Review

Cancer Research

Combining Epigenetic and Immunotherapy to Combat Cancer

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Abstract

The most exciting recent advance for achieving durable management of advanced human cancers is immunotherapy, especially the concept of immune checkpoint blockade. However, with the exception of melanoma, most patients do not respond to immunotherapy alone. A growing body of work has shown that epigenetic drugs, specifically DNA methyltransferase inhibitors, can upregulate immune signaling in epithelial cancer cells through demethylation of endogenous retroviruses and cancer testis antigens. These demethylating agents may induce T-cell attraction and enhance immune checkpoint inhibitor efficacy in mouse models. Current clinical trials are testing this combination therapy as a potent new cancer management strategy. *Cancer Res; 76(7); 1683–9. ©2016 AACR.*

Introduction

Arguably, the most exciting recent advance for achieving durable management of advanced human cancers is immunotherapy, especially the concept of immune checkpoint blockade (1–7). This immunotherapy explosion is a result of elegant fundamental discoveries of ligand receptor interactions that control the immune activity of T cells against tumor cells (8–12). These basic advances and resulting translational applications constitute a key component of a paradigm that has been termed tumor "immune evasion" (13, 14). Interactions between the series of defined ligands and receptors on tumor cells and host immune cells render the latter immunologically inert or "tolerant". This recognition and molecular dissection of the tolerant state completely resurrected the concept of targeting cancer immunologically and provided the tools to modulate immune signaling from both tumor and host immune cells, reversing a key element of immune evasion and promoting tumor elimination (14).

To this end, a growing body of clinical trials has shown exceptional promise. Antibodies blocking CTLA-4, an inhibitory molecule on T cells, produce durable responses for treatment of melanoma (6, 7) and are currently in clinical trials for lung, prostate, and other cancers (15–17). Antibodies targeting human PD-1 (receptor on T cells) and PD-L1, the inhibitory ligand for PD-1 that is expressed at varying levels by cancer cells, have produced exceptionally durable responses in patients with highly aggressive, treatment-resistant metastatic cancers. The effects may be most apparent in patients whose tumors express PD-L1 (1–5, 18). While melanoma has been the most responsive solid tumor (5), exciting results have been achieved in the most lethal of cancers, advanced non–small cell lung cancer (NSCLC; refs. 1, 2, 5). This is of special interest as this cancer was previously considered not to be immune responsive. FDA approval for melanoma and NSCLC has resulted from the above trials (5).

While these advances are very exciting, with the exception of melanoma, the majority of patients do not respond to immune checkpoint therapy alone (5, 13). This raises the obvious question as to whether the combining of immunotherapy with other agents could robustly extend clinical response and efficacy in a larger spectrum of cancer subtypes. Indeed, such concepts are evolving. First, combining immune checkpoint targeting agents in trials giving both anti-PD1 and anti-CTLA4 to patients, while mandating specialized care of toxicities, shows great promise for melanoma (7). Second, combination strategies with standard chemotherapy and targeted therapy approaches can be considered. In this regard, we consider the exciting possibility, gleaned from a signal seen by our group in the clinic and a growing body of preclinical data, that epigenetic therapy could robustly sensitize patients to immune checkpoint therapy.

Definition of Epigenetic Therapy

Although the term epigenetic therapy is now widely used, what defines and constitutes this term is a shifting concept. There has been an explosion over the last decade in our understanding of what constitutes the normal and cancer "epigenome" and how it is regulated (19–21). New insights are constantly emerging into functionally significant histone modifications, importance of DNA methylation patterns, and understanding of nucleosome occupancy dynamics (21). Epigenetic discoveries continually define not only promising new targets for cancer therapy but also ways to "reuse" older drugs already in use in the clinic (22). The above regulatory features, as they participate in abnormal epigenetic alterations in cancer, represent potentially reversible targets for existing drugs and an increasing repertoire of new drugs.

