

Immune microenvironments in solid tumors: new targets for therapy

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Leukocytes and their soluble mediators play important regulatory roles in all aspects of solid tumor development. While immunotherapeutic strategies have conceptually held clinical promise, with the exception of a small percentage of patients, they have failed to demonstrate effective, consistent, and durable anti-cancer responses. Several subtypes of leukocytes that commonly infiltrate solid tumors harbor immunosuppressive activity and undoubtedly restrict the effectiveness of these strategies. Several of these same immune cells also foster tumor development by expression of potent protumor mediators. Given recent evidence revealing that immune-based mechanisms regulate the response to conventional cytotoxic therapy, it seems reasonable to speculate that tumor progression could be effectively diminished by combining cytotoxic strategies with therapies that blunt protumor immune-based effectors and/or neutralize those that instead impede development of desired anti-tumor immunity, thus providing synergistic effects between traditional cytotoxic and immune-modulatory approaches.

Despite expanded appreciation for the diversity of cellular mechanisms fostering solid tumor development, anti-cancer therapy remains heavily reliant on cytotoxic modalities—including chemotherapy (CTX) and radiation therapy (RT)—that kill rapidly proliferating (neoplastic) cells within tumors. Emerging clinical and experimental data indicate that clinical responses to cytotoxic therapy can be improved if immunogenic cell death pathways are also concurrently activated (Ma et al. 2010). Evidence for simultaneous engagement of immunogenic cell death programs has been provided for some tumors following conventional cytotoxic therapy, based on the increased presence of molecules released by dying cells thought to be “sensed” by leukocytes (Kepp et al. 2011), the result of which leads to enhanced “killing” of target cells. While an obvious clinical strategy has been to bolster these anti-tumor mechanisms, achieving clinical

success has been limited. Possible mechanisms underlying these clinical failures include the underappreciated properties of some immune cell types that can harbor both immunosuppressive activity—e.g., blunting malignant cell killing by CD8⁺ cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells—simultaneously with protumor activities that promote survival, invasion, and dissemination of malignant cells (Ruffell et al. 2010). Experimental studies in immune-competent murine models of human cancer have provided support for this concept by revealing that blockade of some protumor immune-based pathways effectively bolsters anti-tumor immunity (neoplastic cell killing) when combined with cytotoxic therapy (DeNardo et al. 2011).

Cancer and chronic inflammation

In homeostatic tissue, resident immune cells serve as sentinels that safeguard tissue and organ integrity. Following acute damage (e.g., infiltration/infection by pathogens or physical trauma), one activity of resident leukocytes is to limit tissue damage while engaging tissue repair programs (e.g., activation of stromal fibroblasts and vasculature for matrix resynthesis and angiogenesis, respectively, and recruitment of leukocytes from peripheral blood to remove damaged cells and debris) and facilitate re-epithelialization, all without inducing autoimmunity. Following resolution of wound responses, tissue damage is (hopefully) minimal and homeostatic maintenance programs return such that organ physiology is unperturbed.

In cancer, immune cells play dual roles with potential to either eliminate or promote malignancy. Premalignant tissues contain proliferating cells harboring genomic damage (e.g., “initiated” cells) that typically activate critical proliferation/survival pathways. In these tissues, chronic engagement/activation of immune cells, stromal fibroblasts, and vascular and mesenchymal support cells together fosters survival of “initiated” cells, culminating in tissue expansion and development of premalignant lesions via a process reminiscent of typical “inflammatory-type” responses observed in tissues responding to acute damage/trauma (Coussens and Werb 2002). When these chronic inflammatory-type events are sustained, neoplastic progression can ensue. Unresolved chronic immune responses thus resemble the resolution phase of wound healing, where the tumor microenvironment contains

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significant infiltrations of cells with immunosuppressive activity akin to a wound failing to heal (Coussens and Werb 2002).

Consistent with this, studies evaluating leukocyte complexity by flow cytometry in human (and murine) tumors have identified multiple immune cell types that variably contain immunosuppressive activity (e.g., block anti-tumor CTL- or NK T-cell-mediated killing of malignant cells)—including regulatory T cells (T_{reg} s), immature monocytes (iMCs), alternatively activated macrophages (AAMs), mast cells, neutrophils, Tie2⁺ monocytes, dendritic cells (DCs), and T helper 2 (T_H2)-CD4⁺ effector T cells (DeNardo et al. 2011; Rolny et al. 2011; Ruffell et al. 2011)—and thus afford developing malignancies a mechanism to escape killing by T cells. Mouse modeling studies indicate that the net effect of these assemblages results in favoring tumor expansion (Fig. 1; DeNardo et al. 2010; Grivennikov et al. 2010; Qian and Pollard 2010; Ruffell et al. 2010). Three

types of leukocytes in particular have emerged as playing significant roles in suppressing anti-tumor immune responses: T_{reg} cells, iMCs, and AAMs.

Immune-based programs that blunt anti-tumor immunity

T_{reg} cells

T_{reg} cells, a subset of the CD4⁺ T-cell population, constitutively express the high-affinity interleukin-2 (IL-2) receptor (CD25), CTL antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), and the lineage-specific transcription factor Foxp3 and play an important physiological role in suppressing responses to self-antigens, thereby preventing autoimmunity (Hori et al. 2003). As many malignant cell types express self-antigens (Kawakami and Rosenberg 1997), it follows that T_{reg} cells in their physiologic capacity

