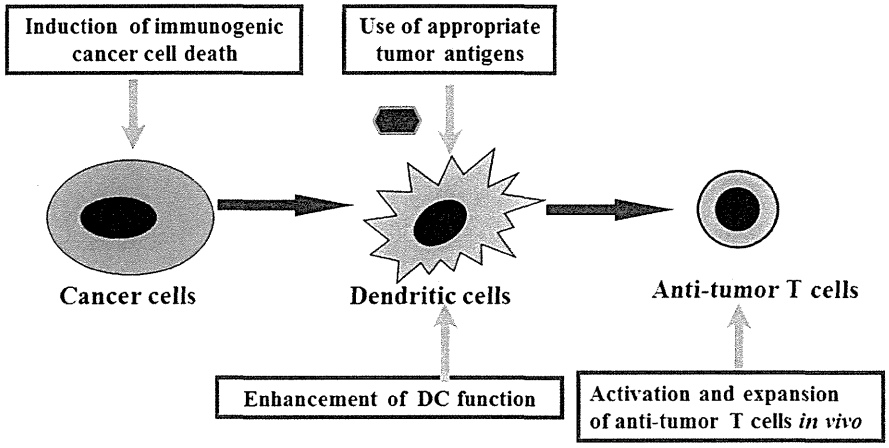

FIGURE 1 | Development of effective immunotherapy by comprehensive regulation of anti-tumor immune network. Comprehensive regulation of anti-tumor immune network, including induction of immunogenic cancer cell death, use of appropriate tumor antigens, enhancement of DC function, activation and expansion of anti-tumor T cells *as well as* reversal of cancer-induced immunosuppression (**Figure 2**) is important for development of effective cancer immunotherapy.

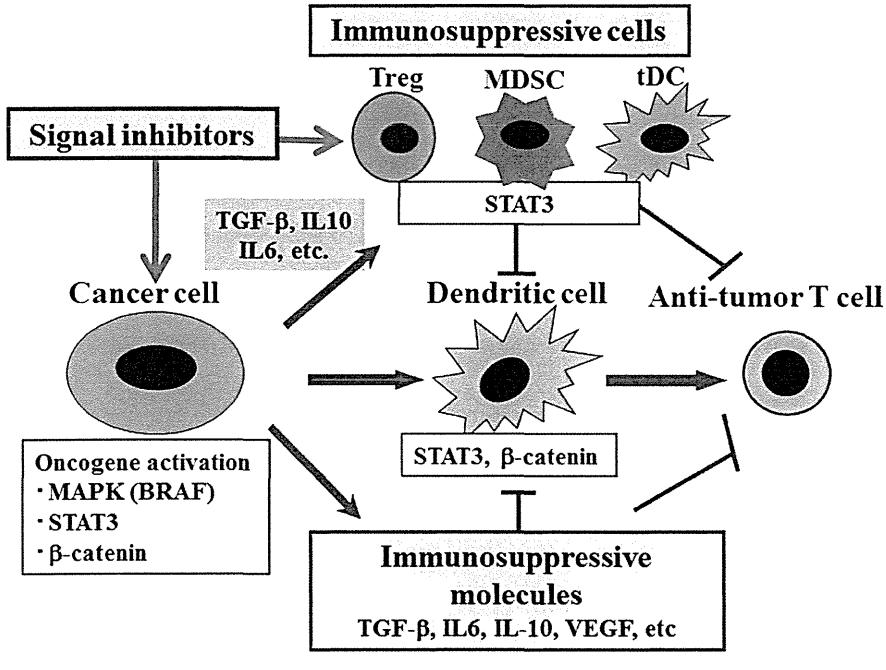

FIGURE 2 | Reversal of cancer-induced immunosuppression by targeting both cancer cells and immune cells using molecular targeted drugs. Cancer cells not only trigger anti-tumor immune responses but also induce various immunosuppressive molecules and cells through oncogene and signaling activation, leading to impaired anti-tumor immune responses. Molecular targeted drugs including various signal inhibitors may be useful for augmentation of anti-tumor immune responses by acting on both cancer cells and various immune cells such as DC, MDSC, and Treg.

