Immunosuppressive networks and checkpoints controlling antitumor immunity and their blockade in the development of cancer immunotherapeutics and vaccines

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Vaccines that promote protective adaptive immune responses have been successfully developed against a range of infectious diseases, and these are normally administered prior to exposure with the relevant virus or bacteria. Adaptive immunity also plays a critical role in the control of tumors. Immunotherapeutics and vaccines that promote effector T cell responses have the potential to eliminate tumors when used in a therapeutic setting. However, the induction of protective antitumor immunity is compromised by innate immunosuppressive mechanisms and regulatory cells that often dominate the tumor microenvironment. Recent studies have shown that blocking these suppressor cells and immune checkpoints to allow induction of antitumor immunity is a successful immunotherapeutic modality for the treatment of cancer. Furthermore, stimulation of innate and consequently adaptive immune responses with concomitant inhibition of immune suppression, especially that mediated by regulatory T (Treg) cells, is emerging as a promising approach to enhance the efficacy of therapeutic vaccines against cancer. This review describes the immunosuppressive mechanisms controlling antitumor immunity and the novel strategies being employed to design effective immunotherapeutics against tumors based on inhibition of suppressor cells or blockade of immune checkpoints to allow induction of more potent effector T cell responses. This review also discusses the potential of using a combination of adjuvants with inhibition of immune checkpoint or suppressor cells for therapeutic vaccines and the translation of pre-clinical studies to the next-generation vaccines against cancer in humans.Keywords: immunotherapy; vaccine; antitumor immunity; immune checkpoint; regulatory T cell; Toll-like receptor agonist

INTRODUCTION

It is well established that the immune system can restrain tumor growth. Adaptive immune responses, in particular IFN-γ-secreting T cells, play a central role in tumor immune surveillance.[1] However, tumor growth is also associated with immune escape, immune subversion and immune suppression. Immunoediting gives rise to tumor variants that can escape immune surveillance.[2] Immune subversion strategies include downregulation of MHC class I or co-stimulatory molecules, such as CD80/86 on antigen presenting cells (APC) or enhancement of co-inhibitory inhibitory molecules such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) or programmed death-1 (PD-1) on T cells.[3] Furthermore, the development of cancer is associated with the induction or recruitment of regulatory cells and the production of molecules that suppress antitumor effector T cell responses.[4] These immune suppression networks include regulatory T (Treg) cells, myeloid derived suppressor cells (MDSC) and type 2 (M2) macrophages, as well as the immunosuppressive cytokines, IL-10 and TGF-β.

The potential of using of immunotherapy, where the patient's own immune system is enhanced to attack tumors, is gaining momentum as a viable approach for the treatment of cancer. Immunotherapeutic approaches include strategies that directly enhance anti-tumor immunity or that block immune checkpoints or suppressor networks, thus allowing the development of effector immune responses that eliminate the tumor (Figure 1). Immune-activating approaches include non-specific stimulation of innate or adaptive immune responses against the tumor or specific stimulation of tumor-specific immune responses using vaccines that include tumor antigens. The aim is to selectively promote effective cytotoxic T-lymphocyte (CTL) and Th1 responses against the tumor.[5] Prophylactic vaccines against infectious disease are among the most effective and least expensive interventions in modern medicine and function by generating protective adaptive immune responses with the help of adjuvants that activate innate immune responses. However, the application of these approaches against cancer is still in its infancy and is compromised by the fact that they must break tolerance to self antigens, they must work therapeutically and they must overcome the immunosuppressive environment of the growing tumor.

Dendritic cells (DCs) are key antigen APC, which play a vital role in activating and directing adaptive immune responses and thus DCs are being exploited in the development of vaccines against a range of cancers.[6] The first licensed DC-based cancer vaccine Provenge[7] induces antitumor responses *in vivo* by the adoptive transfer of DCs pulsed *in vitro* with tumor antigens and stimulated to mature with cytokines and immunomodulatory molecules. Nevertheless, this DC-based tumor vaccine confers only modest survival advantage and has limited success in mediating tumor regression.[8,9]