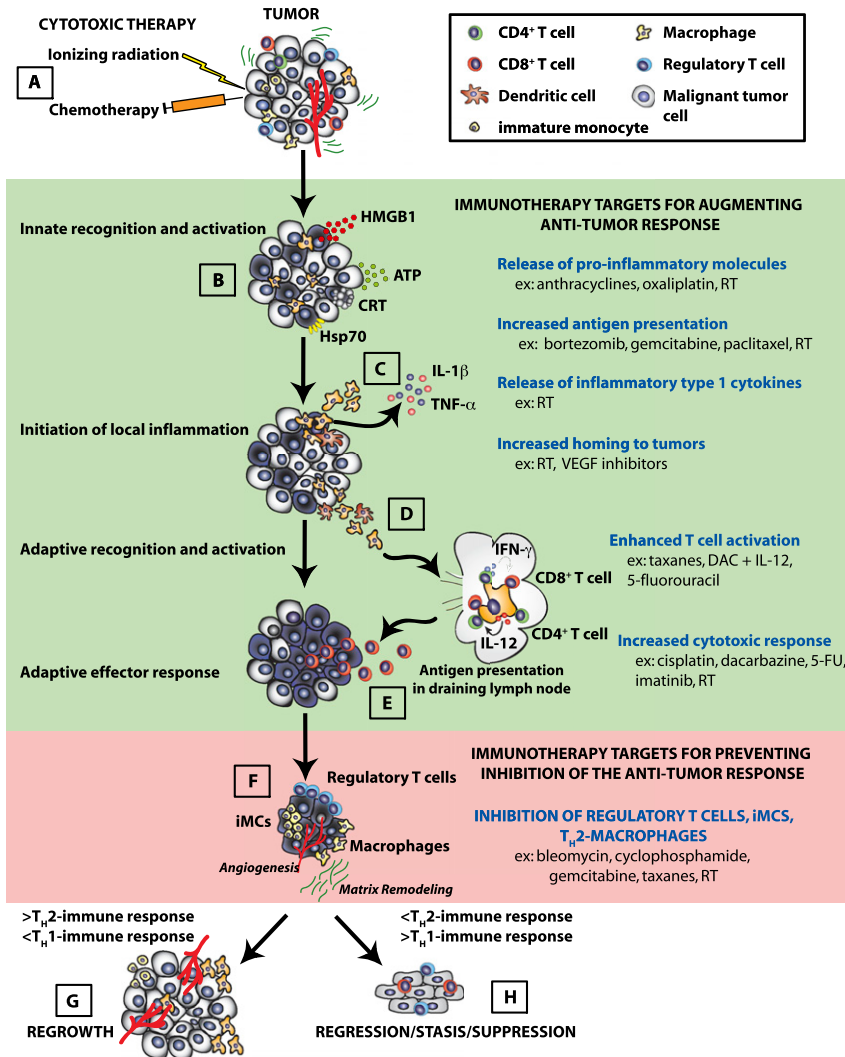


Figure 1. Schematic of immune response pathways induced following cytotoxic therapy. Traditional cytotoxic therapies (e.g., CTX and RT) trigger an immune response in tissues (A), leading to the release of inflammatory mediators (including HMGB1, calreticulin, ATP, and Hsp70) from tumor cells (B). (C) These molecules activate resident immune cells such as DCs and tissue macrophages through cognate receptors, including TLR4 and P2RX7, which triggers the release of TNF- α and IL-1, which further recruits peripheral blood leukocytes (PBLs) from the circulation. (D) Activation of resident DCs and tissue macrophages stimulates their migration to the lymphoid tissue bearing tumor antigens. In the lymphoid tissue, the DCs and macrophages present antigen to CD4⁺ and CD8⁺ T cells, leading to their activation. (E) Activated CD4⁺ and CD8⁺ T cells then re-enter the circulation and return to the tumor to eliminate tumor cells. (F) Throughout this process, a portion of the PBLs recruited from the circulation into the tumor also possess suppressive function (e.g., T_{reg} cells and various subtypes of myeloid cells), and these become increasingly dominant as the tumor is cleared by a cytotoxic response (primarily CD8⁺ T cells and NK cells) and function to reduce the anti-tumor cytotoxic response by a diverse array of mechanisms. (G) If the malignant cells are completely eradicated, the tissue can return to a normal, homeostatic state. (H) However, if incomplete eradication of malignant cells occurs, then, over time, tumor regrowth is evident in the form of recurrent disease at the primary site or metastases at distal sites. Events in the immune response that might serve as targets to enhance the immune response (shaded green) or prevent suppression of the immune response (shaded red) are outlined on the right with examples of cytotoxic agents that can mediate each of these events.

hamper anti-tumor immunity and that tumors may evade immune detection by engaging or activating T_{reg} cell-based pathways. This notion has been borne out by studies evaluating peripheral blood, tumor-draining lymph nodes, and tumors—e.g., breast (Bates et al. 2006) and gastrointestinal (Sasada et al. 2003)—where increased presence of T_{regs} is prominent. Importantly, increased frequency of T_{regs} also correlates with poor outcome for several cancer types (Sasada et al. 2003; Curiel et al. 2004; Bates et al. 2006). Further support for the notion of tumors activating development of T_{reg} cells comes from studies showing that stromal cells produce chemokines such as CCL22 (Curiel et al. 2004) and cytokines such as transforming growth factor- β (TGF β) (Ghiringhelli et al. 2005) that enhance T_{reg} infiltration.

The ability of T_{regs} to block anti-tumor immunity has been confirmed *in vivo*, where adoptive transfer of $CD3^+CD25^-$ T cells from patients into NOD/SCID mice was found to retard tumor growth, while simultaneous transfer of T_{regs} abrogated the protective effect (Curiel et al. 2004). Mechanistically, *in vitro* studies have revealed that leukocytes isolated from melanoma and ovarian cancer patients depleted of T_{reg} cells *ex vivo* enabled remaining leukocytes to respond to selective tumor antigens (Nishikawa et al. 2005). Also significant is the observation that T_{regs} directly promote malignant cell proliferation and dissemination via soluble mediators they express (Tan et al. 2011). Given evidence demonstrating that T_{regs} block anti-tumor immunity (Dunn et al. 2004), it stands to reason that, in order to augment anti-tumor immunity therapeutically, neutralizing pathways that bolster the presence or activity of T_{regs} would likely provide a survival advantage.