We concentrate in this review on the use of drugs already in the clinic that can induce epigenetic effects modulating immune parameters of tumor or host immune cells. These drugs are emblematic of the principal that epigenetic therapy generally targets three protein categories: Writers, enzymes that establish epigenetic marks; Readers, proteins that recognize histone

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modifications or DNA methylation, are recruited to these marks, and may bring in other protein complexes to change gene expression; and Erasers, enzymes that remove epigenetic marks (23). We will focus on drugs that inhibit writers of DNA methylation, DNA methyltransferases (DNMT), and erasers (histone deacetylases or HDAC) that regulate histone lysine acetylation. The actions of DNMTs and HDACs are generally associated with transcriptional repression. Thus, the drugs targeting these proteins can augment expression of involved genes with many consequences for pathways downstream of this gene activation.

DNMT inhibitors (DNMTi) are cytidine analogues that, when incorporated into DNA, not only directly block the catalytic actions of DNMTs to trigger DNA demethylation but also cause their degradation (24). This latter loss of the protein, often not taken into account when considering use of DNMTis, can remove key scaffolding properties that may function for repression of transcription (25–27). Cancers almost universally exhibit profound changes in DNA methylation of cytosines at CpG dinucleotides. These changes include global loss of methylation at regions such as repetitive elements that must be silenced for genome stability and gain of methylation at the promoter regions of tumor suppressor and other genes (19). DNMTis cause expression of genes that are silenced by promoter DNA methylation, reactivating tumor suppressor genes (28). Transient exposure of multiple types of tumor cells to low doses of DNMTis promotes induction of apoptosis, reduced cell cycle activity, and decreased stem cell functions in cancer cells (29). Clinical efficacy of DNMTis, such as 5-azacytidine and 5-aza-2′-deoxycytidine (29, 30) for treating hematologic neoplasms has led to FDA approval for the preleukemic disorder myelodysplastic syndrome (MDS; ref. 31).

HDAC inhibitors (HDACi) are approved for the treatment of cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL; refs. 32, 33). It is, as yet, not clear why these tumors are so sensitive to HDACis (23). HDACis have pleiotropic effects, often very dose and compound dependent. Some of these affect histone acetylation and clearly induce epigenetic effects while others influence the acetylation status of nonhistone and/or nonnuclear proteins, or cause off-target effects including DNA damage (23, 34). Administered to tumor cells after low doses of DNMTis, HDACis can augment the reexpression of genes with promoter DNA hypermethylation (35). This combination is in clinical trials but it remains to be firmly established that it has clinical efficacy above the use of DNMTis and/or HDACis alone.

The Intersection of Epigenetic Therapy with Immunotherapy

Over the past several years, within the context of a Stand up to Cancer (SU2C) project to implement epigenetic therapy for cancer, our group has brought a low dose concept for use of DNMTis (5-azacytidine or 5-aza-2′-deoxycytidine) with or without HDACis to clinical trials for multiple tumor types. Signals for potential efficacy have particularly appeared for advanced, multiply pretreated non–small cell lung cancer (NSCLC; refs. 36, 37). One result in particular has driven much further emphasis in the clinic and the laboratory. A small number of patients with advanced NSCLC who progressed after receiving low-dose epigenetic therapy entered a trial for immune checkpoint therapy. Approximately 20% of the patients responded to the immune checkpoint therapy alone, passing 24 weeks without progression, with most achieving high-grade RECIST criteria responses (1, 38). This is an astounding result for immunotherapy in NSCLC. All 5 patients who had received the prior epigenetic therapy passed the 24-week point without progression with subsequent immune checkpoint therapy and three of these developed high-grade partial RECIST criteria responses that have all been durable over 2.5 years (36, 37). These findings have prompted initiation of a larger clinical trial, which is now ongoing. Moreover, our laboratory group pursued studies to determine the mechanism(s) that might account for epigenetic sensitization to immunotherapy. Our findings to date, and those of others, support the hypothesis that there may be extraordinary potential for combined epigenetic and immunotherapy to increase the frequency of durable responses for immune checkpoint therapy in not only NSCLC but also other common tumor types.