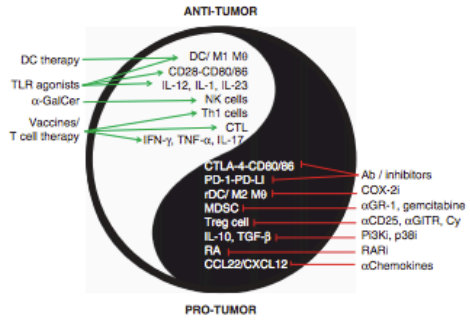

Figure 1. The pro- and anti-tumor arms of the immune response and the targets for the development of cancer immunotherapeutics and vaccines. The induction of antitumor immunity is dependent on activating DCs to express co-stimulatory molecule and innate inflammatory cytokine such as IL-12, IL-23 and IL-1 that promote Th1 and CTL responses. T cells secrete IFN-γ, TNF-α and IL17, which have antitumor effects. However, these effector responses are suppressed by co-inhibitory molecules, such as PD-1/PD-Ll, CTLA-4 and expansion of regulatory DCs (rDC), M2 macrophages and MDSC. These innate suppressor cells secrete IL-10 and TGF-β, which together with retinoic acid (RA), enhance induction of Treg cells that are recruited to the tumor under the influence of the chemokines, including CCL22 and CXCL12. Treg cells and innate regulatory cells suppress effector immune responses and thereby promote the growth of tumors. TLR agonists, αGalCer and vaccines promote innate and adaptive immune responses, while cell-based therapies with DCs or T cells can also enhance antitumor immunity. Conversely, or in combination with these immune activating strategies, antibodies or inhibitors of the suppressive pathways and molecules can block immune checkpoints and suppressor cells and thereby enhance antitumor immunity. DC, dendritic cell; TLR, Toll-like receptor; αGalCer, alpha-galactosylceramide; MI Mθ, type I macrophage, NK, Natural killer; Th1, IFN-γ-secreting CD4 T cell; CTL, cytotoxic T lymphocyte, MDSC, myeloid-derived suppressor cell; Treg cell, regulatory T cell; RA, retinoic acid; Ab, antibody; i, inhibitor; Cy, cyclophosphoamide; Pi3Ki, Phosphatidylinositol 3-kinase inhibitor; p38i, p38 MAP kinase inhibitor; RARi, retinoic acid receptor-alpha inhibitor.

Toll-like receptor (TLR) agonists are potent activators of innate immune cells and also direct adaptive immunity and thus have been employed as adjuvants in a number of experimental cancer vaccines and have been tested in clinical trials. Although the TLR7 agonist, imiquimod is being used in humans for the treatment of superficial basal cell carcinoma,[10] clinical trials with other TLR agonists have not been so effective. One reason for the relatively modest success with TLR agonist-based immunotherapies and vaccines is that TLR agonists can promote regulatory as well as effector T cells and this is compounded in a therapeutic setting by the high prevalence of Treg cells infiltrating the tumor.

Depletion of Treg cells has been shown to promote antitumor responses *in vivo* in mouse tumor models.[11–13] Furthermore, Ipilimumab, a monoclonal neutralizing antibody that blocks CTLA-4 has shown good efficacy in melanoma patients, has been approved by the FDA.[12] Another therapeutic mononclonal antibody, Nivolumab (BMS-936558) that specifically blocks PD-1 has shown some efficacy against a range of human cancers.[14,15] However, persistent blockade of these inhibitory receptors has lead to the breakdown in immune self tolerance, thereby increasing susceptibility to autoimmune or auto-inflammatory side effects, including rash, colitis, hepatitis and endocrinopathies.[16] Alternatively, cancer vaccines that are rationally designed to specifically block tumor-associated immune checkpoints may have reduced side effects. These could potentially involve combination therapy approaches, including inhibition of immunosuppressive cells, blockade of co-inhibitory molecules and the simultaneous activation of immune signaling pathways via TLR agonists to promote effector immune responses.

TARGETING TUMOR INFILTRATING IMMUNOSUPPRESSIVE CELLS

The induction of effective antitumor immune responses is hindered by the high number of infiltrating immunosuppressive cells in the tumor micro-environment, which in turn leads to poor effector immune responses. Therefore, targeting immunosuppressive cells, including Treg cells, MDSC and M2 macrophages has emerged as leading approach in the development of new immunotherapeutics and vaccines against cancer.

Regulatory T cells

Immunosuppressive cells, such as Treg cells, play a crucial role in maintaining the immune homeostasis, which depends on the balance between the immune responses that control infectious pathogens and tumors and the reciprocal immune responses that prevent inflammation and autoimmune diseases.[17] It is now well recognized that the natural Treg cells expressing FOXP3 and inducible antigen-specific Treg cells that secrete IL-10 and TGF-β play a protective role in immunity to infection by controlling pathogen-induced immunopathology and also prevent the development of autoimmune diseases.[18] However, in the tumor environment the frequency of Treg cells versus effector T cells is greater than in the general circulation, and their recruitment and activation is associated with tumor growth because of the local inhibition of the effector immune responses.[19,20] Therefore, depletion of Treg cells can enhance the development of protective T cell responses and induction of antitumor immunity.[12,13] Indeed, the effective immunosuppressive effects of Treg cells may in part explain the failure of many immunotherapeutic approaches to cancer.[6,21] Inhibition of immunosuppressive cells using cyclophosphamide treatment has been shown to enhance antitumor immunity induced by vaccination in melanoma patients.[22] Furthermore, systemic Treg cell depletion in melanoma patients induced regression of metastases.[23] However, it is now accepted that more precise strategies are required to inhibit Treg cells to enhance effector cells that mediate antitumor immunity.