AAMs

Unlike T_{regs} , macrophages derived from circulating immature myeloid precursors play a more complex role in regulating immune responses owing to their ability to possess both pro- and anti-tumor bioactivity, depending on the cytokine milieu they encounter once within tissue (Qian and Pollard 2010). Classically activated macrophages (CAMs) regulated by T_H1 cytokines—e.g., interferon γ (IFN γ), tumor necrosis factor α (TNF α), or granulocyte/monocyte colony-stimulating factor (GM-CSF)—possess enhanced cytotoxic activity, produce proinflammatory (T_H1) cytokines, and have antigen presentation capability (Mosser and Edwards 2008). In contrast, macrophages exposed to T_H2 cytokines (IL-4, IL-13, etc.), immune complexes, or immunosuppressive cytokines become alternatively activated (AAMs) (Qian and Pollard 2010) and instead typically lack cytotoxic activity, block $CD8^+$ T-cell proliferation or infiltration, and express a diverse assortment of proliferative, proangiogenic, and tissue remodeling mediators (DeNardo et al. 2009, 2011; Andreu et al. 2010; Qian and Pollard 2010; Ruffell et al. 2010). Experimental data from murine models indicate that AAMs become T_H2 -skewed due to high levels of type 2 cytokines (IL-4 and IL-13) released by infiltrating $CD4^+$ T cells and neoplastic epithelial cells (DeNardo et al. 2009; Gocheva et al. 2010) or TSLP (thymic stromal lymphopoietin) also produced by

neoplastic epithelial cells (Pedroza-Gonzalez et al. 2011). While AAMs are typical constituents of tissue repair processes, in solid tumors, rather than aiding in “healing,” they instead foster neoplasia (Qian and Pollard 2010). AAMs produce a multitude of factors—including epidermal growth factor (EGF), TGF β , and cathepsin proteases—that together provide a survival advantage to malignant epithelia and regulate their response to cytotoxic therapies (DeNardo et al. 2011; Shree et al. 2011). Data from human tumors support this hypothesis, since the presence of AAMs that are $CD163^+CD204^+$ correlate with reduced survival for patients with breast cancer, non-small-cell lung cancer, and Hodgkin's lymphoma (Kawai et al. 2008; Steidl et al. 2010; DeNardo et al. 2011). Owing to lack of specificity for CD68 as a macrophage-specific marker, however, some of these findings may need to be revisited (Ruffell et al. 2011).

The importance of macrophages in tumor progression is further underscored by mouse modeling data revealing that genetic loss of *CSF1/CSF1 receptors* (Lin et al. 2001) or blockade of M-CSF-induced signaling cascades (DeNardo et al. 2011) reduces macrophage presence in tumors and correlates with reduced mammary tumor metastasis. Thus, AAMs, through their ability to differentially regulate immunity and express molecules that support angiogenesis/tissue remodeling and proliferation, profoundly affect the development, maintenance, and dissemination of malignant tumors.

Immunosuppressive monocytes

Sharing the same common myeloid progenitor as macrophages, immunosuppressive monocytes in rodent tumor models encompass a diverse population of cells characterized by expression of surface markers, including CD11b and Gr1 (Ostrand-Rosenberg 2008; Gabilovich and Nagaraj 2009), and include monocytes variably referred to as myeloid-derived suppressor cells (MDSCs), iMCs, inflammatory monocytes, and neutrophils (Ostrand-Rosenberg 2008). Human equivalents have been identified as $LIN^{-/Lo}$ human leukocyte antigen (HLA)-DR $^-$ CD33 $^+$ CD11b $^+$ and CD14 $^+$ HLA-DR $^{-/Lo}$ cells (Serafini et al. 2006); however, as with mice, these share markers with multiple mature granulocytic subtypes and thus likely represent a mixed population in which some cells contain immunosuppressive properties. MDSCs and iMCs are functionally characterized by their T-cell-suppressive activity; e.g., the ability to suppress T- and NK cell proliferation via arginase I, inducible nitric oxide synthase expression, and peroxynitrite, and, at the same time, promote generation of T_{reg} cells (Mazzoni et al. 2002; Gabilovich and Nagaraj 2009; Doedens et al. 2010; Lu et al. 2011).

In mice, systemic increases in the presence of MDSCs and iMCs have been observed when syngeneic mice are transplanted with or develop spontaneous tumors (Ostrand-Rosenberg 2008). Significant increases in MDSCs in peripheral blood are also a common feature for patients with several types of cancer (Almand et al. 2001). Moreover, in murine models of cancer, MDSCs/iMCs have also been found to mediate resistance to some forms of anti-angiogenic

therapy (Shojaei et al. 2007; Priceman et al. 2010). Thus, strategies aiming to eliminate MDSCs/iMCs may result in shifting the immune microenvironment to instead favor anti-tumor type responses that improve survival.

Cytotoxic therapy and immune cells

Cytotoxic therapy and immunogenic cell death

Cytotoxic therapy (CTX and RT), in combination with surgery, forms the cornerstone of systemic treatment for most clinically detectable solid tumors. Significantly, most cytotoxic therapies result in immune suppression due to a higher sensitivity of bone marrow-derived stem cells and many leukocyte subsets, especially lymphocytes, to their cytotoxic effects. Through specialized cell death pathways, including Fas–FasL, lymphocytes respond to DNA damage induced by CTX and RT by undergoing early apoptosis at doses significantly lower than other cell types, especially epithelial or neural cell types. Bone marrow-derived stem cells are also uniquely sensitive to CTX and RT (Apetoh et al. 2007; Ghiringhelli et al. 2009), and their early destruction is likely a dose-limiting toxicity for many of these modalities; thus, administration of cytotoxic agents can lead to systemic immune suppression. That said, there is increasing evidence that within tumors, cell death generated by these agents also triggers activation of other immune response pathways that serendipitously also regulate therapeutic efficacy of the particular cytotoxic agent/modality (Table 1).

