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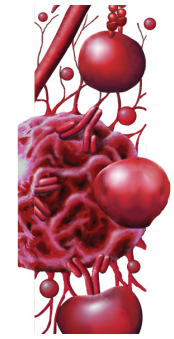

Epigenetic modifiers in immunotherapy: a focus on checkpoint inhibitors

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Immune surveillance should be directed to suppress tumor development and progression, involving a balance of coinhibitory and costimulatory signals that amplify immune response without overwhelming the host. Immunotherapy confers durable clinical benefit in 'immunogenic tumors', whereas in other tumors the responses are modest. Thus, immune checkpoint inhibitors may need to be combined with strategies to boost immune response or increase the tumor immune profile. Epigenetic aberrations contribute significantly to carcinogenesis. Recent findings suggest that epigenetic drugs prime the immune response by increasing expression of tumor-associated antigens and immune-related genes, as well as modulating chemokines and cytokines involved in immune system activation. This review describes our current understanding regarding epigenetic and immunotherapy combination, focusing on immune response priming to checkpoint blockade.

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Epigenetics modifications overview

Epigenetic modulations encompass a broad range of heritable and reversible changes in gene expression, without involving changes in DNA sequence. The haploid human genome encodes approximately three billion DNA base pairs organized within 23 chromosomes, which are tightly wrapped around a histone core. Histones consist of a family of small, positively charged proteins termed histone H1, H2A, H2B, H3 and H4 that bind to the negatively charged DNA [1]. Unlike genetic modification, epigenetic events regulate the fluid balance of chromatin maintained either in an open conformation (euchromatin), associated with active transcription, or in a closed conformation (heterochromatin), associated with gene-repression, and thereby allow the necessary adaptation prompted by environmental influences [2].

Epigenetic regulation of gene expression is controlled through modifications directly on DNA and on the N-terminal histone tails, which include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization and ADP ribosylation [3–5]. Multiple enzymes are responsible for these modifications, including DNA methyltransferases (DNMTs), histone methyltransferases (HMTs) and histone demethylases, histone acetyltransferases and histone deacetylases (HDACs), ubiquitin ligases and deubiquitinases, kinases, phosphatases, small ubiquitin-related modifier ligases and proteases [5].

Epigenetic downregulation of tumor suppressor genes and upregulation of oncogenes are central steps in the development of tumors, directly affecting carcinogenesis [6], metastasis [7], drug resistance [8] and relapse [9]. DNA hypermethylation and

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hypoacetylation of histones H3 and H4 have been linked to cancer progression and, being potentially reversible mutations, they have become an attractive target for cancer therapy [2]. The most advanced epigenetic modulators in clinical use are the DNMT inhibitors (DNMTi) and the HDAC inhibitors (HDACi), which have been investigated with the aim of reversing cancer-promoting epigenetic changes [2,10,11].

HDACi and DNMTi have been extensively studied in solid tumors; however, the optimal clinical setting has been less clear since their single agent activity is modest. In solid tumors, preclinical data suggest that HDACi and DNMTi are effective when used in combination with other anticancer therapies and in reversing therapeutic resistance. Moreover, epigenetic modulators have recently gained interest for their effects on immunomodulatory cells.

This review will focus on the present preclinical and clinical advances that combine epigenetic interventions with checkpoint inhibitors as a new antitumor strategy.

HDAC & HDACi

Class-I HDACs (1–3, 8) are the major mediators of histone deacetylation and are mainly expressed in the nucleus. Class-IIa HDACs (4, 5, 7 and 9) and class-IIb HDACs (6, 10) either shuttle between the nucleus and the cytoplasm, or are restricted to the cytoplasm where their main targets are nonhistone proteins. HDAC11 has been recently reclassified and is the only known class IV HDAC. It is also located in both the nucleus and the cytoplasm and, together with the first two groups, requires Zn^{2+} as a cofactor. Class III enzymes, known as Sirtuins (SIRT1, 2, 3, 4, 5, 6 and 7), differ in structure and function from other deacetylases and are NAD^{+} rather than Zn^{2+} dependent enzymes [12].

Although histone hyperacetylation is commonly associated with transcriptional activation, the broader effects of HDACs on other post-translational modifications and activation of repressor genes involve simultaneous upregulation and downregulation of genes [13].

Divided into several structural classes including hydroxamates, cyclic peptides, short-chain fatty acid and benzamides, several HDACi are in clinical development. The hydroxamates include vorinostat, givinostat, abexinostat, panobinostat, belinostat, rocilinostat and the prototypical HDACi trichostatin A. The cyclic peptides include compounds such as romidepsin (depsipeptide) and trapoxin. Benzamides include entinostat and mocetinostat; while valproic acid and butyrate are short-chain fatty acids [14]. In general, HDACi can be either specific against some HDACs (HDAC isoform-selective inhibitors), or against all types of HDACs (pan-HDACi). Vorinostat and panobinostat are two pan-HDACi, while other compounds can inhibit specifically class I and IIa HDACs (e.g., VPA), or exclusively class I HDACs (e.g., entinostat). The structural difference and selectivity in agents have not resulted in clear clinical distinctions.

The cellular response to HDACi is complex and is likely to involve transcriptional and nontranscriptional phenomena [15]. HDACi modulate gene expression indirectly by mediating the post-translational acetylation of various histone proteins. In addition to histones, nonhistone protein substrates are post-translationally modified by acetylation resulting in their altered function, stability and subcellular localization. Indeed, beyond the classical nuclear HDAC targets, many nonhistone proteins, such as transcription factors (e.g., p53, Bcl6, E2F1 and STATs), DNA repair proteins (e.g., Ku70), cytoskeletal proteins (e.g., α-tubulin) and chaperones (e.g., HSP90) are modulated by HDACi either directly or indirectly [16]. Thus, nonhistone protein acetylation plays a significant role in the modulation of multiple pathways such as apoptosis, cell-cycle arrest, necrosis, autophagy, differentiation and migration [12,15,17–21].

To date, several HDACi have been approved by the US FDA due to their preclinical and clinical efficacy as monotherapy, but mainly in combination with other antitumor drugs in hematological malignancies and in solid tumors. In 2006 and 2009, respectively, vorinostat and romidepsin were approved for the treatment of advanced primary cutaneous T-cell lymphoma in patients with progressive, persistent or recurrent disease on or following two systemic therapies [22]. In 2014, belinostat was approved for the treatment of peripheral T-cell lymphoma, a rare and fast-growing type of non-Hodgkin lymphoma [23]. Most recently in 2015, panobinostat was approved in combination with bortezomib and dexamethasone for the treatment of multiple myeloma [24]. Furthermore, entinostat recently received breakthrough status for the treatment of hormone therapy refractory breast cancer.

Preclinically, HDACi showed synergistic antitumor activity in combination with a large number of structurally diverse anticancer agents [25–28], spurring a number of ongoing combination trials. In particular, HDACi have been found to synergize with capecitabine in colorectal and breast cancer through upregulation of thymidine phosphorylase, the key enzyme that regulates capecitabine conversion to its active form 5-fluorouracil [26,27]. Moreover, studies suggest that HDACi can reverse therapeutic resistance. In a Phase II trial, vorinostat showed encouraging activity reversing resistance to tamoxifen in hormone therapy-resistant breast cancer patients [29]. In the expansion cohort of a Phase I trial, a significant number of patients with

sarcoma, who had failed prior anthracycline treatment, again benefited when combined with panobinostat [30]. A preclinical *in vitro* model showed that the HDACi PCI-24781 (abexinostat) synergized with tamoxifen in breast cancer, inducing apoptosis through downregulation of AKT [25]. Furthermore, HDACi are capable of reverting resistance to the tyrosine kinase inhibitors gefitinib and erlotinib by increasing reactive oxygen species in an *in vitro* model of non-small-cell lung cancer (NSCLC) [28].

In addition to their direct effects on tumor cells, HDACi may also have indirect effects on tumor growth by regulating the host immune response and the tumor vasculature [31–33].

DNMT & DNMTi

DNA methylation plays an important role in maintaining genome integrity. Dysregulated DNA methylation and DNMT downregulation are associated with cancer through an unknown mechanism [34]. DNMTs are a highly conserved family of four enzymes that are responsible for the transfer of a methyl group from S-adenosyl-L-methionine, the universal methyl donor, to the carbon-5 position of the pyrimidine ring of cytosine in CpG dinucleotides. Three of them, DNMT1 (the maintenance DNMT), DNMT3A and DNMT3B (that encode for the *de novo* methyltransferases) are active on DNA. The fourth member, DNMT3L, does not have enzymatic activity.