Epigenetic Therapy Drugs Boost Immune Attraction Properties of Epithelial Cancer Cells

DNMTis and HDACis have long been known to upregulate expression of individual components of immune signaling in epithelial cancer cells (39). Perhaps best recognized is induced expression of cancer testis antigens (CTA), including those on the X chromosome (CG-X antigens) and on autosomes (non-X CG antigens). CTAs are expressed in early embryonic and germ cells, but generally silenced in mature somatic cells by promoter CpG island DNA methylation (40). CTAs often remain DNA methylated and silenced in cancer cells although they can also lose methylation and be abnormally expressed (41). The promoter methylation of CTAs is controlled by interactions between DNMT1 and *de novo* DNMTs, principally DNMT3B. Inhibition of DNMTs can cause demethylation and reexpression of CTAs including the MAGE-A1 and NY-ESO-1 antigens (40, 42) in cancer cells but not normal fibroblasts (43). Hypomethylation of CTAs correlates with global hypomethylation in epithelial ovarian cancer (EOC), as well as BORIS upregulation (44). BORIS, a paralog of the CTCF insulator protein, is itself a CTA and is postulated to regulate other CTAs (41).

As CTAs can be recognized by the host immune system, they represent good candidates for immunotherapy, including vaccines. There is thus great potential for DNMT inhibitor treatment to upregulate CTAs on tumors, facilitating targeting by the host immune system (41). Guo and colleagues showed that the DNMTi 5-aza-2-deoxycytidine could demethylate and upregulate the murine CTA P1A in 4T.1 mammary carcinoma cells in syngeneic mice. P1A was presented and recognized by H-2L d)-restricted P1A–specific T cells, and combined therapy with 5-aza-2′-deoxycytidine and adoptive transfer of these T cells significantly reduced lung metastases in this mouse model (45). The novel DNMT inhibitor SGI110, which has longer *in vivo* stability than 5-azacytidine or 5-aza-2′-deoxycytidine and has shown clinical activity in patients with MDS and AML (46), also upregulates CTAs. In AML xenografts, SGI110 upregulates NY-ESO-1 and MAGE-A and induces cytotoxicity by $CD8^+$ T cells specific for NY-ESO-1 (47). Similar results were observed in epithelial ovarian cancer (EOC) xenografts (48). These promising results led to a phase I clinical trial in EOC in which Odunsi and colleagues added 5-aza-2′-deoxycytidine to NY-ESO-1 vaccine combined with doxorubicin chemotherapy in patients with relapsed EOC. They observed DNA hypomethylation at the NY-ESO-1 promoter.

NY-ESO-1 was upregulated and increased serum antibodies to NY-ESO-1 were detected, most importantly in two-thirds of the patients who previously were seronegative for NY-ESO-1 antibodies. They observed specific T-cell responses against NY-ESO-1 and stable disease or partial clinical response in 6 of 10 patients (49).

Our own data (37, 50) validate the upregulation of CTAs by DNMT inhibitors. CTAs were significantly upregulated by 5-azacytidine in the majority of 77 epithelial cancer cell lines. CTAs were most upregulated in colorectal (64% of cell lines) and ovarian (39%) cancer lines and less so for breast cancers (19%). We also noted an upregulation of genes involved in antigen processing and presentation by 5-azacytidine treatment or in DNMT1$^{-/-}$ DNMT3B$^{-/-}$ DKO cells (51) compared with the parental HCT116 cell line (37, 50). These include the MHC class I proteins (B2M, HLA-A, HLA-B, HLA-C) that present antigens on the surface of epithelial cells for host immune cell recognition, as well as proteins involved in processing of antigens by the proteasome (PSMB8, PSMB9, TAP1; ref. 50). This upregulation was previously noted by Karpf and colleagues after DNMTi treatment (42). Unlike CTAs, these genes are not initially methylated at their promoter regions, so a separate mechanism(s), likely downstream of epigenetic changes, is responsible for their upregulation.