Whereas neoplastic cells have long been thought to undergo an “immunologically silent” demise following cytotoxic therapy, whereby apoptotic machinery eliminates them (Albert et al. 1998), recent studies have challenged this notion (Ma et al. 2011) and revealed that nonapoptotic and biochemically distinct cell death pathways are also activated following RT and some forms of CTX (e.g., anthracyclines and oxaliplatin) (Fig. 1). Mechanistically, leukocytes detect cell death through immune-based receptors selective for molecules released by dying cells (often termed “danger signals”), including Toll-like receptor-4 (TLR-4) and its ligand, the high-mobility group box protein 1 (HMGB1) (Apetoh et al. 2007). Detection of danger signals by resident leukocytes results in subsequent activation of both innate (myeloid and NK cells) and adaptive (T and B) cell lineages. Molecular mechanisms underlying immunogenic cell death following cytotoxic therapy involve activation of several critical sequential checkpoints. These include (1) exposure of endoplasmic reticulum (ER)-resident protein complexes, comprised of calreticulin/ERp57 on plasma membranes of neoplastic cells that serve as “eat me” signals for DCs; (2) release of the chromatin-binding HMGB1 protein, which by a TLR4/MyD88-dependent mechanism inhibits degradation of DC phagosomes, thereby facilitating antigen presentation (Apetoh et al. 2007); (3) ATP release from dying neoplastic cells and subsequent engagement of DC P2RX7 purinergic receptors, leading to IL-1 β release (Ghiringhelli et al. 2009); and (4) effective antigen cross-presentation by DCs with increased production of IFN γ /IFN γ receptors and CD8⁺

CTL-dependent killing responses. Experimental evidence supporting these pathways emanates from in vivo evaluation in murine tumor models where the immune response induced by CTX or RT efficiently prevents tumor growth dependent on activation of these pathways (Apetoh et al. 2007; Ghiringhelli et al. 2009). Clinical evidence for the importance of these mechanisms is provided by human breast cancer patients harboring Asp299Gly *TLR4* polymorphisms or loss-of-function mutations in the *P2RX7* gene, both of which disrupt DC–T-cell functional interactions by impairing DCs’ ability to sense HMGB1 and ATP release by dying cells, and correlates clinically with resistance to CTX (anthracyclines) and RT (Ghiringhelli et al. 2009).

Recognition that immune-based mechanisms modulate the response to cytotoxic therapy implies that the ultimate effectiveness of cytotoxic modalities could be improved by combinatorial approaches that also engage immunogenic death programs. Thus, strategies improving antigen presentation (to T cells) and/or increasing macrophage cytolytic activity would theoretically impede tumorigenesis if the protumorigenic properties of those leukocytes following cytotoxic therapy could also be effectively blunted. Requisite for success of this scenario is that T_H1-based immune programs would be fostered, and dominant T_H2-type programming would be blunted. T_H1 programming in response to increased expression of type 1 cytokines (TNF α , IFN γ , and IL-2) activates cell-mediated responses that are “anti-tumor” in nature. T_H2 programming, on the other hand, is mediated by expression of type 2 cytokines (IL-4, IL-10, and TGF β) that instead initiate tissue remodeling, angiogenesis, and (sometimes) humoral immunity, and together foster a protumorigenic state (Yang et al. 2008; DeNardo et al. 2010; Ruffell et al. 2010).

Evidence that forced T_H1 polarization of tumor microenvironments can improve response to cytotoxic therapy has been observed. For example, immunization with plasmacytoma supernatant plus IL-1 resulted in decreased tissue/tumor levels of IL-10 and TGF β and increased levels of IFN γ and IL-2, thus favoring T_H1 immunity and tumor regression (Li et al. 1998). Other studies reported that combined CTX or RT with DC vaccination, which augments the initial T_H1 response through enhanced antigen presentation, also resulted in tumor regression (Koike et al. 2008; Matsumura et al. 2008). A general conclusion from these studies is that cytotoxic therapy indeed fosters an anti-tumor immune microenvironment; however, this response tends not to be durable, likely due to protumor, immunosuppressive programs that become dominant, thereby fueling tumor recurrence and subsequent resistance to therapy.

Cytotoxic therapy and cancer immunotherapy—a different approach

Harnessing the body’s own immune system to fight cancer has long been considered the ultimate treatment for cancer because of its potential to specifically and durably target antigen-positive neoplastic cells while limiting damage to

Table 1. Immune effects of cytotoxic agents

Cytotoxic agent	Example	Effect on tumor immune response							References
		Release of proinflammatory molecules	Antigen presentation	Release of inflammatory cytokines	Increased homing of inflammatory cells	Enhanced T-cell activation	Enhanced cytotoxic immune response	Inhibition of immunosuppressive cells	
Alkylating agents	Cyclophosphamide, ifosfamide, busulfan, melphalan, dacarbazine				●				Bracci et al. 2007; Robert et al. 2011
Anthracyclines	Daunorubicin, doxorubicin	●							Ma et al. 2010; Ramakrishnan et al. 2010
Anti-metabolites	Methotrexate, 5-fluorouracil, fludarabine, gemcitabine		●						Suzuki et al. 2005; Ma et al. 2010
DNA methyltransferase inhibitors	2'-Deoxy-5-azacytidine (decitabine or DAC)						●		Ma et al. 2010
Platinum agents	Carboplatin, cisplatin, oxaliplatin	●							Ghiringhelli et al. 2009; Ma et al. 2010; Ramakrishnan et al. 2010
Spindle poisons	Paclitaxel, docetaxel, vincristine, vinblastine (<i>Vinca</i> alkaloids)		●				●		Apetoh et al. 2007; Ma et al. 2010; Ramakrishnan et al. 2010; DeNardo et al. 2011
Topoisomerase inhibitors	Etoposide, irinotecan								
Tyrosine kinase inhibitors	Imatinib, dasatinib, sunitinib							●	Kao et al. 2009; Oza-Choy et al. 2009
Radiation	γ /X-rays, electrons, Protons, α particles	●	●	●	●		●	●	Matsumura et al. 2008; Ahn et al. 2010; Meng et al. 2010; DeNardo et al. 2011

Common classes of cytotoxic agents are listed, with their known effects on immune responses indicated.