DNMT1 is the most abundant DNMT and binds to hemimethylated DNA at CpG sites. After DNA replication, the parent strand remains methylated, while the new strand is not. This allows DNMT1 to recognize the newly synthesized strand, bind to it and methylate these hemimethylated CpG sites, maintaining methylation patterns through mitosis. The *de novo* DNMTs are essential for early development since these enzymes mediate DNA methylation after embryo implantation [35–37].

Affecting protein/DNA interactions through chromatin remodeling, DNMTs determine DNA accessibility to transcriptional factors. In this way, these enzymes regulate transcriptional silencing of different genes, essential for genome stability, particularly in repetitive DNA sequences [38–40]. If the promoter region is methylated, the corresponding gene is repressed, as methylation prevents the recognition of the gene by transcription factors. Aberrant hypermethylation may lead to potent transcriptional silencing that inactivates tumor suppressor gene expression and crucial cellular pathways, such as DNA repair [35]. Hypermethylation is indeed linked to specific types of tumor such as colorectal, breast, lung cancers and glioma [41], highlighting its role in tumor progression.

The DNMTi 5′-azacytidine (azacitidine) and 5-aza-2′-deoxycytidine (decitabine), also described as hypomethylating agents (HMAs), are currently approved for the treatment of several hematological cancers. Their application in cancer is limited by their modest clinical activity and relative toxicity [42] as single agents. Moreover, a majority of patients that benefit from HMAs will develop resistance due to unknown mechanism(s) [43,44]. Azacitidine is approved by the FDA for the treatment of myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia, and by the EMA for the treatment of acute myeloid leukemia (AML). In advanced US clinical studies, azacitidine is being evaluated for efficacy in AML [42,45]. Decitabine is approved by the FDA for the treatment of MDS [46], and by the EMA for AML [47].

Initially used at much higher doses, later studies suggested that HMAs may be used more efficiently at lower concentrations. Acting as epigenetic modifying drugs, DNMTi reactivate silenced genes without high toxicity (Figure 1) [42]. Currently, Phase I clinical trials are evaluating HMAs for the treatment of solid tumors [48]. In a small study, azacitidine in combination with entinostat has shown activity in a restricted number of extensively pretreated patients with recurrent metastatic NSCLC. Notably, the dose of azacitidine received by patients in this trial was below the maximal tolerated dose, allowing epigenetic activity of the drug and long treatment, but reducing toxicity [49]. Moreover, this trial supported prior preclinical data that DNMTi re-express epigenetically silenced target genes. These effects are prolonged and more robust when combined with HDAC inhibition. Early data suggest better long-term survival when decitabine was followed by chemotherapy [50,51]. These observations suggest that epigenetic drugs could prime cancers to respond to other antitumor therapy (Figure 1).

A more recent agent, zebularine, with better stability and lower toxicity, has shown promising results *in vitro* and *in vivo* targeting tumor cells preferentially [42]. A recent report showed that zebularine exerts an antitumor effect on cholangiocarcinoma cells by both decreasing DNMT protein concentrations and altering the Wnt signaling pathway [52]. Another report showed that zebularine reduced viability and DNA synthesis of head and neck cancer cells *in vitro* by inducing cell-cycle arrest and apoptosis. This effect was mediated by p21, checkpoint kinase 1 and caspase 3/PARP-dependent pathways, without involving p21 or checkpoint kinase 1 promoter demethylation [53]. Due to minimal toxicity, prolonged treatment with zebularine may be tolerated. These characteristics have prompted its current investigation in combination with other therapeutic strategies, including chemotherapy, immunotherapy and radiotherapy.

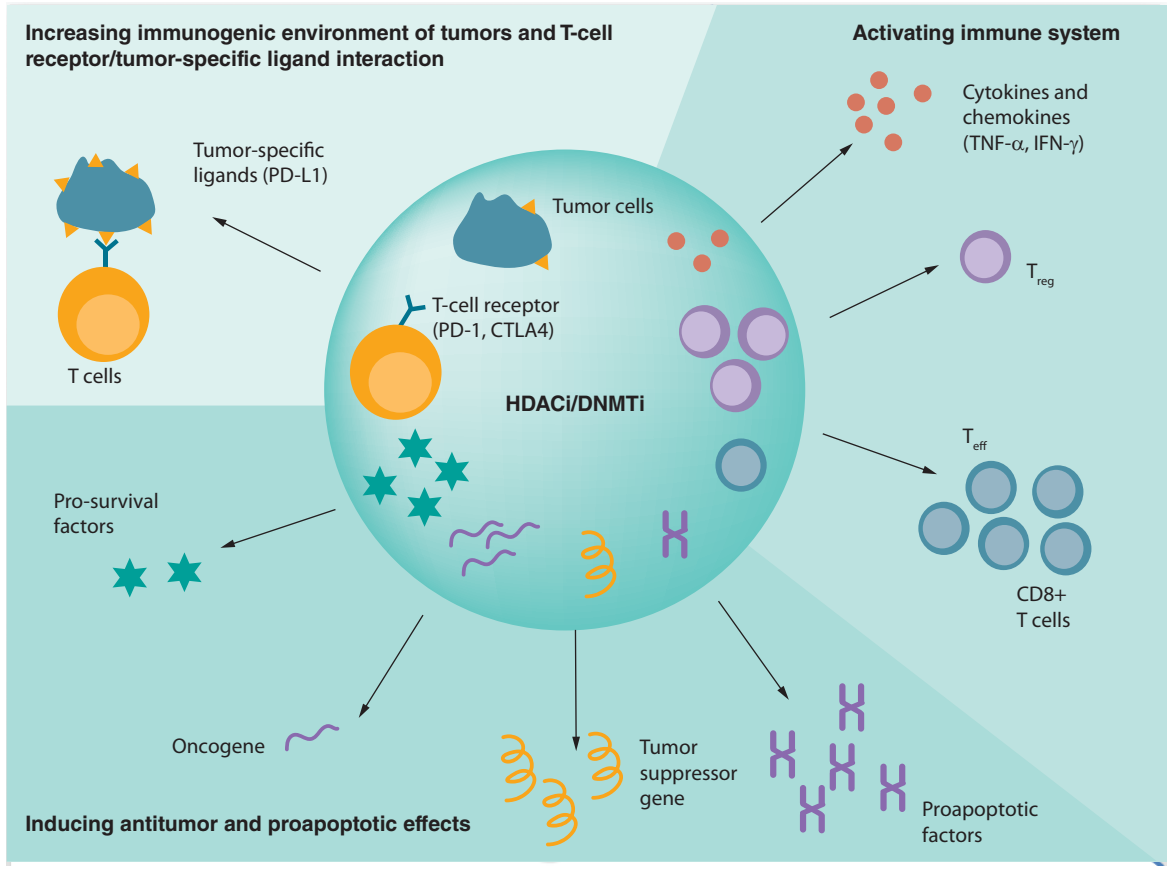

Figure 1. Epigenetic modulation of immune response.

Among the various histone modifications, methylation events catalyzed by HMTs have been of particular interest due to their association with cancer [54]. Several HMT inhibitors have recently been developed and have shown antitumor and antiproliferative effects in tumors, especially those with genetic HMT alterations [55,56]. The HMT EZH2 represents the catalytic core protein of the polycomb repressive complex (PRC2) that predominantly catalyzes the trimethylation of histone-3 lysine-27 (H3K27). EZH2 activity leads to the transcriptional silencing of target genes involved in cell-cycle regulation, differentiation and proliferation. EZH2 has been found mutated, amplified or overexpressed in different cancer types and thus has garnered attention as an anticancer drug target [55,57]. Several EZH2 inhibitors are currently in the process of clinical development [58,59].

Immune checkpoint inhibitors & cancer

The successful introduction of immune checkpoint inhibitors into the therapeutic arsenal has improved the lives of patients with cancer, often with considerably longer disease control than with other therapies. Immune checkpoints are important factors in the balance of self-tolerance and response to pathogens. Emerging data point to the importance of dysregulated immune response and self-tolerance as a mechanism for cancers to evade immune surveillance. Hence, there has been an increased interest in developing T cells as a therapeutic target not only for their ability to mount an immune response to tumor components, but also for their direct ability to eliminate tumor cells [60].