The IFN response, upstream of antigen processing and presentation genes, is activated by DNMT inhibitors. This was first described by Karpf and colleagues (52); they observed an induction of STAT signaling and type I IFN genes in colon cancer cells treated with 5-aza-2′-deoxycytidine and showed that 5-aza-2′-deoxycytidine could sensitize cells to treatment with IFNα. This activation was confirmed in a later study (42). Matei and colleagues observed upregulation of cytokines as well as JAK/STAT and IFN signaling pathways in tumor biopsies from ovarian cancer patients treated with a combination of 5-aza-2′-deoxycytidine and carboplatin (30). High doses of DNMTi (10 μmol/L- 5-aza-2′-deoxycytidine) induced an IFN response, apoptosis, and increased endogenous retroviral (ERV) transcripts and repetitive satellite RNAs in p53-null mouse fibroblasts (53). Leonova and colleagues attributed these latter responses to concordant regulation of satellite repeats by P53 and DNMTs and a buildup of repetitive RNAs that triggered the IFN response.

Against this above background, our group has observed a robust concordance for 5-azacytidine and 5-aza-2′-deoxycytidine induced increases in virtually all of the above immune parameters. We observed increased IFN signaling and concordant upregulation of surface antigens and their assembly proteins in 77 epithelial cancer cell lines treated with 5-azacytidine (37, 50). We defined a 300 gene expression signature that we termed 5-azacytidine–induced immune genes or AIM (50). In general, AIM genes were not induced by HDACi (TSA) treatment alone, but 5-azacytidine plus TSA caused higher expression than 5-azacytidine alone (50). We noted the highest AIM activation in EOC and NSCLC (50). Expression of AIM separated primary tumor samples from The Cancer Genome Atlas (EOC, NSCLC, and other cancers) into high and low expression groups (50). We hypothesize that the "low AIM" tumors represent an "immune evasion/immune editing" pattern (54, 55) that 5-azacytidine could reverse to sensitize patients to subsequent immunotherapy (50).

Recent work from our group and by de Carvalho and colleagues (56, 57) shows that one key way in which DNMTis upregulate immune signaling in cancer is through the viral defense pathway. In ovarian cancer cell lines, DNMTis activate a canonical IFN signaling pathway, inducing IFNβ and JAK/STAT signaling, through upregulation of dsRNA that activates the cytosolic dsRNA sensors TLR3 and MDA5. One type of RNA triggering this response is transcribed from hypermethylated endogenous retroviruses (ERVs) (56). Roulois and colleagues showed similar involvement of dsRNA and the MDA5 sensor in colon cancer cells and demonstrated that this IFN response was essential to the inhibition of colon cancer stem cells by DNMTis (57). Blocking the IFN response rescued about half of the DNMTi-induced apoptosis in ovarian cancer cells (56).

The ERVs that trigger the above DNMTi-induced immune response represent a significant fraction of repetitive elements in the human genome that are silenced in somatic cells by DNA methylation. In fact, up to 90% of methylated CpGs are located in the 45% of the human genome represented by repetitive sequences (58). ERVs are generally silenced in normal cells to promote genome stability, but are demethylated and reexpressed in some tumors. ERV demethylation and reexpression by DNMTis has been shown in human embryonic stem cells to cause upregulation of IFITM1, a protein involved in viral defense signaling (59). In melanoma, the ERV-K (HML-2) 5′LTR shows CpG hypomethylation and increased transcriptional activity (60). ERVs can be targeted as tumor-associated antigens on melanoma cells (61). Thus, ERV activation promotes viral signaling and presents possible tumor-specific antigens to target.

While hypomethylation and upregulation of methylated regions encoding double-stranded RNA is a major contributor to the DNMTi-induced immune response, other target sites of demethylation can be important. *IRF7*, which encodes a master transcription factor activating the IFN response, is frequently silenced in association with promoter CpG island DNA hypermethylation in lung and other cancers (37, 50, 56, 57, 62). This loss of function can diminish interferon responses in tumor cells. Indeed, when this gene is methylated, its expression can be upregulated by 5-azacytidine in squamous NSCLC (37) and EOC cells (50). Our group (56) and De Carvalho and colleagues (57) found that when *IRF7* is hypermethylated, knockdown of this gene significantly reduces the DNMTi-induced IFN response in ovarian (56) and colon (57) cancer cells, respectively.

