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The Tumor Immunoenvironment

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Chapter 12
Roles of Signaling Pathways in Cancer Cells and Immune Cells in Generation of Immunosuppressive Tumor-Associated Microenvironments

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Abstract Cancer cells trigger multiple immunosuppressive cascades and generate immunosuppressive tumor-associated microenvironments including tumor and sentinel lymph nodes. Constitutive activation of various signaling pathways (e.g., MAPK, STAT3, NF-κB, β-catenin) in human cancer cells was found to trigger the multiple immunosuppressive cascades through the production of immunosuppressive cytokines, such as TGF-β, IL-10, IL-6, and VEGF, and induction of immunosuppressive immune cells, such as regulatory T cells, tolerogenic dendritic cells, and myeloid derived suppressor cells. Some of these cancer-derived cytokines impair various immune cells through activation of their signaling molecules such as STAT3 and NF-κB. Inhibitors for these activated signals could inhibit the multiple immunosuppressive cascades by acting on both cancer cells and immune cells. Since common signaling mechanisms are often utilized for some of the hallmarks of cancer (e.g., cell proliferation/survival, invasion/metastasis, and immunosuppression), targeting these common signaling pathways may be an attractive strategy for cancer therapy, including immunotherapy.

Keywords Immunosuppression · BRAF · STAT3 · β-catenin · NF-κB · TGF-β · IL-10 · MAPK · MDSC · Regulatory T cells

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12.1 Introduction

We have previously identified various human tumor antigens recognized by T cells, and developed various antigen specific immunotherapies (Kawakami et al. 2004; Kawakami et al. 1994; Kawakami et al. 1994; Rosenberg et al. 1998). The vaccine using gp100 melanoma antigenic peptide plus IL-2 resulted in 16 % objective response with 9 % CR in the recent multicenter randomized trial (Schwartzentruber et al. 2011), while the recent adoptive immunotherapy using cultured melanoma specific T cells after the myeloablative treatment, which depletes various immunosuppressive cells, resulted in more than 70 % objective response with 21 % durable CR for advanced melanoma patients with multiple metastases (Rosenberg et al. 2011). Immunological analysis of the clinical trials indicated that immunosuppression in cancer patients is one of the major obstacles for development of effective immunotherapy. Thus, understanding of the mechanisms for the immunosuppression in cancer patients and development of strategy to overcome it is important for improvement of cancer therapy.

12.2 Immunopathology of Cancer-Associated Microenvironments

Roles of the immune system in cancer have recently been extensively exploited. The murine studies analyzing the interaction between cancer cells and immune system (and other stromal cells) during cancer development revealed that innate cells such as macrophages and mast cells rather have tumor promoting activity though increase of cancer cell proliferation and invasion ability, as well as induction of angiogenesis. In contrast, T cells and NK cells have the ability to eliminate cancer cells (Immunosurveillance). However, cancer cells having intrinsic genetic instability subsequently evade the immune defense system by losing highly immunogenic tumor antigens and acquiring various immunoresistant and immunosuppressive mechanisms (Immune evasion). This process is also known as Immunoediting (Schreiber et al. 2011). In fact, cancer cells developed from immunocompromised hosts are sensitive to immune cell attack, and cancer cells obtained from patients have a variety of immune-suppressive and resistant features (Zou 2005; Gajewski et al. 2006; Yaguchi et al. 2011). Therefore, the immunological characteristics of cancer cells are defined by both cancer cells' intrinsic nature and immune reactivity of patients.

When investigating the mechanisms of the immunosuppression in cancer patients, it is important to consider tumor-associated microenvironments, including tumor tissues where effector immune cells should eliminate cancer cells, sentinel lymph nodes (SLNs) where tumor specific T cells should be primed, and bone marrow where is a source of various immunosuppressive cells including myeloid-derived suppressor cells (MDSCs) and mesenchymal stem cells (MSCs), as well as

a reservoir for tumor specific memory T cells. Analysis of tumor tissues obtained from patients revealed that tumor appears to be under immunosuppressive conditions suggested by the expression of various immunosuppressive molecules in cancer cells (e.g., soluble molecules such as TGF-β, IL-10, IL-6, VEGF, GM-CSF, IL-13, PGE2, sMICA, membrane molecules such as PD-L1, FasL, ILT7L, intracellular molecules such as IDO, COX2), and by accumulation of various immunosuppressive cells (e.g., Treg, MDSCs, M2-like macrophages, tolerogenic dendritic cells (DCs), plasmacytoid DCs, cancer-associated fibroblasts (CAFs), MSCs). Similarly, in the sentinel lymph nodes of cancer patients, accumulation of such immunosuppressive cells was also observed (Kim et al. 2006; Swartz and Lund 2012; Fridman et al. 2011). However, the comprehensive analysis of the molecular and cellular mechanisms for the immunosuppression in the tumor-associated microenvironments remains to be performed.

It has recently been reported that levels of spontaneous $CD8^+$ T cell responses (infiltration of memory $CD8^+$ T cells in the tumor tissue prior to the cancer treatment) are different among patients with various cancers, including colon cancer, ovarian cancer, and melanoma. High infiltration of memory $CD8^+$ T cells in tumor significantly correlated with better prognosis, and its prognostic predictive value appeared to be better than TNM staging (Fridman et al. 2011; Mlecnik et al. 2011). It was also correlated with response to immunotherapy in melanoma and even chemotherapy in colon cancer (Gajewski et al. 2011). Therefore, international collaborative study "Immunoscore validation task force" is currently in progress to confirm the diagnostic value of infiltration of $CD8^+$ T cells in colon cancer (Galon et al. 2012). However, it has not yet been understood what makes the difference of spontaneous $CD8^+$ T cell response among patients. It may be regulated by both cancer cell characteristics and immunological constitution of hosts.