normal tissue. Given the immunogenic potential of cytotoxic therapies alone, it follows that strategies augmenting the immune response to cancer would synergize with anti-tumor immunity generated by cytotoxic therapy. That said, current cancer immunotherapies use a variety of strategies, including therapeutic monoclonal antibodies (mAbs) and adoptive cell transfer (ACT) involving transfer of ex vivo expanded autologous or allogeneic tumor-reactive lymphocytes and cancer vaccines, and thus attempt to stimulate anti-tumor immunity (Table 2). A critical appraisal of these approaches reveals limited overall objective response rates (3.6%) across several early-phase trials (Klebanoff et al. 2011). Although positive results with surrogate immunological endpoints have been reported, the vast majority of phase III immunotherapy trials in patients with solid tumors have failed to demonstrate improved overall survival. Analysis of these reveals that the strategies do indeed initiate and/or prime anti-tumor immunity; however, their limited success lies in their failure to also inhibit the pathways that block CTL and NK T-cell-mediated killing. As new data emerge expanding on our understanding of these complex immune-based mechanisms, new approaches are sure to develop that not only enhance generation of anti-tumor immunity, but also prevent its suppression.

Cancer immunotherapy I: augmenting the anti-tumor immune response

ACT ACT has been reported to induce objective tumor regression and long-term responses for a small fraction of melanoma patients (Rosenberg et al. 2008). Although first described in the 1980s, therapeutic efficacy and increased patient survival were only reported following addition of immuno-depleting CTX prior to ACT, which was subsequently further improved by myeloablative lympho-depleting regimens (Dudley et al. 2008). Mechanistically, (limited) removal of endogenous lymphocytes that act as "sinks" for homeostatic cytokines and elimination of immunosuppressive T_{regs} and iMCs underlay these objective clinical responses (Gattinoni et al. 2005; Dudley et al. 2008).

Gabrilovich and colleagues (Ramakrishnan et al. 2010) evaluated several cancer vaccines with ACT in murine models in combination with several widely used chemotherapeutic drugs. These researchers reported that CTX rendered tumor cells more susceptible to the cytolytic effects of CTLs via increased perforin-independent permeability to granzyme B, mediated by up-regulation of mannose-6-phosphate receptors on malignant cells (Ramakrishnan et al. 2010). When combined with CTX, CTLs raised against specific tumor antigens induced apoptosis in neighboring tumor cells that did not express the antigens. Thus, small numbers of CTLs can mediate potent anti-tumor effects when combined with CTX and provide a rationale for combining these modalities for treatment of patients with advanced cancer.

Cancer vaccines Inspired by success with vaccination against bacterial and some viral pathogens, a variety of

approaches have been explored in an attempt to immunize patients against their own cancers, some of which include use of whole (killed) tumor cells, proteins, peptides, or DNA vaccines (Giaccone et al. 2005; Testori et al. 2008; Dougan and Dranoff 2009; Amato et al. 2010). In spite of limited success with these, there is renewed interest following recent positive clinical results in prostate cancer and lymphoma. Sipuleucel-T (Provenge), a cellular vaccine comprising autologous antigen-presenting cells (APCs) cultured with a fusion protein of prostatic acid phosphatase with GM-CSF, extended median survival in two independent phase III trials, leading to FDA approval for treatment of advanced prostate cancer (Small et al. 2006; Kantoff et al. 2010). Other encouraging results have been reported, most notably idotype vaccination for follicular lymphoma in a phase III trial that demonstrated a prolonged period of CTX-induced remission (Neelapu et al. 2005).

DCs link innate and adaptive immunity and can induce contrasting states, including immunity and tolerance to self. Multiple populations of DCs are recognized in vivo in both human and murine tumors, each with distinct properties that variably regulate humoral and cellular immunity (Hashimoto et al. 2011). While antibody responses are preferentially mediated by CD14⁺ dermal DCs, CTL responses are instead preferentially mediated by Langerhans cells (Hashimoto et al. 2011), thus indicating that DC-mediated mechanisms inducing humoral and/or cellular immunity are fundamentally distinct. Early clinical trials testing vaccination with ex vivo generated DCs pulsed with tumor antigens provided proof-of-principle evidence that therapeutic immunity could be elicited; however, clinical benefit measured by regression of established tumors in patients with stage IV cancer was observed in only a small percentage of patients (Palucka et al. 2008). Patients with soft tissue sarcoma who received fractionated external beam radiation in combination with administration of intratumoral DCs demonstrated an increased T-cell infiltration, with tumoral CD4⁺ T cells positively correlating with tumor-specific immune responses (Finkelstein et al. 2011). Thus, new-generation DC vaccines are needed that generate large numbers of high avidity effector anti-tumor T cells able to overcome suppressive mechanisms in the tumor microenvironment. These, combined with therapies blunting T_H2 -based pro-tumor immunity and CTX or RT, would thus be anticipated to provide much more durable tumor repression.

Cancer immunotherapy II: targeting immunosuppressive pathways and cells

T_{reg} cells Evidence for the role of T_{regs} in anti-tumor immunity was first provided by Sakaguchi and colleagues (Shimizu et al. 1999; Sakaguchi 2005) using a syngeneic murine heterotopic transplant model. This was later reproduced in several murine tumor models, all of which demonstrated that depletion of T_{regs} via anti-CD25 mAb prior to tumor inoculation led to syngeneic tumor rejection (Casares et al. 2003). In conjunction with cytotoxic therapy, strategies targeting CD25, such as depleting mAbs or an IL-2-diphtheria toxin fusion protein, enhanced

