properly migrating to lymph nodes, can initiate and expand tumor-specific $CD4^+$ and $CD8^+$ T cell responses to induce meaningful therapeutic responses. So far, despite induction of robust tumor-specific T cell responses in many patients and occasional spectacular complete tumor regressions, particularly in patients with melanoma, (Steinman and Banchereau, 2007; Palucka et al., 2007; Lesterhuis et al., 2008), this hope has not been fulfilled. On one hand, this could be due to many of the problems inherent to any immunotherapeutic approach of cancer, such as the presence of various types of suppressive leukocytes with or without constitutive P-STAT3 signaling (see above), immunoediting (Dunn et al., 2006; Koebel et al., 2007), abnormal tumor vasculature inhibiting effector T cell entry (Hamzah et al., 2008a; Hamzah et al., 2008b), or other factors inherent to the tumor cell biology in its interaction with the stromal environment. On the other hand, the attempts at DC therapy have been plagued by a lack of knowledge of the ideal antigen-loaded DC for optimal therapy and by a lack of standardization (Figdor et al., 2004; Steinman and Banchereau, 2007). In many instances, the DCs were loaded with a limited number of exact HLA-binding peptides with few if any T helper cell peptides. Such a scheme is suboptimal for induction of long-lived CTL responses, because these need to be supported by specific $CD4^+$ T cell responses (Smith et al., 2004; Melief and van der Burg, 2008). Also, in most instances, such peptides only bound to one or a limited number of HLA class I molecules, mainly HLA-A2. Ideally, one would like to properly load all the available HLA class I- and class II-presenting molecules on the DC with tumor antigen-derived peptides (Melief and van der Burg, 2008). Several strategies have been tried to achieve this, such as DC loading with tumor-derived RNA (Gilboa and Vieweg, 2004; Su et al., 2005) or immune complexes (Kalergis and Ravetch, 2002; Rafiq et al., 2002; Schuurhuis et al., 2002; Schuurhuis et al., 2006b). The latter strategy works well in mouse models but has not yet been tried in patients. Loading of DCs with immune complexes consisting of a tumor-associated protein, complexed to a specific IgG antibody, has the double advantage of not only efficiently promoting MHC class I and class II presentation to the available MHC molecules on the DC but also activating the DC through the same Fc receptor(s) (Kalergis and Ravetch, 2002; Rafiq et al., 2002; Schuurhuis et al., 2002; Schuurhuis et al., 2006b), a powerful DC activation method. Fc-receptor-mediated activation of DC works best via ex vivo loading of the DC, probably because this allows the use of optimal concentrations of immune complexes in the absence of immune complex uptake and destruction by macrophages and granulocytes (Schuurhuis et al., 2006b).

Other DC targeting strategies have proven to operate well both in vitro and directly in vivo. An attractive DC targeting approach is the docking onto C-type lectin receptors on DC such as DEC-205 (Steinman et al., 2003; Trumpfheller et al., 2006; Bozzacco et al., 2007), DC-SIGN (Tacken et al., 2005; Tacken et al., 2008), or DNGR-1 (Sancho et al., 2008). Tumor antigens equipped with the sugar moieties docking the antigen to the DC receptors or tumor antigens, coupled to or incorporated in monoclonal antibodies directed against the C-type lectin receptors (Trumpfheller et al., 2006; Bozzacco et al., 2007; Sancho et al., 2008), effectively target the antigens into the MHC class I and II processing pathways of the targeted DC. Because targeting of these receptors is not associated with DC activation, additional DC-activating compounds such as agonistic antibody directed against CD40 or TLR ligands need to be given as well (Trumpfheller et al., 2006). In fact, recent evidence shows that without such DC-activating compounds, this type of DC targeting can be used in vivo to induce disease-specific tolerance to beta cells of pancreatic islets (Mukhopadhaya et al., 2008), reminding us that a very similar mechanism drives DC-mediated tolerance to tumor-associated antigens in the absence of proper DC activation by growing tumors. A similar highly effective targeting of tumor antigens to DC in vivo can be achieved by injecting peptides coupled to TLR ligands (Khan et al., 2007). The efficiency of C-type lectin and TLR-ligand-conjugate-mediated antigen targeting to DCs in vivo raises the question of whether this is not the way to go, instead of the laborious and expensive ex vivo loading of DC with tumor antigens.