One important point is that immune condition in cancer patients is regulated by complex immune networks and it is first triggered by cancer cells, more specifically genetic or epigenetic alterations in cancer cells. Cancer cells trigger multiple immunosuppressive cascades in which various immunosuppressive molecules such as TGF-β, IL-10, IL-6, VEGF, PD-L1, COX2, and IDO, and immunosuppressive cells, such as tolerogenic DCs, MDSCs and Treg cells, are involved, and finally immunosuppressive conditions are established in the tumor-associate microenvironments.

12.3 Immunosuppressive Cascades Triggered by Gene Alterations in Cancer Cells

To understand the immunosuppressive cascades triggered by cancer cells, we have evaluated the role of TGF-β, which is produced by most human cancer cells, infiltrated immune cells and stromal cells, in the regulation of immunological conditions in tumor and SLN. In our mouse tumor model, increase of TGF-β in the

tumor microenvironment by implantation of the TGF-β gene-transduced tumor cells resulted in increased accumulation of $CD11b^+$ $Gr\text{-}1^+$ MDSCs and $FoxP3^+CD4^+$Treg cells in both tumor and SLN. Numbers of DCs infiltrated the tumor tissue were decreased. Interestingly, numbers of DCs were increased in SLN compared to non-SLN in mice implanted with either TGF-β-transduced tumor cells or control tumor cells, but function of DCs from mice with abundant TGF-β expression in the tumor microenvironment was significantly impaired as assessed by their T cell stimulatory activity. Implantation of TGF-β-producing tumor cells also induced M2-like macrophages, which produced abundant CCL22 in SLN; CCL22 appeared to recruit $CCR4^+$ Tregs into SLN (Tsujikawa et al. 2012). Consequently, induction of tumor antigen specific T cells from SLN was significantly reduced, and, finally, infiltration of $CD8^+$ T cells in tumor appeared to be reduced in the mice with abundant TGF-β expression. It has been reported that inhibition of TGF-β signaling by injection of plasmid DNA encoding TGF-β type II receptor near the tumor sites was reported to enhance tumor antigen specific T cells accompanied by decrease of Treg cells (Fujita et al. 2009). Therefore, these mouse models recapitulate the observations in the analysis of clinical samples and indicate that immunosuppressive molecules, such as TGF-β, may be one of the factors to define the immune status in tumor and SLN, including spontaneous $CD8^+$ T cell response.

We have previously reported that TGF-β-induced-Snail stimulated not only epithelial-to-mesenchymal transition (EMT) of cancer cells, but also production of immunosuppressive cytokines and chemokines, including TGF-β, IL-10, CCL2, and TSP-1, which caused DC impairment and Treg induction. The impaired DCs could also induce Tregs. CCL2 impairs DCs and recruits immunosuppressive MDSCs into tumor. The blockade of Snail in the tumor microenvironment by intratumoral administration of Snail-specific siRNA restored immunocompetence of mice having Snail-transduced tumor and resulted in enhanced induction of tumor antigen specific T cells in vivo (Kudo-Saito et al. 2009). These results illustrate that TGF-β production in the tumor microenvironment by either cancer cells or infiltrated stromal cells, including various immune cells, triggers multiple immunosuppressive cascades involving various immunosuppressive cytokines/chemokines and cells. This reemphasizes that the TGF-β cascade is an attractive target for reversal of cancer-induced immunosuppression (Fig. 12.1).

The molecular mechanisms of the increased production of TGF-β by human cancer cells have not been well understood. In human melanoma, production of TGF-β was not mainly regulated by MAPK and STAT3 pathways as described below. We have recently found that one of the intracellular kinase, which is frequently phoshphorylated in various cancer cells, is involved in TGF-β production by human melanoma cells. This was assessed by screening signaling molecules in melanoma cells involving suppression of DC function by using kinase siRNA library (manuscript in preparation). Therefore, this activated kinase in cancer cells can be an upstream target to inhibit the TGF-β-triggered immunosuppressive cascades. We are currently searching for small molecular drugs which efficiently inhibit this kinase. This is one example of immunosuppressive