Antigen-specific response is a complex and highly regulated process that can be specifically directed against cancer cells. T-cell activation occurs through antigen-specific stimulation via the T-cell receptor and requires costimulator signals to fully obtain T-cells responses [61–63]. Different immunomodulatory signals regulate antigen-specific immune response, involving stimulatory and inhibitory receptor/ligand pairs that tightly control the process. Preclinical and clinical studies have identified several immunomodulatory receptors and ligands expressed on T cells, tumor cells and APC, whose manipulation can amplify, inhibit or reawaken T-cell response against tumors [64–66]. This may be achieved by blocking coinhibitory molecules, such as CTLA-4, PD-1, LAG-3, or by enhancing the signaling of costimulatory molecules, such as glucocorticoid-induced TNF receptor family-related gene (GITR) and tumor

necrosis factor receptor superfamily member 4 (OX-40) [66–68]. An alternative target is represented by the killer-cell immunoglobulin-like receptor family, a class of transmembrane proteins that negatively regulate antigen-specific and cytotoxic activity of natural killer (NK) cells [69].

This class of molecules, which are known as immune checkpoints inhibitors, provides new strategies for cancer treatment (Table 1). The most extensively studied are CTLA-4 and PD-1, which when inhibited have elicited clinical efficacy in many tumor types like melanoma, renal cell carcinoma and NSCLC alone and more pronounced when combined [70,71]. Whereas both drugs function as negative regulators, each plays a nonredundant role in modulating immune response. While CTLA-4 is essential during early activation of T cells in lymphatic tissue, PD-1 is primarily involved in modulating T-cell activation in peripheral tissues, including the tumor microenvironment, leading to apoptosis and downregulation of T-cell effectors (Teff) [64,72].

CTLA-4, expressed on the T-cell surface, competes with the stimulatory receptor CD28 for binding to its ligands CD80/CD86. In advanced melanoma, adjuvant treatment with the CTLA-4 targeting monoclonal antibody ipilimumab improved overall survival and durability of objective tumor response, leading to its approval in 2015 [73,74]. The PD-1 receptor is expressed on T cells, B cells, NK cells, monocytes, macrophages and dendritic cells (DCs). PD-1 binds to two ligands, PD-L1 and PD-L2. PD-L1 is expressed on a variety of cell types including epithelium, muscle, mesenchymal stem cells, T and B cells, DCs, macrophages and cancer cells, while PD-L2 expression is restricted to immune-related cells such as DCs, macrophages and mast cells [75]. Under normal conditions, the interaction between PD-1 and its ligand PD-L1 downregulates cytotoxic T-cell activity to maintain immune homeostasis. When a PD-1 expressing T cell engages PD-L1, its cytotoxic activity is downregulated, thereby protecting normal cells from T-cell-mediated damage [76,77].

PD-1/PD-L1 overexpression has been found in melanoma, ovarian, triple negative (TNBC) and HER2+ breast cancer, NSCLC and other tumors such as AML and chronic lymphatic leukemia (CCL) [78]. Colorectal cancers that displayed high tumor-infiltrating lymphocytes (TILs) and had defects in mismatch repair, evidenced by microsatellite instability, overexpressed PD-1, PD-L1, CTLA-4 and LAG-3 [79] and were sensitive to PD-1 inhibition. Frequently, high PD-L1 tumor expression correlates with TILs, suggesting that in the tumor microenvironment, tumor cells co-opted these mechanisms to avoid immunological surveillance and facilitate cancer growth [64,66]. In breast cancer, PD-L1 expression is less frequent in estrogen receptor positive (ER+) disease and more commonly associated with TNBC, ER negative and progesterone receptor (PR) negative tumors. Increased PD-L1 expression appears to correlate with higher TILs and those data together correlate with better response [80,81]. Moreover, increased TILs are associated with decreased distant recurrence in patients with ER-/Her2+ tumors or TNBC [82].

Table 1. Checkpoint inhibitor antibodies in clinical development.

Antibody	Target	Clinical development
Ipilimumab	CTLA-4	US FDA-approved
Tremelimumab	CTLA-4	Phase III
Nivolumab	PD-1	US FDA-approved
Pembrolizumab	PD-1	US FDA-approved
Pidilizumab (CT-011)	PD-1	Phase I/II
AMP-224	PD-1	Phase I completed
BMS-936559	PD-L1	Phase I completed
Atezolizumab (MPDL3280A)	PD-L1	Phase III/IV
Durvalumab (MEDI4736)	PD-L1	Phase I/II
Lirilumab	KIR	Phase I/II
BMS-986016	LAG-3	Phase I
TRX518	GITR	Phase III/IV
MEDI6469	OX40	Phase I/II
CC-90002	CD47	Phase I
Hu5F9-G4	CD47	Phase I

Interfering with PD-L1 activity on tumor cells and tumor-infiltrating immune cells may potentially prevent suppressive signaling and block T-cell suppression throughout the tumor microenvironment [83]. Several agents that block either PD-1 or PD-L1 are under clinical investigation: two monoclonal antibodies, pembrolizumab and nivolumab, were FDA approved in 2014 for the treatment of advanced or unresectable melanoma. More recently, pembrolizumab was further approved as second-line treatment for patients with advanced PD-L1 expressing NSCLC. Nivolumab was subsequently approved for the treatment of renal cell carcinoma and advanced NSCLC in patients who had progressed during or after platinum-based chemotherapy [84–86] (Table 1).

LAG-3 is a regulator of TILs activity. This protein is expressed in both activated and exhausted lymphocytes and DCs. It interacts with MHC-II receptors and its blockade supports Teff activity both *in vitro* and *in vivo*. Recently, it was suggested that combining PD-1 and LAG-3 inhibition enhances antitumor immunity. In preclinical studies, combined inhibition resulted in synergistic activity to control immune homeostasis, tumor growth and antitumor immunity by preventing Teff exhaustion. Thus, this combination is considered a promising strategy for immune-based cancer therapy [67,87].

Another promising immunotherapeutic strategy is the combination of checkpoint and OX40 inhibitors. When used as a single agent, the activating anti-OX40 monoclonal antibody elicits modest clinical and immunological response in patients with metastatic disease. Results from preclinical and early Phase I studies have validated OX40 as a well-tolerated potent immune-stimulating target that enhances cellular immunity, $CD4^+$ and $CD8^+$ T-cell proliferation and increases tumor-specific immune response [87,88].

With encouraging preclinical results in the vaccine and antitumor settings, GITR represents an additional target for combinatory cancer therapy [68,89,90,91]. GITR is widely expressed on T cells, B cells, NK, myeloid and Treg. When upregulated, GITR induces proliferation, activation and cytokine production of $CD4^+$ and $CD8^+$ T cells [89]. In addition, GITR engagement can reduce the immunosuppressive activity of Treg. This phenomenon is associated with loss of Foxp3 expression on tumor-associated Treg [89,90]. In an *in vitro* ovarian cancer model, combining anti-PD-1 and anti-GITR treatment resulted in significant tumor growth reduction, associated with $CD4^+$ and $CD8^+$ cell activation and attenuation of the Treg compartment. This suggested a shift away from an immunosuppressive tumor environment to an immunostimulatory state, which favorably contributes to a durable antitumor effect [91]. Interestingly, anti-PD-1/GITR treatment was found to synergize with classic chemotherapy agents, such as cisplatin and paclitaxel, providing long-term remission in both ovarian and breast cancer-bearing mice [91].

Combining anti-GITR and anti-CTLA-4 antibodies has also been investigated. In combination, their antitumor effects were improved compared with single antibody treatment alone [92]. This enhanced effect was attributed to their distinct activities. Indeed, anti-CTLA4 induced increased tumor infiltrating $CD8^+$ T cells in the mice. Anti-GITR treatment increased cytokine secretion and $CD8^+$ T-cell resistance to Treg suppression. These tumor-specific $CD8^+$ T cells were resistant to Treg and showed an upregulated CD25 expression in mice [92]. Moreover, local delivery of anti-CTLA-4 and anti-GITR to the sites of interaction between T cells and tumor antigen-loaded DC vaccines enhanced the induction of antitumor immunity, while avoiding autoimmunity [93]. Currently, the combination of anti-GITR with anti-PD-1 or anti-CTLA-4 and chemotherapy is under investigation in a variety of clinical settings [68,90,91,93].