Treg cell depletion strategies have utilized monoclonal neutralizing antibodies or ligand-directed toxins targeted to cell surface receptors, such as CD25. Two anti-CD25 antibodies, daclizumab and basiliximab induce Treg cell death by cytokine deprivation (IL-2) and by triggering antibody-dependent cell-mediated cytotoxicity or complement-mediated cytotoxicity.[4] The results of clinical trials have shown that daclizumab depletes Treg cells and thereby enhances CTL responses to tumor antigens induced by vaccination without any autoimmune side effects.[24,25]

In addition to Treg cell depletion strategies, other approaches have also been utilized to eliminate the immunosuppressive effects of Treg cells. For example, intratumoral injection with an agonistic antibody (DTA-1) to the glucocorticoid-induced tumor necrosis factor (TNF) receptor, which is constitutively expressed on Treg cells, invoked potent antitumor immunity and eradicated established tumors in mice.[26,27] Another strategy involves the inhibition of receptor activation of NF-κB (RANK) signaling using an anti-RANK ligand (RANKL) antibody denosumab, which blocks the engagement of RANKL on Treg cells to the RANK receptor on the tumor cells and thus blocks Treg cell-induced metastases of certain cancers.[4,28] Furthermore, targeting the Treg cell transcription factor FOXP3 by RNA interference, such as miR-31 (negative regulator of FOXP3), can modulate Treg cells functionality and abolishes their suppressor activity.[29]

Furthermore, a peptide inhibitor of FOXP3 has been shown to impair Treg cell activity and enhance the efficacy of peptide vaccine against CT26 tumors in mice.[30] However, translating these approaches to clinical applications will be challenging, as they would have to be specifically delivered to tumor infiltrating Treg cells because FOXP3 is transiently expressed in activated effector T cells and in certain cancer cells.[31]

Despite the obvious benefits of increasing effector T cells in the tumor environment, depletion of Treg cells may have a high risk of developing systemic autoimmune diseases due to increased inflammatory responses. Thus, alternative approaches need to be devised that involve selective inhibition or depletion of immune suppressive cells in the tumor with optimal activation of antitumor T cell responses. One approach may be to target Treg cell migration into tumor. Studies in cancer patients have demonstrated significant trafficking of Tregs into tumors and have found an association between the frequency of intratumoral Treg cells and poor survival.[32] Tumor cells and macrophages produce the chemokine C-C motif chemokine ligand 2 (CCL22), which promotes trafficking of Treg cells that express CCR4, the receptor for CCL22. Studies in mice showed that *in vivo* blockade of CCL22 reduced the trafficking of Treg cells into tumors.[33] CXCL12/CXCR4 signals have also been shown to regulate the Treg trafficking to the bone marrow in prostate cancer patients with bone metastasis.[34] Blocking the CXCL12/CXCR4 signals significantly reduces the Treg cell trafficking to bone marrow.[32,34] Therefore, strategies that target chemokines to prevent T cell migration into tumor may be a more tailored and less risky approach than systemic depletion of Treg cells.

Myeloid-derived suppressor cells

MDSC are another class of immunosuppressive cells, which are a heterogeneous population of immature myeloid cells that accumulate under conditions of inflammation and in tumors and exert inhibitory function on immune responses. MDSCs are thought to promote the tumor growth by both enhancement of tumor angiogenesis and metastasis and also inhibition of antitumor effector T cell responses. Under normal physiological conditions, these cells are generated in bone marrow and differentiate into mature macrophages, DCs and granulocytes. However, in pathological conditions, there is a dramatic expansion of these Gr-1$^+$CD11b$^+$ cells, with the same phenotype and immunosuppressive activity in various tissues, and the differentiation into mature myeloid cells is blocked.[35,36] MDSCs regulate innate immune and T cell responses by depleting arginine, modulating cytokine production by macrophages, upregulating the production of immune-suppressive factors, such as nitric oxide and reactive oxygen species,[36–39] and by overexpressing anti inflammatory cytokines, such as TGF-β and IL-10.[40,41] MDSCs suppress proliferation and cytokine production by T cells and natural killer cells, as well as induce apoptosis of CD8$^+$ T cells.[42]