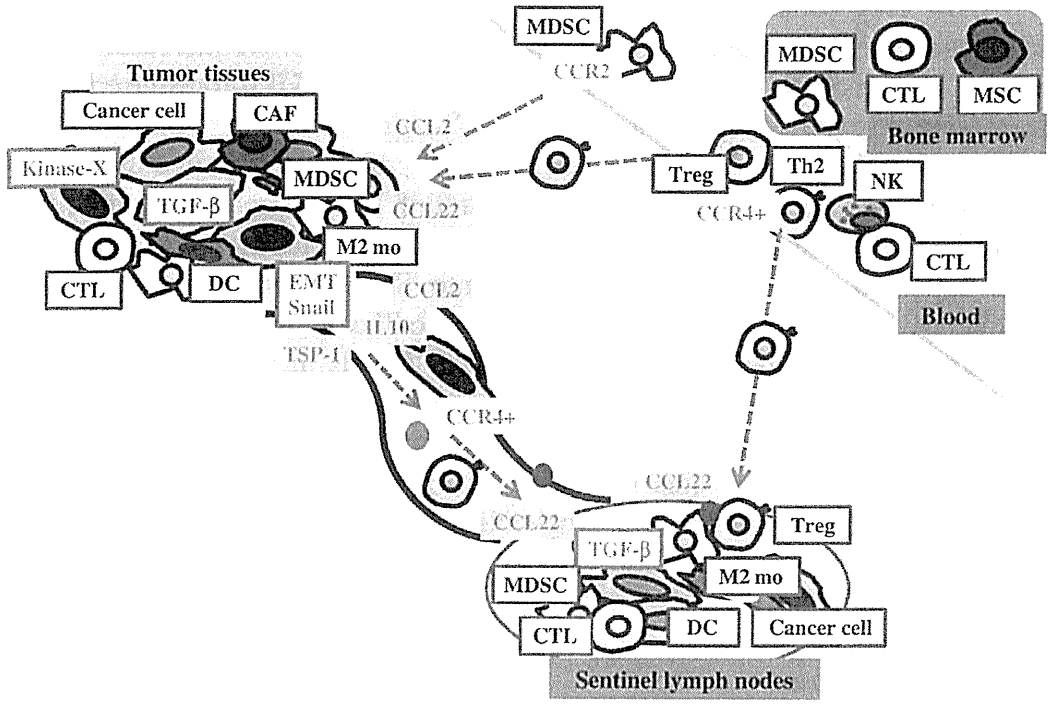

Fig. 12.1 Cancer cell triggered immunosuppressive cascades: TGF-β cascade. TGF-β produced by cancer cells and infiltrated stromal cells including various immune cells triggers immunosuppressive cascade (production of CCL2, IL-10, TGF-β , etc., and subsequent recruitment and induction of various immunosuppressive cells such as MDSC, M2 macropages, Treg cells via CCL2, CCL22, IL-10, TGF-β , etc.), and generates immunosuppressive condition in the tumor and sentinel lymph nodes. TGF-β-Snail axis induces not only epithelial-to-mesenchymal transition (EMT) which enhances invasion ability of cancer cells, but also immunosuppression which may further enhance metastasis of cancer cells

cascades triggered by gene alterations in cancer cells. Since gene and signal alterations in human cancer cells vary among cancer types, even among patients with the same type of cancer, there are multiple immunosuppressive cascades to be investigated.

12.4 Alterations of Gene and Signal Pathways Involved in the Immunosuppression in Human Cancer

Human cancer cells have various genetic and epigenetic alterations. Thus, understanding of immunosuppressive cascades triggered by each gene/signal alteration is important. Here, we describe some of our recent observations on human cancer cells.

12.4.1 RAS/BRAF/MEK/MAPK Signaling Pathway

When common mutation of BRAF (V600E), a molecule in MAPK signal pathway, was discovered by sequencing signaling molecules in human melanoma cells (Davies et al. 2002), we evaluated the role of the mutant BRAF (V600E) for malignant characteristics of human melanoma cells by using BRAF (V600E)-specific lentiviral shRNA. We found that the BRAF mutation was involved in the cell proliferation and invasion ability of melanoma cells (Sumimoto et al. 2004). We have also found that production of multiple cytokines, IL-6, IL-10, and VEGF, which have the ability to suppress function of DCs, were significantly decreased by BRAF(V600E) shRNA without affecting cell survival of the some melanoma cell lines (Sumimoto et al. 2006). These cytokines suppress DC activity to stimulate T cells mainly through the inhibition of IL-12 and TNF-α production, and augmentation of IL-10 production. Treatment of melanoma cells with BRAF (V600E)-specific shRNA or MEK inhibitors resulted in decrease of immunosuppressive activity of melanoma cells, indicating the MAPK pathway is essential for DC impairment by melanoma cells. MEK inhibitors were also reported to increase susceptibility of melanoma cells to CTL lysis partly due to increased expression of melanoma antigens, such as MART-1/melan-A and gp100 (Kono et al. 2006; Boni et al. 2010). "Avoiding immune destruction" resulting from the loss of highly immunogenic tumor antigens and acquiring immunoresistant and immunosuppressive mechanisms is now generally recognized as one of "the hallmarks of cancer" (Hanahan and Weinberg 2011). These results indicate that the BRAF-MAPK axis is commonly involved in the cancer cell proliferation, invasion, and immunosuppression (Fig. 12.2).

These observations indicate that blockade of the BRAF-MAPK axis may not only suppress proliferation and invasion of cancer cells, but also inhibit immunosuppressive activity and increase susceptibility of melanoma cells to T cells. This suggests that it is a common attractive target for melanoma treatment, particularly in combination with various immunotherapies. Since MAPK signal is also important for T cell proliferation, administration of MAPK pathway inhibitors may also suppress anti-tumor T cell response. However, two BRAF inhibitors, which preferentially inhibit mutant BRAF, have recently been developed, and their administration has already been shown to be effective in patients with melanoma (Chapman et al. 2011; Hauschild et al. 2012). These selective mutant BRAF inhibitors actually cause melanoma cell death in vivo resulting in reduction of tumor sizes in some patients. Therefore, the selective mutant BRAF inhibitors may be useful for combination with immunotherapies through the following mechanisms:

1) Tumor destruction causes release of endogenous tumor antigens which include multiple patient's unique mutated antigens, leading to induction of multiple autologous tumor specific T cells,
2) Reduction of tumor burden via inhibition of cancer cell proliferation and cancer cell death results in reduction of immunosuppressive condition,