Another promising immunoregulatory target is CD47. This transmembrane immunoglobulin protein is widely expressed in a variety of cell types, but its overexpression has been found in tumor cells suggesting a role in disrupting antitumor immune response. CD47, known for its essential role in preventing phagocytic removal of healthy cells by the immune system, is involved in several cellular mechanisms, including angiogenesis, cancer cell death and regulation of T-cell immunity through its interaction with thrombospondin-1. Thus, its expression on immune-related cells with dual positive/negative regulatory effects suggests its role in the regulation of immunity. The therapeutic benefit of CD47-targeted immunotherapy consequently relies on all combined effects. Based on these observations, CD47 has drawn clinical attention as a prominent target in cancer immunotherapy. Indeed, several preclinical studies demonstrated therapeutic benefit of anti-CD47 antibodies in hematological and solid cancers, which has lead to the initiation of several clinical studies to evaluate its therapeutic potential (NCT02641002; NCT02367196; NCT02216409 and Table 1) [94].

Finally, other immune-regulating strategies such as targeting CD137 (urelumab) and killer-cell immunoglobulin-like receptors (lirilumab) have shown to be synergic with the anti-CD20 rituximab, revealing promising preclinical efficacy that enhances NK-mediated cytotoxicity in lymphomas [95,96]. Since each of those approaches utilizes a distinct mechanism, they represent novel strategies that need further clinical investigation.

HDACi & immune checkpoints inhibitors

Multiple reports suggest that HDACi enhance the immunogenicity of cancer cells. Modulation of HDACs is involved in the regulation of NK-cell activating ligands, MHC class I and II molecules [97], elevation of NK and $CD8^+$ cytotoxicity and proinflammatory cytokines, modulation of Treg and Treg Foxp3 expression (Figure 1) [97]. The class I-specific HDACi entinostat promotes the reduction of myeloid-derived suppressor cells (MDSCs) and increases human leukocyte antigens-DR expression on monocytes, without altering the CD8/CD4 T-cell ratio when used in combination with the aromatase inhibitor exemestane in a randomized Phase II study in hormone receptor-positive breast cancer [98]. Moreover, low doses of entinostat transcriptionally reduced Treg Foxp3 expression, reducing their suppressive function, without affecting Teff in renal and prostate cancer models *in vitro*, which was associated with improved tumor growth inhibition in combination with either IL-2 or a surviving-based vaccine therapy *in vivo*. Since STAT3-specific inhibition was able to rescue Foxp3 downregulation induced by entinostat, the authors suggested STAT3 involvement in HDACi-dependent Foxp3 modulation [99]. Other studies demonstrated that entinostat induces immune-related genes involved in antigen presentation in breast cancer [100], while panobinostat was able to modulate different serum cytokines involved in T-cell activation in patients with Hodgkin lymphoma [101]. Furthermore, entinostat improved treatment outcomes with complete regression and absence of metastasis in CT26 colorectal tumors and in a 4T1 metastatic breast cancer mouse model in combination with the DNMTi azacitidine, the anti-CTLA-4 and anti-PD-1 antibodies, eliminating tumors in 80% of tumor-inoculated mice. Epigenetic modulators did not increase tumor-infiltrating $CD8^+$ T cells further than checkpoint inhibitors, but were able to decrease tumor-infiltrating FoxP3+ Treg. Moreover, entinostat reduced circulating granulocytic MDSCs that directly inhibit the function of $CD8^+$ T cells [102]. HDACi and DNMTi have shown to restore MHC-I and APM gene expression silenced by epigenetic alterations [103].

Preclinical studies suggest that the upregulation of immune checkpoints is epigenetically regulated through the action of HDACi that modulate PD-L1 expression in melanoma [104,105]. In particular, class I HDAC inhibition causes upregulation of PD-L1, and PD-L2 to a less extent, in human and murine cell lines *in vitro*. This was confirmed *in vivo* in a murine cell melanoma model where mice, receiving a combination treatment with HDACi panobinostat and an anti-PD-1, showed slower tumor progression and increased survival [105]. However, as seen with other signaling pathways, HDAC inhibition may be tissue and HDAC specific. Indeed, HDAC6 is suggested to play a role in the regulation of immunogenicity in chronic lymphocytic leukemia (CLL). In contrast to the effects observed with other HDACi, selective HDAC6 blockade, using the HDAC6 inhibitor rocilinostat or by HDAC6-specific silencing, resulted in downregulation of PD-L1 in primary B cells isolated from CLL patients and restoration of CD4:CD8 ratio [104]. Thus, considering the ability of HDACi to modulate the PD-1/PD-L1 pathway, their capability to revert hormone therapy resistance and to synergize with tamoxifen, our group recently initiated a randomized Phase II clinical trial in patients with ER+ advanced hormone therapy-resistant breast cancer, where tamoxifen was combined with vorinostat and the anti-PD-1 antibody pembrolizumab (NCT02395627 and Table 2). Our primary goal will be to evaluate the overall response rate and progression-free survival in order to test epigenetic immune priming in hormone therapy-resistant breast cancer.

Although several studies have shown that HDAC inhibition can promote immune response, as described above, others demonstrate that HDACi may have additional effects that act to counter this promotion. HDAC inhibition has been shown to exert toxic effects on lymphocytes, decreasing their function, inhibiting $CD4^+$ cytotoxicity, proliferation and viability and reducing antigen-presenting cell function [97]. Panobinostat was found to be cytotoxic to human lymphocytes at concentrations lower than those required for melanoma antitumor effects and was able to dysregulate lymphocyte activation signaling pathways leading to decreased functions [106]. Conversely, several studies showed a stimulation of $CD8^+$ T-cells activation and function following pan-HDACi [107].

Foxp3 is a transcription factor greatly responsible for Treg development and function, whose expression can be modulated by epigenetic modifications [108]. Acetylation, together with methylation, represents an important post-translational modification that regulates stability and activity of Foxp3 [108]. HDAC9 has been found to colocalize with Foxp3 in resting Treg, and this can be reverted by HDACi treatment [109]. By enhancing Foxp3 acetylation, HDACi have been found to increase Treg suppressive functions [110]. HDAC6 inhibition was found to be sufficient to induce Foxp3 hyperacetylation, and reduce proteasomal degradation [111]. A separate report showed that HDAC5 is required for the suppressive formation and function of Treg *in vitro* and *in vivo*. Depletion of HDAC5 prevented conversion of $CD4^+$ T cells to Treg under polarizing conditions and decreased Foxp3 expression. However, less Treg activity was associated with $CD8^+$ T cells inability to produce IFN-γ and, thus, did not

translate in better antitumor immunity [112]. Other reports, as we previously described, instead showed Treg and Foxp3 downregulation following entinostat treatment [99,113].

HDACi can increase tumor-associated antigens and modulate the expression of many components of the tumor antigen processing and MHC presentation pathway resulting in enhanced T-cell antitumor immunity. At the same time, HDACi can enhance tumor cell recognition and elimination by NK increasing the expression of ligands detected by the NKG2D receptor on tumor cells [107]. Vorinostat induces immunogenic cell death in colon cells that are efficiently taken up by DC *in vitro* [114]. However, some reports showed opposite effects with decreased T cells and NK recognition and decreased pro-inflammatory cytokines production after HDACi treatment [107].

These apparent counteracting effects resulting from epigenetic immune modulation and how they translate to immune response are likely dependent on the specific tumor–immune compartment relationship as well as the types and combinations of specific inhibitors employed. A greater understanding of context-specific relationships between the tumor, the tumor microenvironment and the immune compartment is needed to more effectively and rationally employ epigenetic modulators.

DNMTi & immune checkpoint inhibitors

DNMTi produce cancer cell-independent immunomodulatory effects by increasing MHC I and II molecules expression in cancer cells, increasing immunogenicity and critical immunostimulatory cytokines, enhancing NK and T-cell function through activation of genes involved in NK reactivity, IFN-γ production, CD4 T-helper cells behavior and other various mechanisms. The IFN-γ locus is highly methylated in naïve $CD8^+$ T cells and unmethylated in Teff, suggesting a potential DNMTi-associated modulation upon exposure with such agents [115]. Increased cell-surface expression of MHC class I cell molecules were observed in tumors explanted from mice treated with azacitidine [116]. DNA methylation is involved in the regulation of human leukocyte antigens class I, and their downregulation in cancer is associated with promoter hypermethylation [117].