Interestingly, MDSCs have also been shown to indirectly suppress T cell activation by inducing other immunosuppressive cells such as Treg cells and M2 macrophages.[43–47] The MDSCs accumulation in the inflammatory pathogenic setting suggests that increased MDSC numbers play a protective role by providing an anti-inflammatory mechanism for the maintenance of homeostasis; however, in the tumor environment, the anti-inflammatory effect of MDSCs leads to attenuation of antitumor immunity. Thus, targeting the inhibitory functions of MDSCs present a promising approach to enhance therapeutic potential of antitumor vaccines and immunotherapies.

Anti-Gr-1 monoclonal antibodies have been widely used to deplete MDSCs *in vivo* in mice. However, the clinical translation of anti-Gr-1 antibodies to deplete MDSCs in humans is challenging, since they recognize both Ly6G and Ly6C (molecules that express the Gr-1 epitope on MDSCs), which could lead to non-selective depletion of monocytes, T cells, natural killer cells and macrophages and might impair host immunity and lead to opportunistic infections.[48] Recently, pre-clinical and clinical studies have shown that promoting the differentiation of immature suppressive MDSCs into mature, non-suppressive cells such as myeloid DCs, CD4$^+$ and CD8$^+$ T cells using all trans retinoic acid[49–51] and Vitamin D3[52,53] can enhance the antitumor immune responses. A number of strategies have been used to deplete MDSCs, including the use of chemotherapeutic drug gemcitabine,[45,54] sunitinib,[55–57] 5-FU,[58] docetaxel,[59] retinoic acid[50,60] or the debulking of tumors.[61,62] Furthermore, multiple inhibitor molecules, such as CDDO-ME,[63] PDE-5,[63] COX-2[64,65] and nitro aspirin[66] have also been successfully shown to inhibit the suppressive function MDSC, restore T cell proliferation and decrease tumor growth *in vivo*. These MDSCs depletion and inhibitory approaches can improve immune surveillance and promote antitumor immune responses, thus targeting MDSCs may be a promising approach for enhancing the efficacy of cancer vaccines or immunotherapies.

M2 macrophages

Tumor-associated macrophages are a dominant population of immune cells present in the tumor microenvironment and are mostly characterized as alternatively-activated M2-like macrophages, which are anti-inflammatory, immunosuppressive and facilitate tumor progression,[67,68] unlike M1 macrophages, which are highly inflammatory, microbicidal and tumoricidal. M2 macrophages play a significant role in promoting tumor growth, angiogenesis, metastasis, matrix remodeling and facilitate immune evasion in various human and animal tumors.[68–70] M2 macrophages also provide chemotherapy resistance,[71,72] radiotherapy resistance[73] and promote tumor growth. Therefore, targeting M2 macrophages is now considered a promising approach for treatment of cancer.

A number of approaches have been employed to target M2 macrophages in tumors. Firstly, macrophage recruitment into the tumor microenvironment can be suppressed by inhibiting chemoattractants, such as CCL2, macrophage colony-stimulating factor or C-C motif chemokine receptor 2 (CCR2) and macrophage colony-stimulating factor receptor (M-CSFR); this has reduced tumor growth and enhanced prognosis in multiple cancers.[74–80] Secondly, M2 macrophage survival can be suppressed using chemical drugs, such as bisphosphonates, dasatinib,[81–83] or bacterial infection with attenuated *Shigella flexneri*[84] that selectively kill M2 macrophages and result in regression of tumor growth, angiogenesis and metastasis. Alternatively, M2 macrophages can be depleted using immunotoxin-conjugated monoclonal anti-FRβ antibody, which target membrane molecules of M2 macrophages.[85,86] Lastly, the tumor promoting activity of M2 macrophages can be blocked using inhibitors of STAT3 (for example: sunitinib,[87] sorafenib,[88] WP 1066,[89] corosolic acid[90] and oleanolic acid[91]), STAT6,[92] c-Myc,[93] PI3K,[94] KLF4,[95] HIFs,[96] Ets2[97] and Mtor.[98]

BLOCKADE OF IMMUNE CHECKPOINTS

Among the most promising approaches to activate therapeutic antitumor immunity is the blockade of the immune checkpoints. Immune checkpoints are inhibitory pathways employed by the immune system to maintain self-tolerance and thereby prevent the development of autoimmunity.[3] They also help to regulate the duration and amplitude of physiological immune responses against pathogens in order to minimize collateral tissue damage and prevent immunopathology during infection. There is increasing evidence that tumors exploit certain immune checkpoint pathways and thereby subvert antitumor immunity, especially T cell responses specific for tumor antigens. Fortunately, most of the immune checkpoint pathways are initiated by ligand-receptor

interactions, thus they can be readily blocked using neutralizing monoclonal antibodies and inhibitory peptides or modulated using recombinant forms of ligands or receptors. This can help to drive effector immune responses and can enhance the efficacy of tumor vaccines and immunotherapeutics.