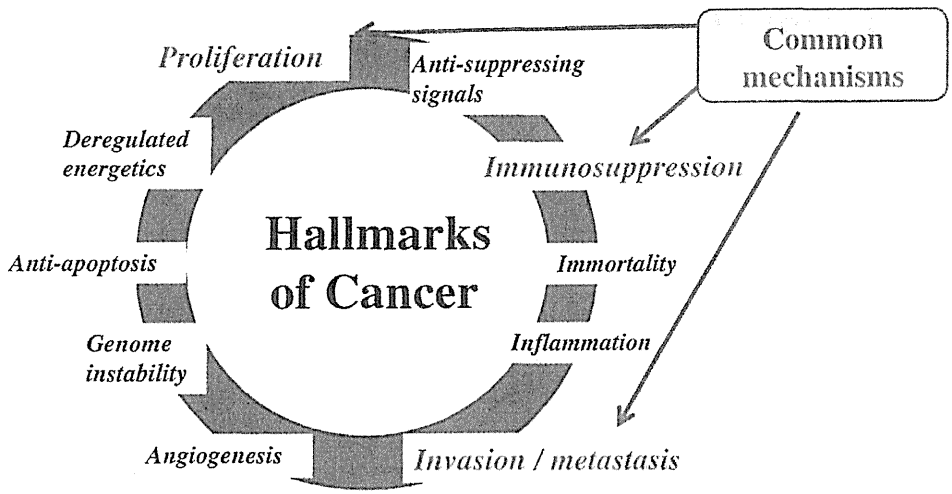

Fig. 12.2 Common mechanisms are sometimes used for hallmarks of cancer such as proliferation, metastasis and immunosuppression. Common signaling pathways such as MAPK signaling are sometimes utilized for some of the hallmarks of cancer including cancer cell proliferation, invasion, and immunosuppression. Therefore, inhibitors against the common pathways may be useful for cancer treatment through simultaneous inhibition of multiple hallmarks of cancer. Combination of molecular targeted drugs and immunotherapy may be an attractive strategy for cancer treatment

3) Decrease of production of multiple immunosuppressive cytokines results in simultaneous inhibition of multiple immunosuppressive cascades,
4) Susceptibility of cancer cells to cytotoxic T cells (CTLs) is increased partly via increased expression of tumor antigens,
5) Mutant BRAF selective inhibitors are less inhibitory for proliferation of anti-tumor T cells, and
6) Invasion and metastatic ability of cancer cells is decreased.

It has recently been reported that administration of mutant BRAF selective inhibitors did not suppress immune response in general (Hong et al. 2012), and actually increased infiltration of T cells, particularly granzyme positive $CD8^+$ T cells, in tumors, which was correlated with tumor reduction and necrosis (Wilmott et al. 2011). In vivo immunological effects of a MEK inhibitor which is also effective for patients with melanoma having mutation of either NRAS or BRAF remain to be investigated (Flaherty et al. 2012). The same strategy may also be applied for other cancers with BRAF mutations, including colon cancer and thyroid cancer.

12.4.2 JAK/STAT3 Signaling Pathway

In human melanoma, in addition to RAS/BRAF mutation, activation of STAT3 is frequently observed. Depletion of STAT3 by lentiviral shRNA in STAT3 active melanoma also resulted in the inhibition of multiple immunosuppressive

cytokines, including IL-6, IL-10, and VEGF (Sumimoto et al. 2006). Interestingly, these tumor-derived cytokines activate STAT3 in various immune cells including DCs, MDSCs, and Tregs, and affect their functions. The cytokine-induced STAT3 activation resulted in the generation of low IL-12 and high IL-10 producing human DCs with decreased T cell stimulatory activity.

In the mouse tumor model, STAT3-depleted DCs obtained from myeloid-specific STAT3-conditional knockout mice, were resistant to these tumor-derived immunosuppressive cytokines, and also had strong T cell stimulatory activity along with sustained high IL-12 production. Injection of the STAT3-depleted DCs into tumor, which is under the immunosuppressive condition, showed strong anti-tumor effects accompanied by induction of higher IFN-γ producing tumor antigen specific Th1 cells compared to the injection of control DCs (Iwata-Kajihara et al. 2011). Similarly, STAT3-depleted macrophages were resistant to tumor-derived immunosuppressive cytokines, and induction of immunosuppressive macrophages and MDSCs were partially inhibited by STAT3 depletion. STAT3 was also reported to be involved in expansion of MDSCs (Wu et al. 2011). STAT3 activation was actually observed in $CD14^{+}HLA\text{-}DR^{negative/low}$ MDSCs in blood of cancer patients (Poschke et al. 2010). STAT3 is also important for Treg cells (Pallandre et al. 2007). STAT3 inhibition of anti-tumor $CD8^{+}$T cells was reported to enhance their effects when adoptively transferred into tumor-bearing mice (Kujawski et al. 2010). These results indicate that constitutive activation of STAT3 in cancer cells triggers induction of various immunosuppressive immune cells, including tolerogenic DCs, MDSCs, and Tregs, partly through activation of STAT3 in these immune cells (Kortylewski et al. 2005; Yu et al. 2007). Therefore, STAT3 inhibitors may be useful for reversal of cancer-induced immunosuppression through not only acting on cancer cells, but also acting on various immune cells.

Recently, molecular targeted therapies acting on various signaling molecules in cancer cells have been used for cancer treatment. STAT3 inhibitors are being evaluated in clinical trials. In murine tumor model, various STAT3 inhibitors have been shown to augment anti-tumor immunity (Lee et al. 2011). In addition to STAT3 inhibitors, inhibitors to molecules present at upstream of STAT3, including inhibitors for direct upstream molecule JAK and further upstream molecules EGF-R/VEGF-R (which have already been available for clinical use), may also be useful for reversal of immunosuppression and combination with immunotherapy. JAK inhibitors have been shown to augment anti-tumor immunity and enhance anti-tumor effects in combination with immunotherapies, such as IL-12 administration (Burdelya et al. 2002). We have observed that EGF-R inhibitors suppress production of some of the immunosuppressive cytokines, such as IL-6 and VEGF, from human lung cancer cells with EGF-R mutations. In the murine tumor model, administration of the EGF-R inhibitors along with cancer vaccines showed synergistic anti-tumor effects through indirect (via decrease of immunosuppressive cytokines from cancer cells) and direct enhancement of DC ability to stimulate T cells. Administration of multikinase inhibitor Sunitinib, which also suppresses downstream STAT3 signaling, to RCC patients was

reported to result in decrease of MDSCs and Tregs along with increase of IFN-γ producing T cells (Xin et al. 2009; Ozao-Choy et al. 2009; Ko et al. 2009). Another multikinase inhibitor Dasatinib was reported to increase response rate in about half of patients with $Ph1^+$CML and ALL accompanied by LGL lymphocytosis and autoimmune like syndrome, such as pleuritis and colitis; it was reported to inhibit STAT3 signaling in immune cells after administration (Mustjoki et al. 2009; Jalkanen et al. 2010). Therefore, there are various ways of STAT3 signal inhibition for reversal of immunosuppression in cancer patients in clinic. We have recently screened natural compounds contained in the Japanese traditional Kampo medicines, and found that some of the compounds are able to inhibit STAT3 and MAPK pathways, possibly by targeting upstream signaling molecules. Their systemic administration augmented tumor specific T cells accompanied by decrease of Tregs in the tumor in tumor-bearing mice (manuscript in preparation).