Treatment with decitabine enhanced expression of antigen processing machinery components like tapasin, TAP1 and TAP2 [117]. Another recent report showed upregulation of immune genes after azacitidine treatment in multiple solid tumor types suggesting that patients with a low basal immune gene expression signature may derive the greatest benefit from epigenetic priming for immune therapy [118]. Several preclinical and clinical studies have shown that epigenetic modifiers such as DNMTi and HDACi increase immunogenicity via the re-expression of numerous tumor-associated antigens including several members of the MAGE, SSX, SPANX, PAGE families [119].

In addition to boosting immune response, DNMTi can decrease immunosuppression. The DNMTi azacitidine has been shown to reduce Treg function in MDS patients [120]. Decitabine reduced MDSCs in a syngeneic murine ovarian cancer model [121], synergizing with anti-CTLA4 therapy, increasing the production of cytokines involved in NK and CD8 cytotoxic T-cells recruitment and their IFN-γ and TNF-α production. Moreover, DNMTi demonstrated the ability to prime immune response in different tumor types [118].

Treatment of leukemia cells with decitabine resulted in a dose-dependent upregulation of PD-1, PD-L1, PD-L2 and CTLA-4 expression, which was confirmed even in a small set of patients with MDS, chronic myelomonocytic leukemia and AML treated with epigenetic therapy. Notably, patients resistant to DNMTi exhibited elevated levels of those genes, thus the authors here suggested a putative role of PD-1 upregulation as one of the mechanisms promoting resistance to hypomethylating agents [43]. This was supported by another study demonstrating that azacitidine-mediated PD-1 promoter demethylation was associated with PD-1 mRNA upregulation and worse overall response in MDS patients. These findings suggest that PD-1 promoter demethylation correlates with poorer clinical response to azacitidine [44]. It has been shown that, during infection, the expression of PD-1 on T cells is regulated by DNA methylation [122]. Despite the presumed role of PD-1 overexpression as a driver to DNMTi resistance, the evidence of causation is yet to be determined. The clinical efficacy of PD-1 inhibitors in MDS and leukemia is currently being investigated (Table 2). Moreover, azacitidine was able to upregulate PD-L1 expression both at the transcriptional level and also directly on cell surface in an *in vitro* model of NSCLC cell lines, in general characterized by low expression of this gene. By matching basal gene expression and DNA methylation patterns of hundreds of primary NSCLC cancers, the authors suggested that a major effect of the azacitidine treatment is the alteration of immune-related pathways that leads tumor cells to be more susceptible to T cell-mediated cytotoxicity. In this way, the authors speculated that PD-L1 could be a useful biomarker to identify patients with NSCLC that could benefit from combined DNMTi and anti-PD-1 blockade, defining a new role for epigenetic therapy as sensitizer to checkpoint inhibitors [123].

Table 2. Current clinical trials where epigenetic drugs are combined with checkpoint inhibitors.

Clinical trials identifier	Status	Phase	Cancer type	Epigenetic drug	Immune checkpoint inhibitor	Additional drugs
NCT02453620	Recruiting	I	Metastatic unresectable HER2-negative breast cancer	Entinostat	Nivolumab and ipilimumab	
NCT02032810	Recruiting	I	Unresectable stage III/IV melanoma	Panobinostat	Ipilimumab	
NCT02635061	Not yet recruiting	I	Unresectable NSCLC	ACY-241	Nivolumab and ipilimumab	
NCT01928576	Recruiting	II	NSCLC	Entinostat and azacytidine	Nivolumab	
NCT02437136	Recruiting	I/II	NSCLC and melanoma	Entinostat	Pembrolizumab	
NCT02538510	Recruiting	I/II	Metastatic unresectable HNSCC and SGC	Vorinostat	Pembrolizumab	
NCT02638090	Recruiting	I/II	Stage IV NSCLC	Vorinostat	Pembrolizumab	
NCT02619253	Recruiting	I/II	Advanced renal or urothelial cell carcinoma	Vorinostat	Pembrolizumab	
NCT02395627	Recruiting	II	Hormone therapy resistant breast cancer	Vorinostat	Pembrolizumab	Tamoxifen
NCT02530463	Recruiting	II	MDS	Azacytidine	Nivolumab and/or ipilimumab	
NCT02399917	Recruiting	II	Refractory/relapsed AML	Azacytidine	Lirilumab	
NCT02599649	Recruiting	II	MDS	Azacytidine	Lirilumab and nivolumab	
NCT02397720	Recruiting	II	AML	Azacytidine	Nivolumab	
NCT02260440	Recruiting	II	Metastatic CRC	Azacytidine	Pembrolizumab	
NCT02546986	Recruiting	II	Advanced/metastatic NSCLC	Oral azacytidine	Pembrolizumab	
NCT02512172	Recruiting	I	MSS advanced CRC	Romidepsin and/or azacytidine	Pembrolizumab	
NCT02508870	Recruiting	I	MDS	Azacytidine	Atezolizumab	

AML: Acute myeloid leukemia; CRC: Colorectal cancer; HNSCC: Head and neck squamous cell carcinoma; MDS: Myelodysplastic syndromes; MSS: Microsatellite stable; NSCLC: Non-small-cell lung cancer; SGC: Salivary gland cancer.

Recent reports suggest a possible mechanism of action where DNMTi upregulate immune signaling in cancer by mimicking the viral defense pathway [124,125]. Particularly in ovarian cancer, DNMTi induced an interferon response in cancer cells by activating cytosolic dsRNA sensing. Remarkably, viral defense pathway signaling levels correlated with improved responses to anti-CTLA-4 immune checkpoint therapy and long-term survival in melanoma patients [125]. Similarly in colorectal cancer, azacitidine significantly reduced the frequency of colorectal cancer-initiating cells without demethylation of aberrantly methylated CpG islands. Low doses of azacitidine induced formation of dsRNA and activation of the cytosolic pattern recognition receptor MDA5, and downstream activation of MAVS and IRF7 [124]. Clinical trials in NSCLC, breast and colorectal cancer with low dose of DNMTi identified upregulation of IFN-responsive genes [118,123], highlighting the clinical relevance of those results. These results suggest that DNMTi could trick cancer cells into behaving as virus-infected cells and trigger dsRNA sensing, a central step to cellular viral defense response. As a consequence, this mechanism could attract lymphocytes to the tumor microenvironment that could be of particular interest in a context of immunotherapy combination strategy.

Recently, the role of HMTs in tumor immunity regulation has been investigated. EZH2-mediated histone H3 lysine 27 trimethylation (H3K27me3) and DNMT1-mediated DNA methylation repress the tumor production of T helper 1 (Th1)-type chemokines CXCL9 and CXCL10 in ovarian cancer. Combination treatment with EZH2 inhibitors and

azacitidine increases Teff tumor infiltration, decreases tumor progression and improves the therapeutic efficacy of anti-PD-L1 therapy [126]. EZH2 and other PRC2 components have been found to repress the expression and subsequent production of Th1-type chemokines CXCL9 and CXCL10 even in colon cancer. PRC2 machinery components expression was inversely correlated to $CD4^+$, $CD8^+$ and Th1-type chemokines in human colon cancer tissue, and this expression pattern was significantly associated with patient survival [127].

These data suggest that PRC2-mediated epigenetic silencing is both a crucial oncogenic mechanism and a key control pathway regulating tumor immunosuppression. Thus, targeting these epigenetic programs may have significant implications in combination cancer immunotherapies improving their efficacy and improving T cell-mediated immunity in the tumor microenvironment.

Conclusion & future perspective

Immune checkpoint blockade represents a new standard for cancer treatment with promising prospects for clinical benefit and enhanced durability of tumor response. Recent advances in the understanding of the mechanism involved in immune regulation provide a strong basis for the development of new combination strategies. Indeed, the future of cancer therapy includes combinatorial approaches with radiotherapy, chemotherapy, vaccines and immunotherapy to improve response in cancer. In this context, the combination of epigenetic drugs, such as HDACi and DNMTi, with immunotherapy found its rational foundation. Preclinical results have prompted multiple clinical trials, listed in Table 2, where DNMTi and HDACi are used to prime immunotherapy. Identifying the optimal biologic dosing strategies may represent the key to the success of these combinatorial therapeutic strategies.

Finally, PD-L1 expression as a predictive biomarker for PD-1 blockade effectiveness is being evaluated in clinical studies, but its ultimate predictive value remains unclear. In the absence of a standardized technique to measure PD-L1 in tumor, variations resulting from differing immunohistochemistry cutoffs, tissue preparation, expression heterogeneity, variations dependent on the site of the biopsy and the use of several different nonreproducible antibody reagents mean that interpretation will remain investigational [128].