T cell—APC interaction involves cell surface co-stimulatory and co-inhibitory molecules,[99] as well as membrane receptors that are involved in adhesion and migration. These include members of the immunoglobulin superfamily, such as CD80/CD86-CD28 molecules and the TNF receptor (TNFR) superfamily, such as CD40-CD40L. Antagonizing the interaction between co-inhibitory cell surface receptors with neutralizing antibodies or inhibitory peptides can block the tolerogenic pathways. For example, blockade of CTLA-4 pathway and programmed cell death protein 1 (PD-1)-PDL-1 (also known as B7 homolog 1; B7H1) pathway can enhance immune responses. DCs from ovarian carcinomas overexpress B7H-1 (PD-1) and blockade of B7-H1 reduced IL-10 and enhanced IFN-γ production by T cells, thereby reduced the growth of human ovarian carcinoma in non-obese diabetic-severe combined immunodeficient mice.[100] Furthermore, B7H4 expression in gastric cancer is associated with poor survival.[101] Expression of B7-H4 by APC is enhanced by Treg cells through IL-10 production,[102] and blockade of B7-H4 enhances antitumor T cell responses.[103] Alternatively, agonists that induce signaling via co-stimulatory cell surface receptors, such as CD28, CD137, CD27 and CD40 have been used to enhance lymphocyte priming to promote antitumor immune responses.[104] Ipilimumab, a CTLA-4-specific monoclonal antibody, developed by Bristol Myers Squibb, approved by the FDA in 2011 for the treatment of metastatic melanoma, was the first immunotherapeutic based on the blockade of immune checkpoints. However, the inhibition of CTLA-4 is associated with immune-related adverse events,[105] including colitis/diarrhea, dermatitis, hepatitis and endocrinopathies.[106] Another CTLA-4-specific monoclonal antibody, tremelimumab, is currently being evaluated for the treatment of several other cancers as a monotherapy, or as an adjuvant in a DC vaccine.[107,108]

An antibody that targets the co-inhibitory molecule PD-1, BMS 936558, has been evaluated in clinical trials and has shown significant and durable responses in several types of refractory tumors.[15] This antibody was designed to represent newer generation of immunomodulatory biologics that stimulate highly effective and long lasting host tumor immunity with controllable autoimmune toxicities. However, endocrine toxicities have been reported with this antibody. Other drugs that target PD-1, including MK3475, CT-011 and AMP-224 are currently in clinical trials for different cancers. Furthermore, Genentech, AstraZeneca and Roche have antibodies against PDL-1 under clinical evaluation for solid tumors, melanoma or renal cell cancer.[109] Additional immune checkpoints inhibitors under evaluation include those that target lymphocyte activation gene 3,[110,111] 2B4 (also known as CD244), B and T lymphocyte attenuator,[112] T cell membrane protein 3[113,114] and adenosine A2a receptor.[115]

A number of immunotherapeutics based on multiple antibody and immunoglobulin fusion proteins targeting co-inhibitory molecules are in pre-clinical and clinical trials (for example, IMP321 against lymphocyte activation gene 3 is in phase III clinical trial in breast cancer). Alternatively, the direct activation of co-stimulatory molecule signaling pathways using agonists, such as the monoclonal antibody TGN1412 specific for CD28 has the capacity to stimulate naïve human T cells without the need for a T cell receptor signal. This has been evaluated in clinical trials. However, within 90 min of infusion of the antibody, severe systemic inflammatory responses were induced, characterized by a massive pro-inflammatory cytokine storm followed by multi-organ injury and lymphocyte depletion. Fortunately, all volunteers survived after receiving immunosuppressive treatments and cardiopulmonary support, and this trial cautioned against the global non-discriminatory stimulation of naïve T cell activation.[116]