signaling molecules in human melanoma cells (Davies et al., 2002). We have evaluated the role of mutant BRAF (V600E) in human melanoma cells by using mutant BRAF (V600E)-specific lentiviral shRNAs, and found that BRAF mutation was involved in enhanced cell proliferation and invasion (Sumimoto et al., 2004, 2005). We also found that inhibition of MAPK signaling pathway in human melanoma cells by genetic depletion of mutant BRAF or specific inhibitors reduced production of multiple immunosuppressive cytokines such as IL-6, IL-10, and VEGF, in most cases without affecting cell viability (Sumimoto et al., 2006). These cytokines

suppress DCs' ability to stimulate T cells through decreased production of IL-12 and TNF-α and increased production of IL-10 by DCs. Treatment of melanoma cells with BRAF (V600E)-specific shRNA or MEK inhibitors resulted in decreased immunosuppressive activity of melanoma cells on DCs, suggesting that MAPK signaling pathway in cancer is associated with impaired DC function in melanoma patients. MEK inhibitors were reported to increase susceptibility of melanoma cells to CTL lysis partly due to increased expression of melanosomal antigens such as MART-1/melan-A and gp100 (Kono et al., 2006; Boni et al., 2010). These results indicate that the BRAF-MAPK axis is important not only in classical malignant features such as cancer cell proliferation and invasion, but also in immunosuppression and immunoresistance. "Avoiding immune destruction" has recently been recognized as one of the "the hallmarks of cancer" (Hanahan and Weinberg, 2011).

The BRAF-MAPK axis may be a common attractive target for melanoma treatment, including immunotherapy. However, MAPK signaling pathway is also important for normal cell functions, such as T cell proliferation. Thus, administration of MAPK inhibitors may also suppress desirable anti-tumor T cell responses. Recently, two BRAF inhibitors that preferentially inhibit mutant BRAF in cancer cells have been developed, and their administration resulted in regression of melanoma in clinical trials (Chapman et al., 2011). These mutant BRAF-selective inhibitors can be particularly useful in combination with immunotherapies for melanoma. Melanoma cell death induced by BRAF inhibitors may lead to release of multiple endogenous tumor antigens including mutated antigens unique to each patient (Melanoma is known to have more frequent mutations than other cancers probably due to UV irradiation). This results in subsequent induction of autologous tumor-specific T cells. Decreased production of multiple immunosuppressive cytokines along with decreased number of melanoma cells may result in simultaneous inhibition of multiple immunosuppressive cascades, and reduce total immunosuppressive activity of melanoma without suppressing anti-tumor T cell expansion. Increased expression of melanoma antigens leads to enhanced susceptibility of cancer cells to CTL lysis (Kono et al., 2006; Boni et al., 2010). Suppression of melanoma cell proliferation and invasion may also enhance total anti-tumor activity of mutant BRAF inhibitors. In fact, it has recently been reported that administration of the mutant BRAF inhibitors alone resulted in the increased infiltration of granzyme positive $CD8^+$ T cells in tumors without inhibiting general immune responses, which was correlated with tumor reduction and necrosis (Wilmott et al., 2011; Hong et al., 2012). In a recent study, mutant BRAF-selective inhibitor and anti-CTLA-4 mAb were used in combination to treat transgenic mice with mutant BRAF and PTEN deletion that spontaneously developed melanoma. Despite their expectation, the combined therapy did not show enhanced anti-tumor effects compared with the treatment with either inhibitor or antibody alone. However, in B16 melanoma model using non-transgenic mice, the anti-CTLA-4 mAb augmented the effects of cancer vaccine (Hooijkaas et al., 2012). Further analysis revealed that BRAF inhibitor did not cause cell death in melanoma of transgenic mouse model, suggesting that *in situ* destruction of cancer cells is an essential step in the enhancement of anti-tumor T cell responses. The mutant BRAF inhibitors may also be useful for treating other cancers that are BRAF mutation positive, such as colon cancer, lung cancer, and thyroid cancer. Although MEK inhibitor is known to suppress proliferation of melanoma with either NRAS or BRAF mutation, it remains to be evaluated whether the inhibitor also has immunological effects, such as stimulating or suppressing activity on anti-tumor T cells (Flaherty et al., 2012).