Additional and more specific predictive biomarkers need to be identified to help stratify which patient will benefit from immunotherapy and what therapeutic combinations should be employed. Indeed, assessing methylation and histone acetylation status in tumor and the immune cell compartment, associated with the modulation of immune-related genes could be integrated in the research of optimal biomarkers helping identify patient-specific dosing strategies targeting the immune system and immune activation. This, together with an improved understanding of the mechanism of action of immune checkpoint inhibitors, will help to further broaden the therapeutic impact of this novel anticancer strategy that has improved the lives of many patients with cancer.

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Executive summary

- Epigenetic modifications are involved in gene expression and cell signaling regulation linked to cancer development and progression.
- Histone deacetylases inhibitors and DNA methyltransferases inhibitors are two major classes of epigenetic drugs used in cancer treatment, and current studies are investigating their ability to prime immune response.
- Checkpoint inhibitors are an emerging class of immune-modulatory agents that, by targeting coinhibitory signals, enhance the immune response and confer durable clinical benefit in solid tumors like melanoma, renal cell carcinoma and non-small-cell lung cancer.
- Recent preclinical and clinical studies have demonstrated that the combination of epigenetic drugs, such as histone deacetylases inhibitors and DNA methyltransferases inhibitors, together with immune checkpoint inhibitors can be beneficial for the treatment of different tumor types.

References

Papers of special note have been highlighted as: • of interest

1 Wolffe AP. Chromatin: structure and function. 1-477, Academic Press, San Diego, CA, USA (1999).

2 Ramachandran S, Ient J, Gottgens EL, Krieg AJ, Hammond EM. Epigenetic therapy for solid tumors: highlighting the impact of tumor hypoxia. *Genes (Basel)* 6(4), 935–956 (2015).

3 Kouzarides T. Chromatin modifications and their function. *Cell* 128(4), 693–705 (2007).

4 Cohen I, Poreba E, Kamieniarz K, Schneider R. Histone modifiers in cancer: friends or foes? *Genes Cancer* 2(6), 631–647 (2011).

5 Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res.* 21(3), 381–395 (2011).

6 Sarkar S, Goldgar S, Byler S, Rosenthal S, Heerboth S. Demethylation and re-expression of epigenetically silenced tumor suppressor genes: sensitization of cancer cells by combination therapy. *Epigenomics* 5(1), 87–94 (2013).

7 Sarkar S, Horn G, Moulton K *et al.* Cancer development, progression, and therapy: an epigenetic overview. *Int. J. Mol. Sci.* 14(10), 21087–21113 (2013).

8 Glasspool RM, Teodoridis JM, Brown R. Epigenetics as a mechanism driving polygenic clinical drug resistance. *Br. J. Cancer* 94(8), 1087–1092 (2006).

9 Housman G, Byler S, Heerboth S *et al.* Drug resistance in cancer: an overview. *Cancers (Basel)* 6(3), 1769–1792 (2014).

10 Song SH, Han SW, Bang YJ. Epigenetic-based therapies in cancer: progress to date. *Drugs* 71(18), 2391–2403 (2011).

11 Nebbioso A, Carafa V, Benedetti R, Altucci L. Trials with 'epigenetic' drugs: an update. *Mol. Oncol.* 6(6), 657–682 (2012).

12 Budillon A, Bruzzese F, Di Gennaro E, Caraglia M. Multiple-target drugs: inhibitors of heat shock protein 90 and of histone deacetylase. *Curr. Drug Targets* 6(3), 337–351 (2005).

13 Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat. Rev. Cancer* 6(1), 38–51 (2006).

14 Arrighetti N, Corno C, Gatti L. Drug combinations with HDAC inhibitors in antitumor therapy. *Crit. Rev. Oncog.* 20(1–2), 83–117 (2015).

15 Marks PA, Xu WS. Histone deacetylase inhibitors: potential in cancer therapy. *J. Cell. Biochem.* 107(4), 600–608 (2009).

16 Thurn KT, Thomas S, Moore A, Munster PN. Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer. *Future Oncol.* 7(2), 263–283 (2011).

17 Ocker M. Deacetylase inhibitors – focus on non-histone targets and effects. *World J. Biol. Chem.* 1(5), 55–61 (2010).

18 Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. *Nat. Biotechnol.* 25(1), 84–90 (2007).

19 Lane AA, Chabner BA. Histone deacetylase inhibitors in cancer therapy. *J. Clin. Oncol.* 27(32), 5459–5468 (2009).

20 Budillon A, Di Gennaro E, Bruzzese F, Rocco M, Manzo G, Caraglia M. Histone deacetylase inhibitors: a new wave of molecular targeted anticancer agents. *Recent Pat. Anticancer Drug Discov.* 2(2), 119–134 (2007).

21 Marks PA. Histone deacetylase inhibitors: a chemical genetics approach to understanding cellular functions. *Biochim. Biophys. Acta* 1799(10–12), 717–725 (2010).

22 Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R. FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. *Oncologist* 12(10), 1247–1252 (2007).

23 O'connor OA, Horwitz S, Masszi T *et al.* Belinostat in patients with relapsed or refractory peripheral T-cell lymphoma: results of the pivotal Phase II BELIEF (CLN-19) study. *J. Clin. Oncol.* 33(23), 2492–2499 (2015).

24 San-Miguel JF, Hungria VT, Yoon SS *et al.* Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: a multicentre, randomised, double-blind Phase 3 trial. *Lancet Oncol.* 15(11), 1195–1206 (2014).

25 Thomas S, Thurn KT, Raha P, Chen S, Munster PN. Efficacy of histone deacetylase and estrogen receptor inhibition in breast cancer cells due to concerted down regulation of Akt. *PLoS One* 8(7), e68973 (2013).

26 Di Gennaro E, Piro G, Chianese MI *et al.* Vorinostat synergises with capecitabine through upregulation of thymidine phosphorylase. *Br. J. Cancer* 103(11), 1680–1691 (2010).

27 Terranova-Barberio M, Roca MS, Zotti AI *et al.* Valproic acid potentiates the anticancer activity of capecitabine *in vitro* and *in vivo* in breast cancer models via induction of thymidine phosphorylase expression. *Oncotarget* doi:10.18632/oncotarget.6802 (2015) (Epub ahead of print).

28 Leone A, Roca MS, Ciardiello C *et al.* Vorinostat synergizes with EGFR inhibitors in NSCLC cells by increasing ROS via up-regulation of the major mitochondrial porin VDAC1 and modulation of the c-Myc-NRF2-KEAP1 pathway. *Free Radic. Biol. Med.* 89 287–299 (2015).

29 Munster PN, Thurn KT, Thomas S *et al.* A Phase II study of the histone deacetylase inhibitor vorinostat combined with tamoxifen for the treatment of patients with hormone therapy-resistant breast cancer. *Br. J. Cancer* 104(12), 1828–1835 (2011).

30 Thomas S, Aggarwal R, Jahan T *et al.* A phase I trial of panobinostat and epirubicin in solid tumors with a dose expansion in patients with sarcoma. *Ann. Oncol.* doi:10.1093/annonc/mdw044 (2016) (Epub ahead of print).

31 Kim MS, Kwon HJ, Lee YM *et al.* Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat. Med.* 7(4), 437–443 (2001).

32 Lin EY, Pollard JW. Tumor-associated macrophages press the angiogenic switch in breast cancer. *Cancer Res.* 67(11), 5064–5066 (2007).

33 Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat. Rev. Drug Discov.* 5(9), 769–784 (2006).

34 Chik F, Szyf M, Rabbani SA. Role of epigenetics in cancer initiation and progression. *Adv. Exp. Med. Biol.* 720 91–104 (2011).

35 Jin B, Robertson KD. DNA methyltransferases, DNA damage repair, and cancer. *Adv. Exp. Med. Biol.* 754 3–29 (2013).

36 Kareta MS, Botello ZM, Ennis JJ, Chou C, Chedin F. Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L. *J. Biol. Chem.* 281(36), 25893–25902 (2006).

37 Bestor TH. The DNA methyltransferases of mammals. *Hum. Mol. Genet.* 9(16), 2395–2402 (2000).

38 Xu GL, Bestor TH, Bourc'his D *et al.* Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* 402(6758), 187–191 (1999).

39 Gonzalo S, Jaco I, Fraga MF *et al.* DNA methyltransferases control telomere length and telomere recombination in mammalian cells. *Nat. Cell Biol.* 8(4), 416–424 (2006).

40 Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* 99(3), 247–257 (1999).