How might epigenetic therapy then be combined with immune therapy to combat advanced cancers? As introduced earlier, we hypothesize that activation of the above viral defense gene signature by drugs like 5-azacytidine might reverse elements of tumor immune evasion and enhance immune checkpoint therapy. In our recent study (56), basal expression levels of the Aza-induced viral defense gene signature in tumor samples correlate with long-term benefit in patients with advanced melanoma treated with the immune checkpoint inhibitor anti-CTLA-4 (63). Importantly, for virtually all of these melanoma patients, treatment benefit, high tumor mutational burden, and basal viral defense signature were all significantly associated (56). Moreover, low dose 5-azacytidine plus anti-CTLA4 were significantly more effective at controlling tumor growth compared with each agent alone in the B16 mouse model of melanoma (56). These results point to the importance of immune/IFN signaling in the tumor cells, as B16 cells treated *in vitro* with 5-azacytidine, then injected into mice who were then treated with anti-CTLA-4, showed the same effects (56). Melanoma has demonstrated the most impressive results for responses to immune checkpoint therapy (5–7). We would thus propose testing whether epigenetic therapy

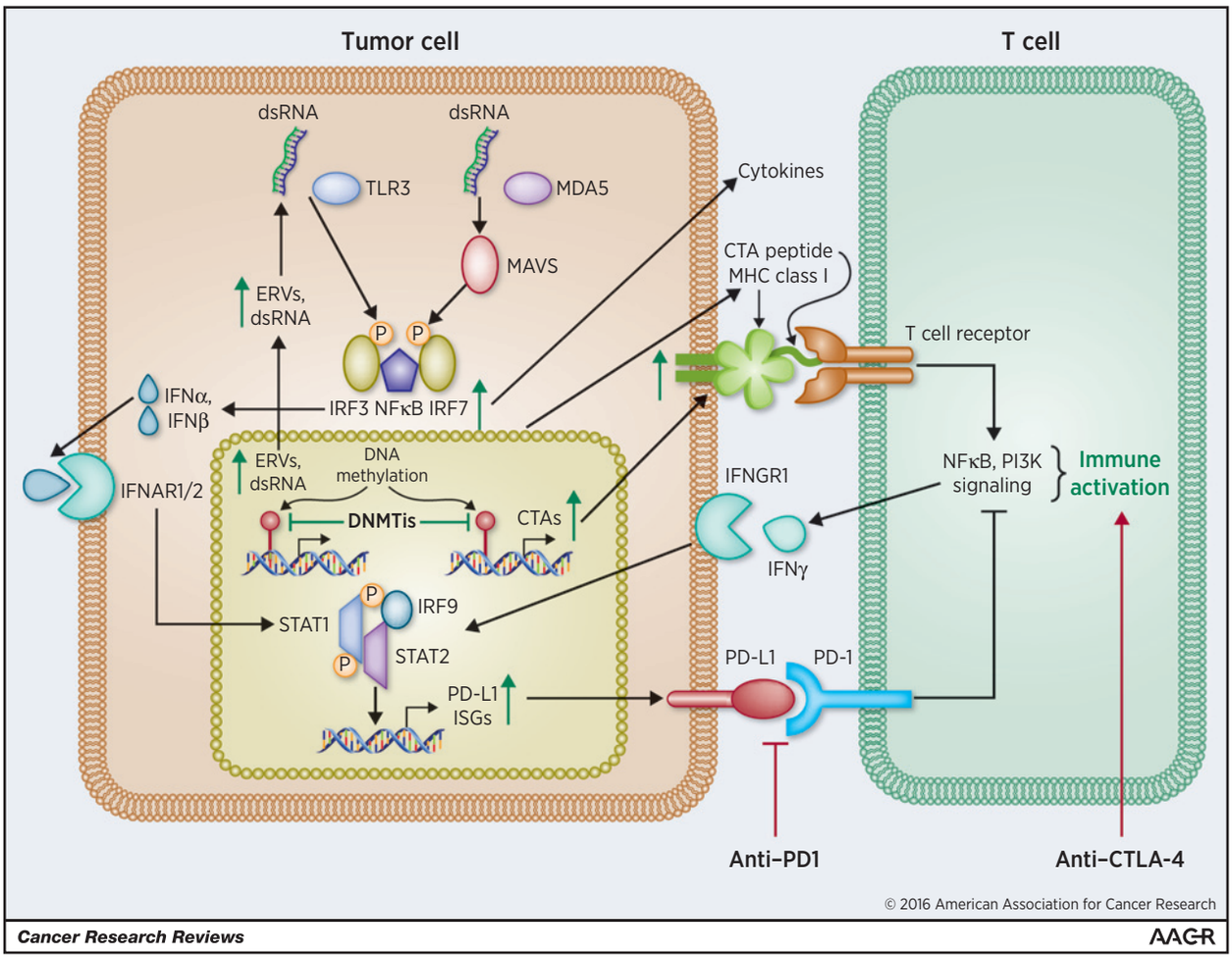

Figure 1.
DNMTis upregulate immune signaling in epithelial cells to synergize with immune checkpoint blockade therapy. DNMTis remove methylation from promoter regions of silenced endogenous retroviruses (ERV), causing double-stranded RNA (dsRNA) to activate sensors including TLR3 and MDA5, which signal through MAVS and IRF7 to cause transcription and secretion of IFNα/β. IFNα/β bind to the IFNAR1/2 receptor, activating JAK/STAT signaling and transcription of IFN-stimulated genes (ISG) that include molecules involved in dsRNA destruction and apoptosis, cytokines that signal to host immune cells, as well as antigen processing and presentation (MHC class I) genes. Separately, CTAs are upregulated by DNMTi removal of methylation from their promoters and are presented by MHC