12.4.3 NF-κB Signaling Pathway

We have also observed similar phenomenon—involvement of the same signaling pathway in both cancer cells and immune cells for generation of immunosuppressive condition, in human ovarian cancers with constitutively activated NF-κB, which causes high production of IL-6, IL-8, and CCL2. High levels of plasma IL-6 and IL-8 were found to correlate with poor prognosis of cancer patients and poor response to various immunotherapies, including vaccinations with cancer antigen peptides and DCs (manuscript in preparation). NF-κB inhibitor inhibited not only production of these immunosuppressive cytokines and chemokines by cancer cells, but also had direct effects on monocytes: they inhibit their differentiation to immunosuppressive macrophages in the presence of cancer cell-derived factors. Although the cross-talk, such as positive feedback loop between IL-6, STAT3 and NF-κB was previously reported to be involved in chronic inflammation (Yu and Pardoll 2009; Murakami and Hirano 2011), significant role of such cross-talk was not observed in these ovarian cancers. Systemic administration of appropriate dose of a NF-κB inhibitor augmented anti-tumor T cell responses possibly through reversal of immunosuppressive condition in a murine tumor model, although NF-κB signal is also essential for induction of anti-tumor T cells.

NF-κB was found to be involved in the intrinsic expression of ILT7 ligand (ILT7L) in some of human renal cell cancers (RCC), although ILT7L can also be up-regulated by IFN-γ from infiltrated T cells. ILT7L inhibits IFN-α production by plasmacytoid DCs and is possibly involved in immunosuppression in the tumor microenvironments, since type-I IFN was reported to be critical for induction of spontaneous anti-tumor T cell response (Fuertes et al. 2005; Gajewski et al. 2012). NF-κB inhibitor suppressed the intrinsic expression of ILT7L on RCC cells (Tsukamoto et al. 2009). It has recently been reported that expression of PD-L1 on

cancer cells was mainly induced by IFN-γ produced by tumor-infiltrating T cells, and the PD-L1 expression on cancer cells and $CD8^+$T cell infiltration correlated with significantly better response to anti-PD-1 antibody treatment (Taube et al. 2012). However, some cancer cells intrinsically express PD-L1 partly due to activation of AKT pathway via PTEN deletion (human glioma) (Parsa et al. 2007) or activated MAPK pathway in some other cancers. We have found new inhibitors, which suppresses intrinsic expression of PD-L1. These observations indicate that signal inhibitors may also be useful for inhibition of these immunosuppressive membrane molecules (e.g., ILT7L and PD-L1) intrinsically expressed through altered signaling in human cancer cells.

12.4.4 Wnt/β-Catenin Signal Pathway

Activation of β-catenin pathway (nuclear staining of β-catenin) was observed in about 30 % of human melanoma, and correlated with expression of IL-10 by immunohistochemical analysis. We found that β-catenin directly activated IL-10 transcription in human melanoma (Yaguchi et al. 2012). Supernatant from cultured β-catenin-accumulating melanoma cells induced high IL-10-, low IL-12-producing DCs with low T cell stimulatory activity in vitro, which was IL-10-dependent; these DCs also had the ability to induce FOXP3-positive immunosuppressive Treg cells. Pretreatment of melanoma cells with shRNA for β-catenin reduced their immunosuppressive activities. Interestingly, supernatant from cultured melanoma also inhibited the effector function of melanoma specific cytotoxic T cells in a β-catenin-dependent, but IL-10-independent manner, indicating that other immunosuppressive molecules are also involved in the β-catenin induced immunosuppression.

When β-catenin-activated human melanoma cell lines were implanted in immunodeficient SCID mice, the level of human IL-10 in blood was increased, and mouse DCs in the spleen and tumor were impaired for T cell stimulatory activity. This was likely because human IL10 can also act on mouse cells and suppress mouse DCs. Systemic administration of a β-catenin inhibitor restored mouse splenic DC activity to stimulate T cells along with decrease of human IL-10 in the serum. Interestingly, a β-catenin inhibitor also had the ability to directly enhance T cell stimulatory activity of human DCs partly due to decreased IL-10 production by DCs. β-catenin was also reported to be involved in generation of regulatory DCs (Fu and Jiang 2010; Manicassamy et al. 2010) and survival of Tregs (Ding et al. 2008). These results indicate again that signal inhibitors may be useful for reversal of cancer-induced immunosuppression by acting on both cancer and immune cells.