In many instances, DCs will acquire exogenous antigen in a very sensitive fashion through specialized receptors. However, synthetic long peptides are also efficiently acquired by DCs (Melief and van der Burg, 2008; Bijker et al., 2008), allowing therapeutic vaccination inducing both $CD4^+$ and $CD8^+$ T cell responses (Zwaveling et al., 2002; Melief and van der Burg, 2008) causing eradication of established high-risk HPV-induced lesions in mice (Zwaveling et al., 2002) and of established cottontail rabbit-papilloma-virus-induced lesions in rabbits (Vambutas et al., 2005). In addition, a complete set of overlapping long peptides of the E6 and E7 oncogenic proteins of HPV16 induced robust immune responses to multiple $CD4^+$ and $CD8^+$ T cell epitopes of these oncoproteins in patients with cervical cancer (Kenter et al., 2008; Welters et al., 2008). Such synthetic long peptides can be easily synthesized under good manufacturing practice (GMP) conditions, required for clinical use, and in contrast to exact MHC class I-binding peptides do not cause specific immune tolerance (Melief and van der Burg, 2008). Receptor-mediated targeting can help to further increase the efficiency of long-peptide DC targeting as shown in mouse experiments (Khan et al., 2007), but clinical application of this requires adaptation of conjugate production to GMP conditions.

Advances in Cancer Immunotherapies

A vivid illustration of the overwhelming importance of intact costimulatory pathways in cancer immunity is the vastly improved resistance against many tumors, including UV-induced skin carcinogenesis and HPV-induced tumor transplants of mice deficient in the E3 ligase Casitas B cell lymphoma-b (cbl-b) (Loeser et al., 2007). Cbl-b-deficient T cells are remarkably independent of costimulation through CD28 on T cells by members of the B7 costimulatory family (such as CD80 and CD86) on antigen-presenting cells, including DCs (Bachmaier et al., 2000; Chiang et al., 2000; Jeon et al., 2004). The phenotype of the tumor model could be recapitulated in normal mice by adoptive transfer of Cbl-b-deficient $CD8^+$ T cells into tumor-bearing wild-type mice (Loeser et al., 2007). These results indicate that if the balance of costimulation in tumor-specific $CD8^+$ T cells is reset, the full power of tumor-reactive T cells can be unleashed and $CD8^+$ T cell-mediated tumor eradication can be achieved. One way to achieve this in normal mice and patients with cancer is to block feedback inhibition of costimulation through CD28 by blockade

of CTLA-4 with monoclonal antibodies (Figure 2). Upon systemic treatment with such antibodies, remarkable therapeutic responses are seen in mice (Sutmuller et al., 2001) and patients with cancer albeit at the expense of severe autoimmune side effects (Phan et al., 2003; Sanderson et al., 2005). In a clinical study of cancer patients treated with CTLA-4-blocking monoclonal antibody, this treatment was observed to expand both effector $CD4^+$ T cells and $Foxp3^+$ Treg cells (Kavanagh et al., 2008), consistent with a second study in which the extent of therapy-induced necrosis in previously vaccinated cancer patients after subsequent treatment with CTLA-4 blockade was related to the ratio of effector cells over Treg cells in posttreatment biopsies (Hodi et al., 2008). Both clinical studies also suggest that the combination of CTLA-4 blockade with depletion of Treg cells has synergistic potential, as indeed observed in a mouse study (Sutmuller et al., 2001). Detailed insights into expression of activating and dampening receptors of B7 family costimulatory molecules and their ligands other than CTLA-4 in cancer and chronic persistent infections have shown that in these conditions, the expression of inhibitory molecules is upregulated (Zang and Allison, 2007; Zou and Chen, 2008; Keir et al., 2008).