TLR LIGANDS AS IMMUNOTHERAPEUTICS AND ADJUVANTS FOR CANCER VACCINES

A new approach to the development of tumor vaccines and immunotherapies has focused on enhancement of effector T cell responses by targeting innate immune cells and the receptors that mediate their activation. Pathogen recognition receptors, in particular TLRs, sense pathogen-associated molecular patterns and are the primary targets for activation of the innate immune cells and are fundamental in the development of effective adaptive immune responses. Innate immune cells not only act as APC for T cells but also provide signals necessary for T-cell activation. Ligand binding to the TLRs, such as lipopolysaccharide to TLR4, flagellin to TLR5, CpG to TLR9, activates downstream intracellular signaling cascades through the transcription factors NF-κB, interferon regulatory factor 3, mitogen-activated protein kinases leading to the production of pro-inflammatory cytokines and type I interferons, which in turn activate co-stimulatory molecules, which are necessary for the antigen-presenting function of DCs and macrophages to activate T cells. Therefore, TLR agonists are potent activators of innate and adaptive immune responses and thus can act as adjuvants to promote immune responses against tumor antigens.

Synthetic ligands for TLR4, TLR7/8 and TLR9 have been through clinical trials against cancer[117,118] and the TLR7 agonist, imiquimod (Aldara) has been licensed for clinical use for the treatment of superficial basal cell carcinoma.[10] However, unsatisfactory results have been obtained from clinical trials with other TLR agonists, and one pharmaceutical company halted its clinical program for PF-3512676, a CpG oligodeoxynucleotide that activates TLR9.[119] Furthermore, the TLR4 agonists OK-432 and OK-PSA have been evaluated as immunotherapeutics for uterine, cervical and non-small lung cancer, but did not show promising results in most patients.[120,121]

In addition to the use of TLR agonists as direct tumor immunotherapeutics, TLR agonists have also been used as adjuvants for co-administration with tumor antigens, peptides, recombinant proteins or killed tumor cells. The detoxified derivative of lipopolysaccharide, monophosphoryl lipid A has shown promise as an adjuvant for vaccines against multiple tumors.[122] Currently, a TLR3 agonist, hiltonol, is being evaluated in clinical trials as an adjuvant for NY-ESO-1 protein vaccine in patients with high-risk melanoma.[123] A study of hiltonol administered with tumor lysate-pulsed DC vaccines is also ongoing in patients with malignant glioma.[123] Immunization of melanoma patients with the melanoma antigen Melan-A/MART-1 formulated in incomplete Freund's adjuvant with CpG-ODN induced strong antigen-specific $CD8^+$ T-cell responses.[124]

Studies in mice have demonstrated mixed results with TLR agonists as adjuvants for tumor vaccines. Prophylactic but not therapeutic immunization with CpG-ODN in a transgenic mouse model expressing SV40 T Ag prevented tumor growth.[125] Furthermore, immunization of Tg mice expressing the Her-2/*neu* gene product with a synthetic peptide specific for Her-2/*neu* in combination with CpG-ODN prevented tumor growth in mice.[126] Furthermore, the efficacy of a DC tumor vaccine was enhanced with the use of CpG as an adjuvant in the presence of a p38 mitogen-activated protein kinases signaling inhibitor.[127] Finally, the therapeutic efficacy of adenovirus expressing human tyrosinase-related protein 2 was enhanced with a peritumoral injection of CpG and poly(I:C) (a synthetic ligand for TLR3).[128]

It has also been demonstrated that TLR agonists have the potential to break tolerance to self-antigens by inhibiting the

function of immunosuppressive cells such as Tregs.[129–131] TLR induced IL-6 production by DCs blocked the suppressive function of $CD4^+CD25^+$ Treg cells.[129] The TLR3 agonist Poly(I:C) has been shown to enhance tumor-suppressing M1 macrophages at the expense of tumor-supporting M2 macrophages and thus inhibit the production of anti-inflammatory cytokines IL-10 and TGF-β.[132,133] Furthermore, lipopolysaccharide and CpG can enhance activation of NF-κB pathways important for the establishment of the M1 phenotype of macrophages and their production of the inflammatory cytokines IL-12, IFN-α/β and TNF-α.[118,134] It is now recognized that TLR agonists are effective adjuvants for cancer vaccines through their ability to promote the differentiation of IFN-γ-secreting Th1 cells. However, TLR2 agonists have the potential to inhibit effector T cell responses[135] and all TLR agonists stimulate IL-10 as well as IL-12 production from DC and thereby promote induction of $IL\text{-}10^+$ Treg as well as Th1 cells.[136] This has lead to the idea of using combinations of TLR activation with inhibitors of Treg cell induction to promote more effective antitumor immunity.[137]

COMBINATION STRATEGIES FOR NEXT-GENERATION CANCER IMMUNOTHERAPEUTICS AND VACCINES

Our increased understanding of the tumor microenvironment and the immune responses that mediate and regulate antitumor immunity has provided a foundation for the rational development of vaccines and immunotherapeutic approaches for the treatment of cancer. Whilst vaccines and immunotherapeutics that target one arm of the immune system have shown some promising results in generating antitumor responses, many of these are subverted by a failure to block the regulatory responses associated with the tumor. Conversely, strategies that globally suppress Treg cells increase the risks for the patient of developing autoimmune diseases. The design of combination vaccines or therapies that activate the inflammatory signaling pathways that target the tumor and specifically block inhibitory pathways in the immunosuppressive tumor microenvironment although challenging may be the way forward (Figure 2).