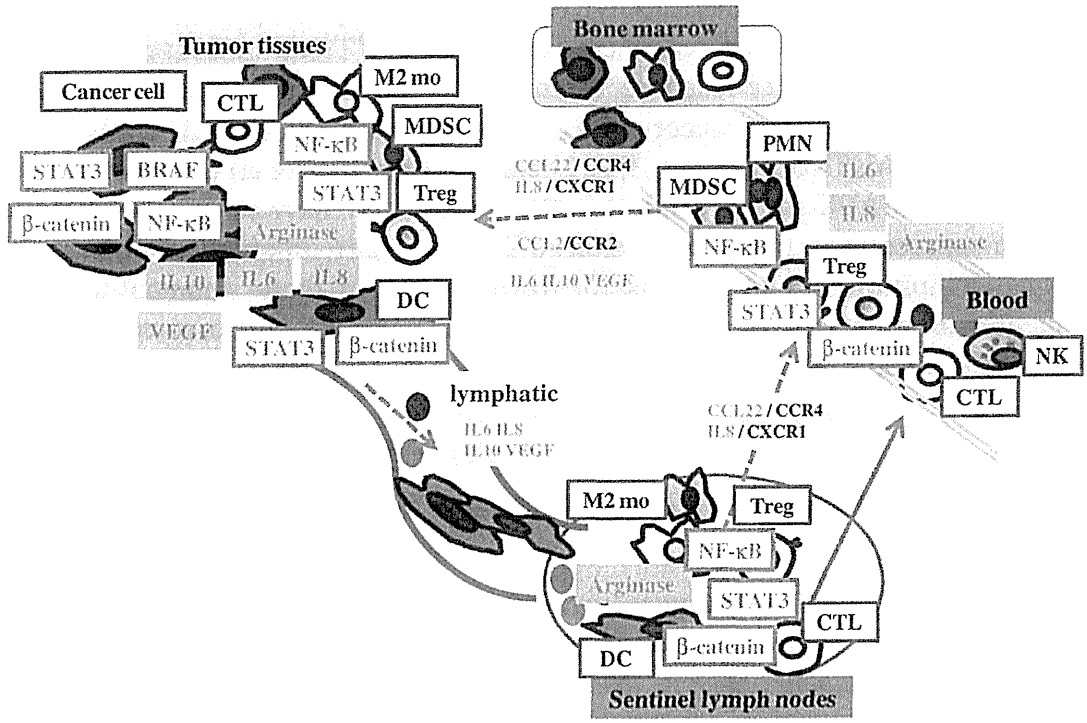

Fig. 12.3 Cancer cell triggered immunosuppressive cascades: MAPK, STAT3, β-catenin, and NF-κB cascades. Alterations of oncogenes and subsequently activated signaling are different among cancer cells even in the same types of cancer. For examples, alterations of MAPK, STAT3, β-catenin, and NF-κB trigger different immunosuppressive cascades via production of immunosuppressive cytokines such as IL-6, IL-8, IL-10, VEGF, etc., and subsequent impairment of DC function, and induction of various immunosuppressive cells such as MDSC and Treg cells

12.5 Clinical Implications of the Immunosuppressive Mechanisms

As described above, multiple immunosuppressive cascades are triggered by gene and signal alternations in human cancer cells and generate immunosuppressive condition particularly in the tumor-associated microenvironments, including the tumor tissues and sentinel lymph nodes (Fig. 12.3). One of the important questions is which molecules and cells, either at upstream or downstream, in the immunosuppressive cascades should be inhibited for the efficient reversal of immunosuppression in patients with cancer. It may depend, at least in part, on cancer types and their genetic alterations.

In general, targeting constitutive active signaling molecules in cancer cells has advantage of direct anti-tumor effects such as inhibition of cancer cell proliferation and direct destruction of malignant cells, which may lead to induction of immune responses to multiple endogenous tumor antigens, including patients' unique antigens (e.g., mutated antigens); this may also lead to simultaneous inhibition of downstream multiple immunosuppressive mechanisms. However, inhibition

of upstream molecules may also cause broad adverse effects, including suppression of anti-tumor immune response, although suppression of anti-tumor immune responses may be avoided by the use of appropriate doses of inhibitors. For instance, as was observed after administration of NF-κB inhibitor or mutated molecule selective inhibitors, such as mutant BRAF selective inhibitors. In contrast, targeting downstream molecules and cells, such as TGF-β, IL-10, PD-L1, IDO, Cox2 or MDSCs and Tregs, by using small molecule inhibitors or antibodies may have advantage of high specificity leading to more efficient blockade with less broad adverse effects. However, inhibition of one molecule or one cell type may not be sufficient to overall reversal of immunosuppression in patients with cancer. The combination of signal inhibitors and blockade of major immuosuppressive molecules or cells (e.g., neutralizing or blocking antibodies for TGF-β or PD-1) may also be attractive strategies for strengthening activity to reverse tumor-associated immunosuppression.

Besides inhibition/blocking of tumor-derived immunosuppressive factors, signal inhibition in immune cells may result in the direct activation of immune cells or inhibition of induction of immunosuppressive cells, including Tregs and MDSCs. Altogether, targeting activated signaling molecules involved in triggering of multiple immunosuppressive cascades may be an attractive strategy for reversal of immunosuppressive conditions in the tumor-associated microenvironments for cancer therapies, particularly immunotherapy (Fig. 12.4). Combination treatments utilizing these molecular targeted drugs and various immunotherapies, including cancer vaccines and check point blockade, are particularly appearing and will be evaluated in future clinical trials. One important point is that an appropriate target may be different among patients, since constitutively activated molecules and signaling pathways vary among patients even with the same type of tumor, indicating necessity of personalized strategy (Table 12.1). In the next 10 years, molecular and cellular basis of cancer-induced immunosuppression in the tumor-associated microenvironments will be further understood, and clinical efficacy of combined immunotherapy with molecular targeted drugs will be clinically evaluated.

In addition to the therapeutic implications of altered gene and signaling involved in cancer-induced immunosuppression, they may play a role in diagnostics. As described above, infiltration of memory $CD8^+$ T cells in tumor mass and serum IL-6/IL-8 levels appear to be prognostic markers and response prediction markers for cancer treatment including immunotherapy. Since the immune status may be a reflection of gene/signal alterations as described above, evaluation of the altered gene/signaling status (e.g., pERK, pSTAT, nuclear translocation of NF-κB, or β-catenin) may also serve as diagnostic biomarkers for cancer patients.