Table 2. Current immunotherapy

Immunotherapy type	Species	Cancer type	Agent	Comment	References
ACT	Mouse	Colon, breast, lymphoma	CTL + paclitaxel or cisplatin or doxorubicin	Significant tumor growth delay seen with combination therapy due to increased sensitivity of tumor cells, Granzyme B via mannose-6 phosphate receptor	Ramakrishnan et al. 2010
	Human	Melanoma	Ex vivo expanded tumor-infiltrating lymphocytes + IL-2; cyclophosphamide + fludarabine + total body irradiation	Objective response rate of 50%–70% in patients with combined regimen (vs. 15% with historical standard of IL-2 + dacarbazine)	Dudley et al. 2008
	Human	Melanoma	Ex vivo expanded CD8 ⁺ T cells against melanoma antigen Melan-A	Tumor-specific immune response detected in 27.2% (three of 11 patients)	Mackensen et al. 2006
Cancer vaccines	Human	Melanoma	Peptide vaccine (Hsp96)	No improvement in overall survival ($P = 0.32$)	Testori et al. 2008
	Human	Kidney	Poxvirus encoding tumor antigen 5T4	Specific antibody responses detected, but no improvement in overall survival ($P = 0.55$)	Amato et al. 2010
	Human	Lung	Antibody that mimics ganglioside GD3	Specific antibody responses detected, but no improvement in overall survival ($P = 0.28$)	van Meerbeeck et al. 2005
	Human	Follicular lymphoma	Idiotypic vaccination	Induction of prolonged remission	Neelapu et al. 2005
	Human	Sarcoma	Intratumoral DCs + RT	Tumor-specific immune responses detected in 52.9% of patients (nine of 17 patients)	Finkelstein et al. 2011
	Human	Prostate	Tumor antigen (prostatic acid phosphatase) coupled to GM-CSF (Sipuleucel-T)	4-mo improvement in median survival compared with no treatment ($P = 0.02$)	Small et al. 2006; Kantoff et al. 2010
Inhibition or depletion of T _{reg} cells	Mouse	Lymphoma, colon	Anti-CD25 depleting mAb + attenuated Poxvirus + RT	Increased antigen-specific immune responses with combined modalities	Kudo-Saito et al. 2005
	Mouse	Melanoma	Agonistic anti-OX40 mAb + cyclophosphamide	Improved survival and significantly delayed tumor growth secondary to intratumoral T _{reg} -specific hyperactivation and apoptosis	Hirschhorn-Cymerman et al. 2009
	Human	Melanoma	IL-2 fusion protein with diphtheria toxin (denileukin difitox) + DC vaccine	Increased T-cell and antibody response to tumor antigen CEA	Morse et al. 2008
	Human	Melanoma	Anti-CTLA4 (Ipilimumab) mAbs + dacarbazine	2-mo improvement in median survival versus dacarbazine alone ($P < 0.001$)	Hodi et al. 2010; Robert et al. 2011

(continued)

Table 2. continued

Immunotherapy type	Species	Cancer type	Agent	Comment	References
Depletion or repolarization of macrophages	Mouse	Melanoma	Clodronate liposomes + RT	Delayed tumor growth following RT only when macrophages were depleted	Meng et al. 2010
	Mouse	Breast	CSF-1 receptor (CSF-1R) inhibitor + paclitaxel	Delayed tumor growth with combination of paclitaxel and macrophage depletion but not with macrophage depletion alone	DeNardo et al. 2011
Depletion of iMCs/MDSCs	Human, mouse	Pancreas	Agonistic CD40 mAb + gemcitabine	Tumor response rate of 30% (vs. 5.4% historically for gemcitabine alone) in both humans and mice; mechanism depends on repolarization of tumor-associated macrophages	Beatty et al. 2011
	Human, mouse	Lung (non-small cell)	ATRA + paclitaxel/cisplatin	2.9 mo increase in progression-free survival ($P = 0.008$); mouse model indicates that ATRA mechanism may be combination of differentiation of iMC into macrophages and increased sensitivity of tumor cells to CTX	Arrieta et al. 2010
	Human, mouse	Multiple	Sunitinib (tyrosine kinase inhibitor) + RT	59% (21 of 36) of lesions had complete or partial response; mouse model shows decreased circulating T_{reg} and iMC/MDSC and suppressive function with sunitinib	Kao et al. 2009; Ozao-Choy et al. 2009
	Mouse	Head and neck, breast	Anti-CD11b mAb	Delayed tumor growth with combination of mAb and RT or paclitaxel	Ahn et al. 2010; DeNardo et al. 2011

Results from selected immunotherapy studies in murine models of human cancer and in patients with various types of solid cancers.

anti-tumor immune responses in both murine models and humans (Kudo-Saito et al. 2005; Mackensen et al. 2006; Morse et al. 2008). However, strategies targeting CD25 lack specificity, in that activated T cells also express CD25; thus, these agents may also blunt formation of robust anti-tumor T-cell responses while also depleting T_{reg} cells (Curtin et al. 2008). Therefore, other strategies targeting the T_{regs} —such as the agonistic antibody against OX40, a TNFR receptor (TNFR) family costimulatory molecule expressed on T cells and DCs—in combination with cyclophosphamide minimize this paradox by inducing T_{reg} -specific apoptosis (Hirschhorn-Cymerman et al. 2009).

Targeting T_{regs} with CTLA-4 antagonists has been perhaps the most successful of the strategies targeting an immunosuppressive pathway, although others such as B7-H3, PD-L1, and CD73 are currently under investigation (Yi and Chen 2009; Ascierto et al. 2010; Jin et al. 2011). CTLA-4 is a negative costimulatory molecule expressed on both activated T cells and T_{reg} cells that helps dampen ongoing immune response, is frequently up-regulated on chronically activated and exhausted T cells (Engelhardt et al. 2006; Wherry et al. 2007), and not only inhibits T-cell activation, but also promotes T_{reg} function (Teft et al. 2006). Results from a phase III trial evaluating the CTLA-4 blocking mAb ipilimumab, recently approved for the treatment of advanced malignant melanoma by the FDA, revealed extended overall survival of previously treated melanoma patients, correlating with increased $CD8^+$ T-cell activation and T_{reg} inhibition (Hodi et al. 2010). A subsequent landmark study demonstrated improved survival in patients with untreated advanced melanoma who received ipilimumab combined with the CTX agent dacarbazine, as compared with those receiving CTX alone (Robert et al. 2011). This study supports the hypothesis that the combination of CTX with a reduction in the suppressive environment—in this case, elimination of T_{regs} —is a strategy that can lead to more effective anti-tumor immunity.