There have already been a number of studies in mice that have demonstrated the benefits of using combination approaches that

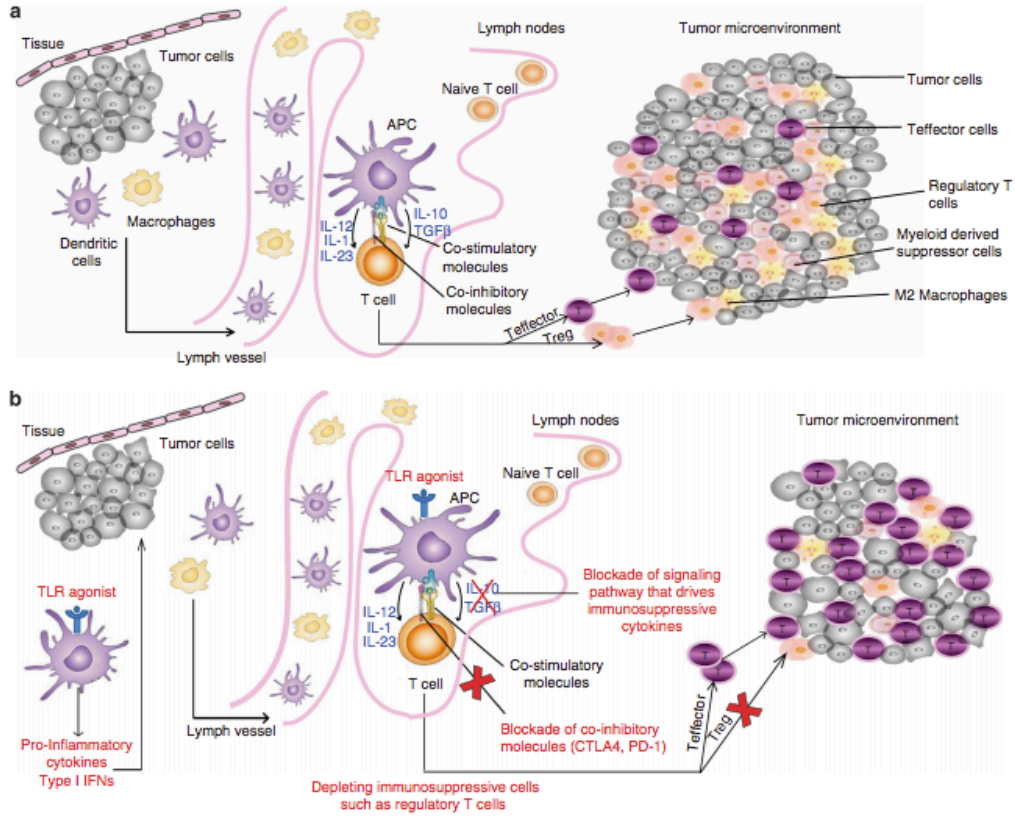

Figure 2. Combination immunotherapeutic/vaccine approaches against tumors. (**a**) In the initial stages of immune response against tumors, the local tissue resident innate immune cells, including macrophages and DCs recognizes the tumor antigens, are activated and migrate to regional lymph nodes via the lymphatic vessels, where they activate the naïve T cells by presenting the tumor antigens. Co-stimulatory and co-inhibitory molecules on APC interact with ligands on T cells, leading to the proliferation of effector and regulatory T cells respectively. A high percentage of immunosuppressive cells, including Treg cells, MDSCs, M2 macrophages, infiltrate the tumor microenvironment, which inhibit the effector immune responses against tumor. (**b**) Combination vaccine or immunotherapeutic approaches that target the immune response pathway are effective means of enhancing antitumor immunity. Strategies that combine innate cell activation with TLR agonists with depletion or inhibition of Tregs and MDSCs, or blockade of co-inhibitory molecules can enhance effector T cell responses against the tumor.