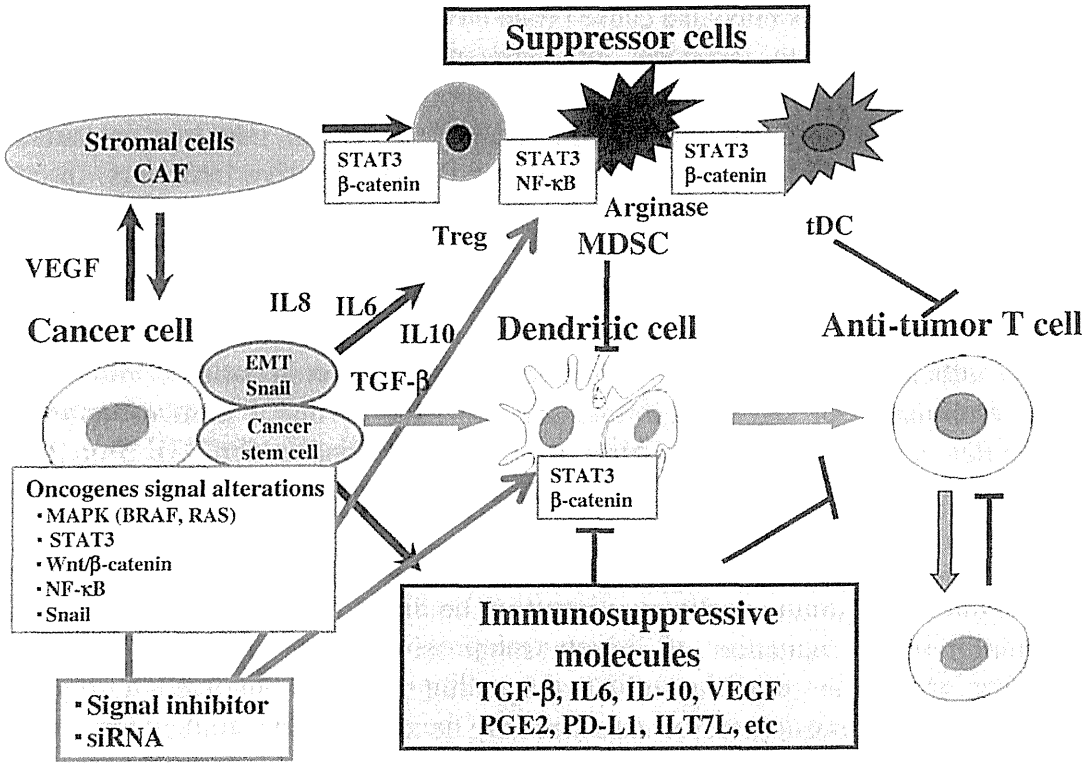

Fig. 12.4 Reversal of immunosuppressive conditions by targeting both cancer cells and immune cells using signal inhibitors. Cancer cell derived factors induce activation of signaling in various immune cells to become immunosuppressive cells. Signal inhibitors (inhibitors for STAT3, β-catenin, NF-κB, etc.) may be useful for reversal of cancer induced immunosuppression by acting on both cancer cells and various immune cells such as DC, MDSC, and Treg cells

Table 12.1 Appropriate targets for reversal of cancer-induced immunosuppression may be different among cancer patients

Active signaling molecules	Immunosuppressive molecules	Cancer type
NRAS/BRAF/MAPK	IL-10, VEGF, IL-6	Melanoma
BRAF/MAPK	IL-10	Colon cancer
KRAS/MAPK	IL-8, VEGF	Pancreatic cancer
MAPK	PD-L1	Ovarian cancer
MAPK	VEGF, IL-8	Renal cell cancer
EGFR/MAPK	IL-6, VEGF	Lung cancer
PI3K/AKT	VEGF, IL-8	Renal cell cancer
PTEN/AKT	PD-L1	Glioma
STAT3	IL-10, IL-6, VEGF	Melanoma
β-catenin	IL-10	Melanoma
β-catenin	IL-10	Colon cancer
NF-κB	IL-6, IL-8, CCL2	Ovarian cancer
NF-κB	IL-6, IL-8, ILT7L	Renal cell cancer
Kinase-X	TGF-β, IL-10, CCL2	Melanoma

12.6 Concluding Remarks

Understating of molecular mechanisms of the immunopathological features of the tumor-associated microenvironments is critical for further development of cancer diagnostics and therapy; not only immunotherapy but also other types of cancer treatments including chemotherapy. In particular, combination therapy utilizing molecular targeted drugs, which are currently used as single agents, and immunotherapy, such as cancer vaccine and check point blockers, is a promising strategy to be exploited in near future clinical trials.

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Cancer-induced immunosuppressive cascades and their reversal by molecular-targeted therapy

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Immunological status in tumor tissues varies among patients. Infiltration of memory-type $CD8^+$ T cells into tumors correlates with prognosis of patients with various cancers. However, the mechanism of the differential $CD8^+$ T cell infiltration has not been well investigated. In general, tumor-associated microenvironments, including tumor and sentinel lymph nodes, are under immunosuppressive conditions such that the immune system is not able to eliminate cancer cells without immune-activating interventions. Constitutive activation of various signaling pathways in human cancer cells triggers multiple immunosuppressive cascades that involve various cytokines, chemokines, and immunosuppressive cells. Signaling pathway inhibitors could inhibit these immunosuppressive cascades by acting on either cancer or immune cells, or both. In addition, common signaling mechanisms are often utilized for multiple hallmarks of cancer (e.g., cell proliferation/survival, invasion/metastasis, and immunosuppression). Therefore, targeting these common signaling pathways may be an attractive strategy for cancer therapy including immunotherapy.