AAMs CTX and RT stimulate recruitment of macrophages (and monocytes) into tissues through direct induction of myeloid cell chemoattractant molecules. Epithelial cells rapidly respond to CTX (paclitaxel, cisplatin, and carboplatin) and RT by direct mRNA induction of monocyte-promoting chemokines such as *csf-1*, *IL-34*, *ccl2/MCP-1*, *ccl5*, *cxcl10*, *cxcl11*, *cx3cl1*, and *HIF1* (Kioi et al. 2010; DeNardo et al. 2011; Ruffell et al. 2011). Cyclophosphamide, oxaliplatin, and RT induce T_H1 (Bracci et al. 2007; Ghiringhelli et al. 2009) as well as T_H2 cytokines (Gremy et al. 2008), indicating that CTX and RT have the potential to skew macrophage phenotypes to either anti- or protumorigenic states. Thus, while CTX and RT may initially mediate a cytotoxic/cytolytic macrophage response (Lambert and Paulnock 1987), enhanced presence of T_H2 cytokines may contribute to ongoing skewing and maintenance of AAMs in tumors and subsequent repulsion of $CD8^+$ T-cell-mediated anti-tumor immunity (Doedens et al. 2010).

The duality of macrophages as mediators of cytotoxic therapy responses has been demonstrated in experimental

murine models showing that macrophage depletion significantly slows tumor growth, but only when provided in combination with either CTX or RT. Selective depletion of macrophages using clodronate liposomes in an orthotopic murine melanoma model given before RT increased latency and slowed tumor regrowth, whereas coimplantation of malignant cells along with bone marrow-derived macrophages increased tumor radioresistance mediated by $TNF\alpha$ signaling pathways (Meng et al. 2010). Macrophage depletion strategies in combination with CTX or RT slow tumor development in murine models of sarcoma and melanoma in part by altering, or perhaps “normalizing,” tumor vasculature (Meng et al. 2010; Rolny et al. 2011). Vascular normalization in this context likely improves tumor hemodynamics, thereby increasing delivery of chemotherapeutic agents and oxygenation of tumor parenchyma, and thereby reducing hypoxia.

Given the evidence that cytotoxic agents are also potent immune adjuvants, it would not be surprising that strategies abolishing or reprogramming AAMs would enhance both cell killing by cytotoxics and immunogenic cell death. Thus, in order to overcome the immunosuppressive barriers established by tumors, it may be important to not only provide antigenic stimulus in the form of cytotoxic therapy, but also neutralize myeloid-based pathways established in the tumor that blunt effective anti-tumor immune responses. To address this possibility, we recently used a mouse model of mammary carcinogenesis (MMTV-PyMT mice) and reported that CSF1R blockade depleted $CD11b^+Ly6G^-Ly6C^{Lo}F4/80^+$ macrophages, but not the less abundant population of granulocytic $CD11b^+Ly6G^+$ -expressing myeloid cells, and resulted in slowed primary tumor growth and diminished metastasis, but only when given in combination with CTX, by $CD8^+$ T-cell-dependent mechanisms (DeNardo et al. 2011). Cathepsin protease-expressing macrophages have been found to mediate many of these effects, and cathepsin B and S protect malignant mammary epithelial cells from Taxol-induced (as well as etoposide and doxorubicin) tumor cell death in coculture (Shree et al. 2011). Combining Taxol with cathepsin inhibition in vivo significantly enhanced efficacy against primary and metastatic mammary tumors, supporting the therapeutic relevance for this effect (Shree et al. 2011). These experimental studies provide a compelling rationale for clinical evaluation of combinatorial approaches inhibiting macrophage recruitment or altering macrophage response pathways and mediator expression/activity in combination with “standard of care” CTX for treatment of some solid tumors in order to overcome inherent resistance to CTX. These strategies are an active area of clinical research in the phase I and II setting, testing a variety of agents designed to either block macrophage recruitment or stimulate alternative macrophage programming (Anthony et al. 2011) with the hope that combinations will provide improved clinical outcomes.

In addition to targeting macrophage recruitment, it is also possible to target macrophage polarization in an attempt to elicit the presence of more favorable T_H1 -polarized cytotoxic macrophages in tumors. One such strategy currently being explored is via targeting CD40, a member of the TNFR

superfamily and a costimulatory molecule expressed on a diverse assortment of cells, including DCs, B cells, and macrophages, as well as endothelial, mesenchymal, and epithelial cells. Binding of the CD40 ligand (CD40L) CD154 to CD40 mediates distinct effects on cells, depending on cell type and the tissue and microenvironment in which they reside. On immune cells, CD40 regulates humoral and cellular immunity, while apoptotic and anti-proliferative pathways are regulated by CD40 on some neoplastic cells (Fonsatti et al. 2010). Activation of APCs requires binding of CD40L on T_H cells to CD40, whereas macrophage activation requires IFN γ produced by T_H1-CD4⁺ T cells in addition to CD40L–CD40 interaction. This results in macrophage up-regulation of CD40 and TNFR and induction of cytotoxic activity, including increased expression of nitric oxide and reactive oxygen species (Fonsatti et al. 2010). To investigate whether agonist CD40 mAbs would thwart tumor-induced immune suppression and instead invoke productive T-cell-dependent anti-tumor immunity, Beatty et al. (2011) treated 21 patients with pancreatic ductal adenocarcinoma (PDA) with a fully humanized agonistic CD40 mAb in combination with gemcitabine and reported tumor regression in some patients. Using a mouse model of PDA to reveal the molecular/cellular mechanisms underlying the improved response, tumor regression was found to be dependent on CD40-activated MHC-II^{hi}CD86⁺ tumoricidal macrophages as opposed to CD8⁺ T cells (Beatty et al. 2011).