target multiple points in the immune system to enhance the antitumor immunity. For example, a study has demonstrated that the efficacy of DC vaccine against B16 melanoma in mice can be enhanced by the gene silencing of TGF-β1, which reduced the tumor-associated Treg cells.[138] Furthermore, it has been shown that a single dose use of cyclophosphamide can reduce the numbers of Treg cells and enhance immune responses to the renal cell cancer vaccine IMA901, thus leading to prolonged survival of cancer patients after vaccination.[139] Also, a combination of the TLR agonist flagellin and a class I PI3 kinase inhibitor has been shown to block induction of Treg cells and promote effector T cells that mediate rejection of tumors in mice.[136] While PI3 kinase inhibitors are already in clinical trials based on their ability to arrest cell proliferation and induce tumor cell death, this study demonstrated that PI3 kinase inhibitors also attenuated TLR-induced IL-10 and TGF-β and thereby inhibited induction of Treg cells. Furthermore, gemcitabine has been shown to reduce the numbers of splenic MDSC in tumor-bearing mice and enhance antitumor immunity when used in combination with IFN-β.[54] In a HER-2/neu tumor model, therapeutic administration of anti-glucocorticoid-induced TNF receptor monoclonal antibody with a HER-2/neu vaccine enhanced vaccine efficacy and protected against pre-existing tumors.[140]

The benefits of combination approaches to inhibit immune checkpoints has recently been demonstrated with the report that the blockade of PD-1/PD-L1 pathway using anti-PD-L1 neutralizing antibodies or depletion of Treg cells alone failed to prevent recurrence of tumors, whereas the combination of PD-L1 blockade with Treg depletion effectively mediated disease regression.[141] Furthermore, immune checkpoint blockade using anti-PD-L1 and anti-LAG-3 antibodies overcame the requirement to deplete tumor-specific Tregs.[141] Double immune checkpoint blockade with ipilimumab (anti-CTLA-4) and nivolumab/BMS-936558 (anti-PD-1) has recently been evaluated in a phase I clinical trial in patients with advance melanoma.[142] The results revealed that the combination treatment induced clinical activity in 65% of patients, with tumor regression of 80% or more in 53% of patients at the maximum doses associated with acceptable side effects. This combination may prove to be powerful for unleashing immune responses to melanoma, provided the incidences of autoimmune adverse effects are manageable.

Blockade of immune checkpoints or the inhibition of immunosuppressive cells are clearly promising approaches for the treatment of cancer. However, global blocking of the anti-inflammatory arm of the immune system alone significantly enhances inflammatory responses, which can lead to the breaking of immune self-tolerance, thereby inducing autoimmune/auto-inflammatory side effects including rash, colitis, hepatitis and endocrinopathies (summarized by Corsello *et al.* 2013).[143] Generalized non-specific activation of the immune system as a result of blockade of immunosuppressive cells or checkpoints when combined with immune stimulators also has the potential to generate systemic inflammation. Thus a major challenge in the blockade of immune checkpoints alone or in combination with immune activators is to avoid exceeding the limits of safety associated with removing the natural breaks on the immune system that may lead to unacceptable toxicities. We believe that this can be achieved by employing strategies that specifically enhance tumor-specific immune responses, while transiently blocking immunosuppressive networks, not systemically but locally in the tumor or the draining lymph nodes. More specific inhibition of tumor associated immunosuppressive cytokines and cells and careful and controlled activation of immune system has the potential to generate antitumor immune responses without excessive systemic inflammation or autoimmune diseases. One approach to achieve this objective involves the use of TLR agonists as immunotherapeutics or adjuvants in tumor vaccines, in combination with transiently depleting immune suppressive cells or blockade of immune checkpoints at the time of immunization in the vicinity of the tumor. For example, it has been shown that the efficacy of TLR agonists as therapeutics or as adjuvants in a DC vaccine were enhanced when Treg cells induction in the tumor microenvironment were attenuated through inhibition of p38 mitogen-activated protein kinases or Pi3K signaling.[127,136] Transient inhibition of Treg cells at the time of immunization generated potent antitumor immunity and immunological memory and is less likely to be associated with systemic autoimmunity. Therefore, combination approaches that specifically inhibit tumor-associated Treg and innate suppressor cells with concomitant enhancement of antitumor effector immune responses have considerable potential as safe and effective cancer immunotherapeutics and vaccines of the future.

CONFLICT OF INTEREST

KHG Mills is a co-founder and shareholder in Opsona Therapeutics and TriMod Therapeutics. TriMod Therapeutics is involved in the development of immunotherapeutics for cancer. Aisha Q. Butt declares no conflict of interest.

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