Class I on the cell surface to aid T-cell recognition of cancer cells. Anti-CTLA-4 further aids activation of T cells and secretion of IFNγ that binds to its receptor IFNGR1 to activate STAT signaling and transcription of ISGs. The PD-L1 ligand is upregulated downstream of DNMTi treatment and the IFN response and binds to PD-1 on T cells to inhibit T cells; this interaction is disrupted by anti-PD-1 to promote T-cell activation.

improves response to anti-CTLA-4 and/or anti-PD-1 therapies in clinical trials for melanoma.

Indeed, synergy of epigenetic and immune therapies was shown in ovarian cancer by Wang and colleagues (64). Treating a syngeneic mouse model of ovarian cancer with low-dose 5-aza-2′-deoxycytidine treatment caused upregulation of chemokines that recruit host natural killer (NK) and effector $CD8^+$ T cells to the tumor. In addition, 5-aza-2′-deoxycytidine boosted the production of IFNγ and TNFα from effector T cells, while combining 5-aza-2′-deoxycytidine with anti-CTLA-4 therapy promoted differentiation of naïve T cells into effector T cells. As a result, this combination reduced tumor burden in the mice and extended their survival.

Another way in which 5-azacytidine may sensitize to immune checkpoint therapy is through upregulation of immune tolerance ligands on tumor cells. In EOC and NSCLC cell lines, transcript and surface protein levels of PD-L1 (37, 50) were upregulated by 5-azacytidine. Activation of this ligand is a downstream consequence of activating the viral/IFN response pathway. Importantly, high tumor cell expression of this ligand for the immune cell receptor PD-1 appears to correlate with good response to anti-PD-1 therapy (1–5, 18). A thorough study of $CD34^+$ blast cells from MDS, chronic myelomonocytic leukemia (CMML), and acute myeloid leukemia (AML) patients treated with 5-aza-2′-deoxycytidine showed upregulation of PD-L1, PD-L2, PD-1, and CTLA-4. PD-1 upregulation was due to demethylation of the gene (65). Thus, immune checkpoint blockade drugs targeting these pathways might benefit MDS/AML patients, especially those receiving 5-aza-2′-deoxycytidine.