In addition to these approaches, others have investigated the efficacy of CD47 blockade to foster macrophage and DC phagocytic activity (Jaiswal et al. 2010). CD47, also known as integrin-associated protein (IAP), encodes a membrane protein mediating intracellular calcium levels following cell adhesion to extracellular matrix. CD47 binds to the SIRP α inhibitor receptor on macrophages and DCs and thereby inhibits phagocytosis; in autoimmune processes, these interactions limit tissue damage (Jaiswal et al. 2010). Expression of CD47 has been found to be significantly increased on some malignant tumor cells, especially in non-Hodgkin's lymphoma, thus rendering malignant cells resistant to macrophage and DC phagocytosis (Chao et al. 2010). Since agonistic CD40 mAb in combination with gemcitabine provides a survival advantage for PDA dependent on tumoricidal macrophages, it seems reasonable to speculate that combining similar approaches with therapies blocking CD47 may be efficacious in solid tumors where CD47 is up-regulated. Taken together, the experience with immunotherapy makes a compelling case for integrating strategies that restrain and/or reprogram tumor immune microenvironments, resulting in bolstering of diverse anti-tumor pathways to achieve meaningful therapeutic gains.

Immunosuppressive myeloid cells Minimizing suppressive iMCs/MDSCs in tumors has been investigated using all-*trans* retinoic acid (ATRA), which induces differentiation of iMCs into macrophages and correlates with enhanced anti-tumor immunity in murine models (Kusmartsev and Gabrilovich 2003). In human clinical trials, addition of retinoic acid to standard CTX improved outcome for

patients with advanced non-small-cell lung cancer (Arrieta et al. 2010). While ATRA decreased accumulation of iMCs in both tumor-bearing mice and humans, exposure to this agent also increased sensitivity of malignant cells to CTX, likely accounting for at least some of its anti-tumor efficacy (Arrieta et al. 2010). Other strategies to eliminate iMCs have used c-KIT antagonists that decrease accumulation of iMCs in murine and human tumors but have only improved anti-tumor immunity when given in the presence of tumor vaccines (Ozao-Choy et al. 2009). A phase I/II clinical study revealed that concurrent administration of sunitinib—an oral, small-molecule, multi-targeted receptor tyrosine kinase inhibitor of the vascular endothelial growth factor receptor (VEGFR), c-KIT, and platelet-derived growth factor receptor approved by the FDA for treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor—with RT in patients with one to five distant oligometastases improved progression-free survival; responses correlated with decreased peripheral blood monocyte levels 7 d following start of therapy (Kao et al. 2009). While evidence supporting the use of c-KIT antagonists and cytotoxic therapy is encouraging, agents that target c-KIT can also have effects on many other cell types, including hematopoietic stem cells, mast cells, and melanocytes, due to activity also against other related kinases, thus posing a significant challenge for interpreting data in terms of effects on iMC subtypes. Although many of the agents used for targeting iMCs lack specificity, data from agents such ATRA and c-KIT antagonists provide suggestive evidence that immature myeloid populations may have important roles in regulating anti-tumor immune responses.

Another approach for depletion of immunosuppressive myeloid cells has been treatment of tumor-bearing mice with α CD11b mAbs. CD11b is an integrin cell adhesion molecule involved in transendothelial migration expressed predominantly by myeloid lineage cells, including neutrophils, macrophages, monocytes, and DCs. Bone marrow-derived CD11b⁺ myeloid cells are recruited to tumors following RT, where they restore vascular programming via VEGF secretion, thus aiding subsequent tumor (re)growth. Neutralizing CD11b mAbs inhibit recruitment of CD11b⁺ myeloid cells into RT-treated tumors, slowing tumor regrowth and thus improving RT response (Ahn et al. 2010). Similarly, mice bearing syngeneic 4T1 mammary tumors treated with CTX and α CD11b mAbs demonstrated significantly slowed primary tumor growth as well as reduced pulmonary metastases (DeNardo et al. 2011). Gr1⁺CCR2⁺CX3CR1^{Lo} iMCs are highly responsive to CCL2 (Zhang et al. 2010), and CCL2/MCP1 is expressed at high levels in mammary tumors and is now mechanistically demonstrated to potentiate pulmonary metastasis (Qian et al. 2011).

A neutralizing antibody specific for human CD11b–CD18 integrin heterodimers, rovelizumab (LeukArrest), has previously been investigated and was found to have an excellent safety profile, but lacked therapeutic efficacy in inflammatory diseases such as multiple sclerosis. However, based on murine studies, it seems reasonable to speculate that a drug like rovelizumab could be

administered safely for transient blockade of myeloid cell infiltration following local RT or systemic CTX and thereby provide a window of opportunity when tumors could be prevented from efficient revascularization and anti-tumor immunity could be bolstered. Thus, extrapolating to the clinical scenario, it will be important to stratify human tumors containing predominately high levels of mature tissue macrophages, as compared with those containing iMCs/MDSCs, as these tumors would likely be less responsive to therapy directed at CSF1R-positive macrophages, but might be expected to instead respond to drugs like rovelizumab.

Conclusions

The relatively modest gains provided by immunotherapy despite intense investigation can be in part attributed to the presence of pathways that suppress anti-tumor immunity. These mechanisms likely evolved as part of tumor development where the local microenvironment contains an immune set point skewed favoring T_H2-type pathways, as compared with homeostatic tissue. Cell types including T_{regs}, AAMs, and iMCs form an inhibitory network that suppresses local immunity, thereby limiting the efficacy of many forms of anti-cancer therapy reliant on formation of productive anti-tumor immune responses.

Studies investigating the mechanism of action for CTX and RT have historically focused on cell-intrinsic molecules regulated by these cytotoxic agents; however, recent evidence indicates the importance of cell-extrinsic factors, particularly for induction of anti-tumor immunity. Given this, inhibitory mechanisms that stymied development of effective immunotherapy may also play an important role in regulating response to cytotoxic agents. Emerging data indicate that targeting immune inhibitory/stimulatory pathways, in conjunction with conventional cytotoxic therapy and current immunotherapy, significantly enhances the effectiveness of cytotoxic therapy by augmenting anti-tumor immunity and preventing its suppression. Further exploration to better characterize and understand inhibitory immune pathways will aid in identification of new targets that redefine our understanding of the anti-tumor mechanism of traditional cytotoxic therapies and direct us to new strategies that improve the efficacy of standard therapy.

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