41 Jacinto FV, Esteller M. Mutator pathways unleashed by epigenetic silencing in human cancer. *Mutagenesis* 22(4), 247–253 (2007).

42 Gnyszka A, Jastrzebski Z, Flis S. DNA methyltransferase inhibitors and their emerging role in epigenetic therapy of cancer. *AntiCancer Res.* 33(8), 2989–2996 (2013).

43 Yang H, Bueso-Ramos C, Dinardo C *et al.* Expression of PD-L1, PD-L2, PD-1 and CTLA4 in myelodysplastic syndromes is enhanced by treatment with hypomethylating agents. *Leukemia* 28(6), 1280–1288 (2014).

44 Orskov AD, Treppendahl MB, Skovbo A *et al.* Hypomethylation and up-regulation of PD-1 in T cells by azacytidine in MDS/AML patients: a rationale for combined targeting of PD-1 and DNA methylation. *Oncotarget* 6(11), 9612–9626 (2015).

45 Huls G. Azacitidine in AML: a treatment option? *Blood* 126(3), 283–284 (2015).

46 Saba HI. Decitabine in the treatment of myelodysplastic syndromes. *Ther. Clin. Risk Manag.* 3(5), 807–817 (2007).

47 Curran MP. Decitabine: a review of its use in older patients with acute myeloid leukaemia. *Drugs Aging* 30(6), 447–458 (2013).

48 Cowan LA, Talwar S, Yang AS. Will DNA methylation inhibitors work in solid tumors? A review of the clinical experience with azacitidine and decitabine in solid tumors. *Epigenomics* 2(1), 71–86 (2010).

49 Juergens RA, Wrangle J, Vendetti FP *et al.* Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. *Cancer Discov.* 1(7), 598–607 (2011).

50 Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J. Pilot Phase I–II study on 5-aza-2'-deoxycytidine (Decitabine) in patients with metastatic lung cancer. *Anticancer Drugs* 8(4), 358–368 (1997).

51 Momparler RL, Ayoub J. Potential of 5-aza-2'-deoxycytidine (Decitabine) a potent inhibitor of DNA methylation for therapy of advanced non-small cell lung cancer. *Lung Cancer* 34(Suppl. 4), S111–115 (2001).

52 Nakamura K, Nakabayashi K, Htet Aung K *et al.* DNA methyltransferase inhibitor zebularine induces human cholangiocarcinoma cell death through alteration of DNA methylation status. *PLoS One* 10(3), e0120545 (2015).

53 Napso T, Fares F. Zebularine induces prolonged apoptosis effects via the caspase-3/PARP pathway in head and neck cancer cells. *Int. J. Oncol.* 44(6), 1971–1979 (2014).

54 Helin K, Dhanak D. Chromatin proteins and modifications as drug targets. *Nature* 502(7472), 480–488 (2013).

55 Daigle SR, Olhava EJ, Therkelsen CA *et al.* Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell* 20(1), 53–65 (2011).

56 Liu F, Li F, Ma A *et al.* Exploiting an allosteric binding site of PRMT3 yields potent and selective inhibitors. *J. Med. Chem.* 56(5), 2110–2124 (2013).

57 Sauvageau M, Sauvageau G. Polycomb group proteins: multi-faceted regulators of somatic stem cells and cancer. *Cell Stem Cell* 7(3), 299–313 (2010).

58 Wu C, Jin X, Yang J *et al.* Inhibition of EZH2 by chemo- and radiotherapy agents and small molecule inhibitors induces cell death in castration-resistant prostate cancer. *Oncotarget* 7(3), 3440–3452 (2016).

59 Song X, Gao T, Wang N *et al.* Selective inhibition of EZH2 by ZLD1039 blocks H3K27methylation and leads to potent anti-tumor activity in breast cancer. *Sci. Rep.* 6 20864 (2016).

60 Park J, Thomas S, Munster PN. Epigenetic modulation with histone deacetylase inhibitors in combination with immunotherapy. *Epigenomics* 7(4), 641–652 (2015).

61 Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* 14, 233–258 (1996).

62 Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J. Exp. Med.* 165(2), 302–319 (1987).

63 Baxter AG, Hodgkin PD. Activation rules: the two-signal theories of immune activation. *Nat. Rev. Immunol.* 2(6), 439–446 (2002).

64 Ott PA, Hodi FS, Robert C. CTLA-4 and PD-1/PD-L1 blockade: new immunotherapeutic modalities with durable clinical benefit in melanoma patients. *Clin. Cancer Res.* 19(19), 5300–5309 (2013).

65 Peggs KS, Quezada SA, Allison JP. Cancer immunotherapy: co-stimulatory agonists and co-inhibitory antagonists. *Clin. Exp. Immunol.* 157(1), 9–19 (2009).

66 Callahan MK, Wolchok JD. At the bedside: CTLA-4- and PD-1-blocking antibodies in cancer immunotherapy. *J. Leukoc. Biol.* 94(1), 41–53 (2013).

67 Woo SR, Turnis ME, Goldberg MV *et al.* Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res.* 72(4), 917–927 (2012).

68 Schaer DA, Cohen AD, Wolchok JD. Anti-GITR antibodies – potential clinical applications for tumor immunotherapy. *Curr. Opin. Investig. Drugs* 11(12), 1378–1386 (2010).

69 Romagne F, Vivier E. Natural killer cell-based therapies. *F1000 Med. Rep.* 3 9 (2011).

70 Hammers HJ, Plimack ER, Infante JR *et al.* Expanded cohort results from CheckMate 016: a Phase I study of nivolumab in combination with ipilimumab in metastatic renal cell carcinoma (mRCC). *ASCO Meeting Abstracts* 33(15_Suppl.), 4516 (2015).

71 Larkin J, Chiarion-Sileni V, Gonzalez R *et al.* Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N. Engl. J. Med.* 373(1), 23–34 (2015).

72 Pentcheva-Hoang T, Corse E, Allison JP. Negative regulators of T-cell activation: potential targets for therapeutic intervention in cancer, autoimmune disease, and persistent infections. *Immunol. Rev.* 229(1), 67–87 (2009).

73 Robert C, Thomas L, Bondarenko I *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N. Engl. J. Med.* 364(26), 2517–2526 (2011).

74 Hodi FS, O'day SJ, Mcdermott DF *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.* 363(8), 711–723 (2010).

75 Kamphorst AO, Ahmed R. Manipulating the PD-1 pathway to improve immunity. *Curr. Opin. Immunol.* 25(3), 381–388 (2013).

76 Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. *Curr. Opin. Immunol.* 24(2), 207–212 (2012).

77 Park JJ, Omiya R, Matsumura Y *et al.* B7-H1/CD80 interaction is required for the induction and maintenance of peripheral T-cell tolerance. *Blood* 116(8), 1291–1298 (2010).

78 Ramsay AG, Clear AJ, Fatah R, Gribben JG. Multiple inhibitory ligands induce impaired T-cell immunologic synapse function in chronic lymphocytic leukemia that can be blocked with lenalidomide: establishing a reversible immune evasion mechanism in human cancer. *Blood* 120(7), 1412–1421 (2012).

79 Llosa NJ, Cruise M, Tam A *et al.* The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov.* 5(1), 43–51 (2015).

80 Wimberly H, Brown JR, Schalper K *et al.* PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer. *Cancer Immunol. Res.* 3(4), 326–332 (2015).

81 Ali HR, Glont SE, Blows FM *et al.* PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes. *Ann. Oncol.* 26(7), 1488–1493 (2015).

82 Loi S, Michiels S, Salgado R *et al.* Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. *Ann. Oncol.* 25(8), 1544–1550 (2014).

83 Chen DS, Irving BA, Hodi FS. Molecular pathways: next-generation immunotherapy-inhibiting programmed death-ligand 1 and programmed death-1. *Clin. Cancer Res.* 18(24), 6580–6587 (2012).

84 Kobold S, Duewell P, Schnurr M, Subklewe M, Rothenfusser S, Endres S. Immunotherapy in tumors. *Dtsch Arztebl. Int.* 112(48), 809–815 (2015).

85 FDA approves Opdivo to treat advanced form of kidney cancer. www.accessdata.fda.gov/drugsatfda_docs/label/2015/125554s012lbl.pdf

86 FDA approves Keytruda for advanced non-small cell lung cancer. www.fda.gov/newsevents/newsroom/pressannouncements/ucm465444.html

87 Linch SN, Mcnamara MJ, Redmond WL. OX40 agonists and combination immunotherapy: putting the pedal to the metal. *Front. Oncol.* 5 34 (2015).