Epigenetic Regulation of Host Immune Cells

While all of the above research has focused on the effects of epigenetic therapy agents on tumor cells, these drugs affect host immune cells as well. Epigenetic regulation during development and differentiation of host immune cells has been well described (39, 66–69). The gene encoding the Foxp3 transcription factor controls regulatory T cell (Treg) development and function (68, 70, 71). Tregs are necessary for control of autoimmunity but also dampen the host immune response against tumor cells. *Foxp3* is methylated and not expressed in naïve $CD4^+CD25^-$ T cells or activated $CD4^+$ T cells, but is unmethylated and expressed in Tregs (72). The Foxp3 protein is stabilized by acetylation by HDAC9, promoting Treg development and preventing transcription of IL2, the cytokine produced by $CD8^+$ T effector cells (23). Thus, DNMTs and HDACs have opposite effects on Treg development.

HDACis boost antitumor immune responses. The HDACis panobinostat and vorinostat reduce tumor burden in immunocompetent mice, but not in immunocompromised $RAG2\gamma C^{-/-}$ and $IFN\gamma R^{-/-}$ mice (73). The authors found significant synergy of HDACi and IFNy treatment in mouse models of colon cancer and lymphoma. IFNy is secreted by cytotoxic T cells and NK cells and in these experiments it increased immunogenicity of tumor cells (73). Interestingly, B cells were a crucial component of the immune system in the response to HDACis (73). In addition, panobinostat significantly increased the effectiveness of adoptive cell transfer therapy (gp100-specific T cells) in the B16 mouse model of melanoma. Panobinostat enhanced gp100-specific T-cell survival and decreased Tregs in the peripheral blood and the tumor microenvironment. This HDACi also induced significantly higher levels of the IL2 receptor (CD25) and the costimulatory molecule OX-40 on T cells in the B16 mice. Taken together, these results suggest that HDACis boost the host immune response to tumors through B and T cells (74). In addition, inhibiting HDACs can also reduce myeloid-derived cells that induce immune tolerance to help prevent the immune system from clearing tumors (75).

Epigenetic agents may also affect the development of NK cells, which recognize virus-infected cells or newly formed tumor cells and release cytokines to kill the infected cells. Specifically, 5-aza-2′-deoxycytidine has been shown to sensitize AML blasts to lysis by NK cells. Kopp and colleagues showed that killer immunoglobulin-like receptors and the activating receptor NKp44 were upregulated on NK cells expanded *in vitro* and treated with low doses of 5-aza-2′-deoxycytidine. However, high doses of 5-aza-2′-deoxycytidine decrease NK cell proliferation and viability (76). These data, along with use of drugs like DNMTis in MDS and recent preclinical studies, suggest that the beneficial immune effects of epigenetic therapy, like the beneficial effects on tumor cells, occur at low doses that avoid toxicities and immunosuppression (22, 29, 56, 57).

From these studies, it is apparent that epigenetic therapies will have effects on the host immune cells as well as the tumor cells. Thus, for full understanding of the potential for epigenetic therapy to sensitize to immune checkpoint therapy, it will be crucial in clinical trials to study biopsies from both the tumor and the peripheral or tumor-infiltrating host immune cells before and after treatment.

Conclusions

We have put forth preclinical evidence to suggest how epigenetic therapy, via several signaling mechanisms involving both tumor cells and host immune cells, might enhance the efficacy of immune checkpoint therapy (Fig. 1). Through coordinated upregulation of tumor antigens and MHC proteins, and IFN pathway induction by dsRNA transcripts including ERVs, DNA demethylating agents may induce T-cell attraction. Immune checkpoint efficacy in this setting may be enhanced when tolerance inducing ligand and receptor interactions are interrupted. Only clinical trials can prove the efficacy of this proposed paradigm. However, successes could establish epigenetic therapy as a relatively well-tolerated addition to immune checkpoint therapy as a potent new cancer management strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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