One pair of B7 family (ligand) molecules with largely inhibitory functions is represented by PD-1, expressed on activated T cells and so-called exhausted T cells and a variety of other cell types, and PD-L1, expressed on many cell types and overexpressed on a variety of human cancers (reviewed in Zang and Allison, 2007; Keir et al., 2008). In chronic persistent virus infection of mice, T cell responses were enhanced by administration of a monoclonal blocking antibody against PD-L1, associated with a marked reduction in viral load (Barber et al., 2006). Similar observations were also made in mouse tumor models. Tumor-associated DCs were observed to overexpress PD-L1 and PD-1 and PD-L1 blockade by anti-PD-L1-augmented DC-mediated T cell activation in this model (Curiel et al., 2003). In the B16 mouse melanoma model, pDCs from tumor-draining lymph nodes were found to activate mature Treg cells via production of indoleamine 2,3-dioxigenase (IDO). The suppressive activity of the Treg cells was blocked by PD-L1 blockade (Sharma et al., 2007). In several mouse tumor models, promising antitumor effects in vivo have been observed with PD-1 blockade and a human PD-1 blocking antibody has been developed and is in phase I clinical studies (reviewed in Keir et al., 2008). Finally, triggering with agonistic antibody of the OX40 molecule on T cells, such as CD40, a member of the TNF receptor family, has a highly favorable effect on tumor-specific T cell immunity, causing local Treg cell depletion and inactivation and tumor-infiltrating DC maturation and migration, decreasing TAMs and MDSCs, and lowering of the expression of TGF-β (Gough et al., 2008; Piconese et al., 2008). This was associated with enhanced infiltration of tumors with effector CD8 T cells and increased survival from tumor challenge (Gough et al., 2008; Piconese et al., 2008) (Figure 2).

Conclusions

Many of the molecular changes that allow a tumor to grow and infiltrate in its environment also create an immunosuppressive milieu that counteracts tumor rejection. TAMs and MDSCs conspire to counteract the generation of fully activated DCs. Full DC activation is a problem in tumor immunology because such activation is more readily accomplished by pathogen-associated molecules such as TLR ligands than by cancer-associated triggers of DC activation, underlining the fact that in many ways, tumors masquerade as self-tissues creating tolerance rather than immunity. In addition, many tumors overexpress STAT3 protein, in turn causing STAT3 expression in tumor-infiltrating leukocytes, associated with production of TGF-β and IL-10 and suppression of Th1 cell responses. In this atmosphere rife with immunosuppressive chemokine and cytokine production, and inadequately activated DCs, regulatory T cells are generated. Together, this conflagration of suppressive leukocytes and improperly activated DCs in many instances is powerless to generate sufficient numbers of properly activated tumor-specific Th1 cells and CTLs, despite ample evidence for expression of tumor-associated antigens in both viral and nonviral cancers. Nevertheless, in many cases tumor-specific effector T cells coexist with the tumor, sometimes even keeping dormant tumors in check, and therapeutic activity of these cells can be rescued by robust tumor vaccination in conjunction with therapeutic measures that tilt the balance in the symbiosis of the tumor with the immune system toward tumor rejection. These insights lead to the inescapable conclusion that no single immunotherapeutic modality can be expected to effectively cure established cancer. Rather, the remarkable beneficial effects of certain forms of low-dose chemotherapy, including DC activation, enhanced crosspresentation of tumor-associated antigens from necrotic or apoptotic cancer cells, lymphodepletion, and reduction of immunosuppressive leukocytes, can be mobilized to act in concert with therapeutic vaccines or adoptive T cell transfer. Promising therapeutic vaccines include ex vivo-activated tumor antigen-loaded DCs, synthetic long peptides targeting DCs and TLR ligand-peptide conjugates, or chimeric antibodies efficiently targeting tumor antigens to C-type lectin receptors on DC.

Clearly, the effects of therapeutic cancer vaccines can be expected to be enhanced further by additional therapies that redress the immunosuppression associated with cancer. Such therapies include administration of STAT3 inhibitors, local or systemic treatment with molecularly defined triggers of DC activation such as TLR ligands and CD40 agonistic antibody and treatment with monoclonal antibodies that block deleterious feedback inhibition pathways, in particular blockers of CTLA-4 and PD-1, or with antibodies that enhance T cell effector function, including agonists of OX-40.

More than ever, development of effective immunotherapy of established cancer calls for unprecedented cooperation among cancer scientists, pharmaceutical companies, and between scientists and the pharma community. At the same time, science in this area is now one of the most challenging and exciting in immunology and medicine.

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