JAK/STAT3 SIGNALING INHIBITORS

STAT3 is frequently activated in various human cancers including melanoma. Similar to the RAS/BRAF/MAPK signaling activation, down-regulation of STAT3 by lentiviral shRNA in STAT3-activated melanoma resulted in inhibition of multiple immunosuppressive cytokines, including IL-6, IL-10, and VEGF, indicating that STAT3 inhibitors may also be useful for immunotherapy (Sumimoto et al., 2006). These suppressive cytokines subsequently activate STAT3 in various immune cells including DCs, MDSCs, and Tregs, and render them immunosuppressive. For example, these cytokines generated low IL-12- and high IL-10-producing human DCs with reduced T cell stimulatory activity. DCs obtained from myeloid-specific STAT3-conditional knockout mice were found to be affected less by cancer-derived immunosuppressive factors (Iwata Kajihara et al., 2011). In addition, these STAT3-depleted DCs produced high and sustained level of IL-12 possibly due to the involvement of STAT3 in a negative feedback mechanism of DC activation via IL-10. These STAT3-depleted DCs have higher T cell stimulatory activity than wild type DCs. When STAT3-depleted DCs were injected into immunosuppressive tumor microenvironment, stronger anti-tumor effects than wild type DCs were observed along with induction of stronger IFN-γ producing Th1 and CTL (Iwata Kajihara et al., 2011). It has been reported that STAT3 is also involved in expansion of MDSCs (Wu et al., 2011), activation of $CD14^+HLA\text{-}DR^{negative/low}$ MDSCs in blood of cancer patients (Poschke et al., 2010), expression of immunosuppressive arginase-1 in human MDSCs (Vasquez Dunddel et al., 2013), survival of Tregs (Pallandre et al., 2007), and anti-tumor activity of $CD8^+$ T cells (Kujawski et al., 2010). These reports suggest that constitutive activation of STAT3 in cancer cells triggers induction of various immunosuppressive immune cells. STAT3 inhibitors are currently being evaluated in clinical trials such as NCT00955812. In murine tumor model, STAT3 inhibitors have been shown to augment anti-tumor immunity (Kortylewski et al., 2005; Yu et al., 2007; Lee et al., 2011). It was recently reported that STAT3 inhibitors also restored drug sensitivity of melanoma cells which had acquired resistance to BRAF inhibitors (Liu et al., 2013). Therefore, STAT3 inhibitors may be useful for reversal of cancer-induced immunosuppression through acting on both cancer cells and various immune cells.

Besides direct inhibition of STAT3, inhibitors of the molecules regulating STAT3 activation may also be effective for the reversal of cancer-induced immunosuppression. An inhibitor of JAKs, upstream molecules of STAT3, was reported to augment anti-tumor effects in combination with immunotherapies such as IL-12 administration (Burdelya et al., 2002). In patients with renal cell cancer (RCC), administration of a multikinase inhibitor Sunitinib capable of suppressing downstream STAT3 signaling resulted in decrease of MDSCs and Tregs along with increase of IFN-γ

producing T cells (Ko et al., 2009; Ozao Choy et al., 2009; Xin et al., 2009). Another multikinase inhibitor Dasatinib, which also inhibit downstream STAT3, increased response rate of the patients with $Ph1^+$ leukemia (CML and ALL) accompanied by LGL lymphocytosis and autoimmune like syndrome such as pleuritis and colitis (Mustjoki et al., 2009; Jalkanen et al., 2010), suggesting that Dasatinib has immunostimulatory activity partly through STAT3 inhibition. Therefore, various ways of STAT3 signal inhibition may be applicable in combination with various immunotherapies.