Keywords: immunosuppression; BRAF; STAT3; β-catenin; NF-κB

Introduction

Human tumor antigens recognized by T cells have previously been identified in our studies and applied to various cancer immunotherapies.[1,2] One such melanoma antigen, gp100, was isolated by cDNA expression cloning using tumor infiltrating T cells (TILs).[3–6] In a recent multicenter, randomized clinical trial, gp100 peptide vaccination combined with interleukin 2 (IL-2) resulted in a 16% objective response with 9% complete response (CR).[7] In contrast, adoptive immunotherapy using cultured TILs following myelo-lymphoablative treatment, which depletes various immunosuppressive cells, resulted in more than 70% objective response with 20% durable CR in advanced melanoma patients with multiple metastases.[8] These studies indicate that immunosuppressive conditions, particularly in tumor-associated microenvironments such as tumor and sentinel lymph nodes (SLNs), are one of the major obstacles for the development of effective immunotherapy. Thus, understanding the mechanisms of cancer cell–induced immunosuppression in tumor-associated microenvironments and developing methods to reverse immunosuppression are important for immunotherapy.

Tumor-associated microenvironments

During cancer development, cancer cells, various immune cells, and other stromal cells, such as fibroblasts and mesenchymal stem cells, interact, and immunoediting via immunosurveillance and immunoescape defines immunological characteristics of cancer cells (e.g., loss of highly immunogenic tumor antigens, acquirement of resistance to immune cells, and ability to suppress immune response).[9] Thus, cancer cells seen in the clinic are generally

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immunosuppressive and generate immunosuppressive conditions in tumor-associated microenvironments. Immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory T (T_{reg}) cells, are increased, while dendritic cells (DCs) appear to be impaired in tumors and sentinel lymph nodes in cancer patients.[10] Interestingly, immune status varies among patients, as reflected by recent findings showing that the level of infiltration of memory $CD8^+$ T cells in tumors differs among cancer patients. More $CD8^+$ T cell infiltration correlated with favorable prognosis in various cancers, including colon and ovarian cancer, and also correlated with the response to immunotherapy or chemotherapy in patients with melanoma or colon cancer, respectively.[11,12] However, the mechanisms underlying differences in immune status among cancer patients remain to be investigated.

Gene and signaling alterations in cancer cells

Immune status in tumor microenvironments may be regulated by stimulating factors for antitumor immune response, including expression of immunogenic tumor antigens and human leukocyte antigen (HLA), spontaneous immune response cascades (such as the pathway from tumor DNA to IFN-producing DCs to $CD8^+$ T cell induction),[13] or by immunosuppressive cytokines such as transforming growth factor β (TGF-β) and IL-10. Since TGF-β is produced by most cancer cells and some infiltrating immune and stromal cells, we have evaluated the role of TGF-β in tumor microenvironments. In a mouse tumor model, increased TGF-β expression in tumor microenvironments via implantation of TGF-β cDNA–transfected tumor cells resulted in increased infiltration of immunosuppressive $CD11b^+$ $Gr\text{-}1^+$ MDSCs and $FoxP3^+$ $CD4^+$ T_{reg} cells in both tumors and SLNs. Infiltration of DCs was decreased in tumors; in SLNs the number of DCs was increased compared to non-SLNs in mice with either TGF-β–transduced or mock-transduced tumor cells, but T cell stimulatory activity of DCs was significantly impaired in mice with TGF-β^+ tumors. M2-like macrophages producing abundant CCL22, which recruits $CCR4^+$ T_{reg} and Th2 cells, were also increased in both tumors and SLNs.[14] Consequently, induction of tumor-specific T cells in SLNs was significantly reduced, which probably led to decreased infiltration of $CD8^+$ T cells in tumors. Therefore, the mouse tumor model recapitulates some of the immune conditions in tumors and SLNs in cancer patients (work in progress). An increase in immunosuppressive factors such as TGF-β may be one of the mechanisms defining the immune status of tumor microenvironments, such as spontaneous $CD8^+$ T cell responses.

We have also found that the TGF-β-induced transcription factor, Snail, which is known to promote metastasis via epithelial-to-mesenchymal transition (EMT) in cancer cells, also enhances production of multiple immunosuppressive cytokines and chemokines, including TGF-β, IL-10, CCL2, and TSP-1, which cause DC impairment and T_{reg} cell induction. The impaired DCs have less T cell stimulatory activity and induce T_{reg} cells. CCL2 not only impairs DC function but also recruits MDSCs into tumors. Intratumoral administration of Snail-specific siRNA restored immunocompetence of mice implanted with Snail-expressing tumor, and resulted in induction of tumor antigen–specific T cells.[15]

In a more recent work in progress, we have identified an upstream signaling molecule of TGF-β production in human cancer cells by screening immunosuppressive activity in DCs using a kinase siRNA library (unpublished data). The identified kinase is significantly phosphorylated in human cancer cells, and its depletion suppressed TGF-β production by cancer cells (manuscript in preparation). These results indicate that TGF-β, produced by either cancer cells or infiltrated stromal cells in tumor microenvironments, triggers immunosuppressive cascades involving various immunosuppressive cytokines, chemokines, and cells, and reemphasizes that TGF-β cascade is an attractive target for reversal of cancer-induced immunosuppression (Fig. 1).

Multiple immunosuppressive cascades in human cancers

Gene and signal alterations in human cancer cells vary among cancer types, and even within the same type of cancer. Therefore, we have evaluated immunosuppressive mechanisms of various human cancers (Fig. 1).

MAPK signal pathway

We have found that activation of the mitogen-activated protein kinase (MAPK) signal pathway via common BRAF mutation (V600E) not only