β-CATENIN-SIGNALING INHIBITORS

In some human cancers including colon cancer, liver cancer, and melanoma, activation of β-catenin pathway (suggested by nuclear staining of β-catenin) is observed. We found that β-catenin directly promote transcription of immunosuppressive cytokine IL-10 in human melanoma (Yaguchi et al., 2012), and protein expression of β-catenin was correlated with expression of IL-10 when evaluated by immunohistochemical analysis of melanoma tissues samples. Culture supernatant of human melanoma cells with accumulated β-catenin-induced high IL-10- and low IL-12-producing DCs in an IL-10 dependent manner. These DCs possessed low T cell stimulatory activity *in vitro*, and induced $FOXP3^+$ immunosuppressive Treg cells. The melanoma derived factors also inhibited the effector function of melanoma-specific CTLs in a β-catenin-dependent, but interestingly IL-10-independent manner, indicating that other immunosuppressive molecules are also involved in the β-catenin-induced immunosuppression. Melanoma cells pretreated with β-catenin-specific shRNA had reduced immunosuppressive activities on both DC and T cells.

When β-catenin-activated human melanoma cell lines were implanted in immunodeficient mice, human IL-10 in mouse serum was increased, and function of mouse DCs in spleens and tumors were impaired for T cell stimulatory activity probably due to increased human IL-10 which is capable of affecting mouse DCs (Yaguchi et al., 2012). Systemic administration of a β-catenin inhibitor restored T cell stimulatory function of the mouse splenic DCs along with decrease of human IL-10 in serum. β-catenin was also reported to be involved in generation of regulatory DC (Fu and Jiang, 2010; Manicassamy et al., 2010a) and survival of Treg (Ding et al., 2008). In addition, β-catenin inhibitor had a direct ability on DC to augment their T cell stimulatory activity partly due to decreased IL-10 production by DC (Manicassamy et al., 2010b). Therefore, β-catenin inhibitors may also be useful for reversal of cancer-induced immunosuppression by acting on both cancer and immune cells.

CONCLUDING REMARKS

As discussed in this article, altered activation of various oncogenes and signaling in both cancer cells and immune cells can be an attractive target to reverse immunosuppressive conditions in tumor-associated microenvironments of cancer patients. Signal inhibitors may augment current cancer immunotherapy, in addition to its possible direct anti-tumor effects through inhibition of cancer cell proliferation and invasion. However, its total *in vivo* activity should be carefully evaluated because it may also cause various adverse effects, including possible inhibition of anti-tumor immune responses. In this regard, mutated-molecule-specific inhibition such as that of the mutant BRAF-selective inhibitors is one of the promising strategies. Activation of STAT3 appears to shift immune response toward cancer's advantage, thus, its inhibition is attractive for possible improvement of anti-tumor immune responses. Altogether, combination therapy using molecular targeted drugs and various immunotherapies such as cancer vaccines and check point blockade is a promising strategy to treat cancer patients. Future clinical trials may demonstrate the proof of concept of this strategy.

However, there are several obstacles to overcome before the benefits of combination therapy can reach the patients. One such obstacle is scientific. Although quite a few signal inhibitors, immunotherapies, and combined therapies have shown promising results in experimental settings, mouse model, and human are different. A successful treatment in mouse models may not work in patients. Therefore, for the selection of appropriate molecular targets and inhibition methods, further understanding of human cancer immunopathology is deeply essential and urgently desired. Another obstacle is a pragmatic one, which may arise when individual therapies in a combination therapy are developed and/or owned by different companies. The issues of company regulations, patents, and logistics could become a barrier between research and clinical translation. The core idea of combination therapy is that by using multiple already-available therapies, cancer patients are able to gain greater-than-sum benefits. Therefore, it is crucial that institutions and companies to look beyond self-interests and work together to reach a common goal. Academic institution may mediate the cooperation between companies and provided combination therapies to patients.

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