88 Curti BD, Kovacsovics-Bankowski M, Morris N *et al.* OX40 is a potent immune-stimulating target in late-stage cancer patients. *Cancer Res.* 73(24), 7189–7198 (2013).

89 Sanmamed MF, Pastor F, Rodriguez A *et al.* Agonists of co-stimulation in cancer immunotherapy directed against CD137, OX40, GITR, CD27, CD28, and ICOS. *Semin. Oncol.* 42(4), 640–655 (2015).

90 Cohen AD, Schaer DA, Liu C *et al.* Agonist anti-GITR monoclonal antibody induces melanoma tumor immunity in mice by altering regulatory T cell stability and intra-tumor accumulation. *PLoS One* 5(5), e10436 (2010).

91 Lu L, Xu X, Zhang B, Zhang R, Ji H, Wang X. Combined PD-1 blockade and GITR triggering induce a potent antitumor immunity in murine cancer models and synergizes with chemotherapeutic drugs. *J. Transl. Med.* 12 36 (2014).

92 Mitsui J, Nishikawa H, Muraoka D *et al.* Two distinct mechanisms of augmented antitumor activity by modulation of immunostimulatory/inhibitory signals. *Clin. Cancer Res.* 16(10), 2781–2791 (2010).

93 Pruitt SK, Boczkowski D, De Rosa N *et al.* Enhancement of anti-tumor immunity through local modulation of CTLA-4 and GITR by dendritic cells. *Eur. J. Immunol.* 41(12), 3553–3563 (2011).

94 Wiersma Valerie R, van Bommel Peter E, De Bruyn Marco, Helfrich Wijnand, Bremer Edwin. CD47, a multi-facetted target for cancer immunotherapy. In: *Atlas of Genetics and Cytogenetics in Oncology and Haematology.* (2014).http://atlasgeneticsoncology.org/Deep/CD47TumAndImmunoID20140.html

95 Kohrt HE, Thielens A, Marabelle A *et al.* Anti-KIR antibody enhancement of anti-lymphoma activity of natural killer cells as monotherapy and in combination with anti-CD20 antibodies. *Blood* 123(5), 678–686 (2014).

96 Kohrt HE, Houot R, Goldstein MJ *et al.* CD137 stimulation enhances the antilymphoma activity of anti-CD20 antibodies. *Blood* 117(8), 2423–2432 (2011).

97 West AC, Smyth MJ, Johnstone RW. The anticancer effects of HDAC inhibitors require the immune system. *Oncoimmunology* 3(1), e27414 (2014).

98 Chumsri S, Lee M-J, Tomita Y *et al.* Epigenetic immune modulation by entinostat in breast cancer: correlative analysis of ENCORE 301 trial. *Cancer Res.* 76(4 Suppl.), Abstract P2–11–10 (2016).

99 Shen L, Ciesielski M, Ramakrishnan S *et al.* Class I histone deacetylase inhibitor entinostat suppresses regulatory T cells and enhances immunotherapies in renal and prostate cancer models. *PLoS One* 7(1), e30815 (2012).

100 Chumsri S, Necela B, Ordentlich P *et al.* Immunomodulatory effects of entinostat on PD-L1 and MHC class I and II in different subtypes of breast cancer. *Cancer Res.* 76(4 Suppl.), Abstract P2–04–02 (2016).

101 Oki Y, Buglio D, Zhang J *et al.* Immune regulatory effects of panobinostat in patients with Hodgkin lymphoma through modulation of serum cytokine levels and T-cell PD1 expression. *Blood Cancer J.* 4 e236 (2014).

102 Kim K, Skora AD, Li Z *et al.* Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc. Natl Acad. Sci. USA* 111(32), 11774–11779 (2014).

- **Authors show that the addition of epigenetic modifiers (the histone deacetylases inhibitors entinostat and the DNA methyltransferases inhibitors [DNMTi] azacitidine) to checkpoint inhibitors (anti-PD-1 and anti-CTLA-4) eradicates tumors with 80% of survival in tumor-bearing mice, primarily targeting myeloid-derived suppressor cells.**

103 Lampen MH, Van Hall T. Strategies to counteract MHC-I defects in tumors. *Curr. Opin. Immunol.* 23(2), 293–298 (2011).

104 Powers JJ, Maharaj KK, Sahakian E *et al.* Histone deacetylase 6 (HDAC6) as a regulator of immune checkpoint molecules in chronic lymphocytic leukemia (CLL). *Blood* 124(21), 3311 (2014).

105 Woods DM, Sodre AL, Villagra A, Sarnaik A, Sotomayor EM, Weber J. HDAC inhibition upregulates PD-1 ligands in melanoma and augments immunotherapy with PD-1 blockade. *Cancer Immunol. Res.* doi:10.1158/2326–6066. CIR-15–0077-T (2015) (Epub ahead of print).

106 Wong DJ, Rao A, Avramis E *et al.* Exposure to a histone deacetylase inhibitor has detrimental effects on human lymphocyte viability and function. *Cancer Immunol. Res.* 2(5), 459–468 (2014).

107 Kroesen M, Gielen P, Brok IC, Armandari I, Hoogerbrugge PM, Adema GJ. HDAC inhibitors and immunotherapy; a double edged sword? *Oncotarget* 5(16), 6558–6572 (2014).

108 Pan F, Fan H, Liu Z, Jiang S. T cell signaling targets for enhancing regulatory or effector function. *Sci. Signal* 5(235), doi:10.1126/scisignal.2003364 (2012) (Epub ahead of print).

109 Akimova T, Beier UH, Liu Y, Wang L, Hancock WW. Histone/protein deacetylases and T-cell immune responses. *Blood* 119(11), 2443–2451 (2012).

110 Akimova T, Ge G, Golovina T *et al.* Histone/protein deacetylase inhibitors increase suppressive functions of human FOXP3+ Tregs. *Clin. Immunol.* 136(3), 348–363 (2010).

111 Beier UH, Wang L, Han R, Akimova T, Liu Y, Hancock WW. Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. *Sci. Signal* 5(229), doi: 10.1126/scisignal.2002873 (2012) (Epub ahead of print).

112 Xiao H, Jiao J, Wang L *et al.* HDAC5 controls the functions of Foxp3 T-regulatory and CD8 T cells. *Int. J. Cancer* doi:10.1002/ijc.29979 (2015) (Epub ahead of print).

- **Authors demonstrate that in absence of HDAC5, Treg showed reduced suppressive function with a poor conversion of CD4$^+$ T cells in Treg, but this did not associate with better antitumor immunity due to the inability of CD8$^+$ T cells to produce specific cytokines.**

113 Bridle BW, Chen L, Lemay CG *et al.* HDAC inhibition suppresses primary immune responses, enhances secondary immune responses, and abrogates autoimmunity during tumor immunotherapy. *Mol. Ther.* 21(4), 887–894 (2013).

114 Christiansen AJ, West A, Banks KM *et al.* Eradication of solid tumors using histone deacetylase inhibitors combined with immune-stimulating antibodies. *Proc. Natl Acad. Sci. USA* 108(10), 4141–4146 (2011).

115 Saleh MH, Wang L, Goldberg MS. Improving cancer immunotherapy with DNA methyltransferase inhibitors. *Cancer Immunol. Immunother.*doi: 10.1007/s00262-015-1776-3 (2015) (Epub ahead of print).

116 Simova J, Pollakova V, Indrova M *et al.* Immunotherapy augments the effect of 5-azacytidine on HPV16-associated tumours with different MHC class I-expression status. *Br. J. Cancer* 105(10), 1533–1541 (2011).

117 Serrano A, Castro-Vega I, Redondo M. Role of gene methylation in antitumor immune response: implication for tumor progression. *Cancers (Basel)* 3(2), 1672–1690 (2011).

118 Li H, Chiappinelli KB, Guzzetta AA *et al.* Immune regulation by low doses of the DNA methyltransferase inhibitor 5-azacitidine in common human epithelial cancers. *Oncotarget* 5(3), 587–598 (2014).

119 Heninger E, Krueger TE, Lang JM. Augmenting antitumor immune responses with epigenetic modifying agents. *Front. Immunol.* 6 29 (2015).

120 Costantini B, Kordasti SY, Kulasekararaj AG *et al.* The effects of 5-azacytidine on the function and number of regulatory T cells and T-effectors in myelodysplastic syndrome. *Haematologica* 98(8), 1196–1205 (2013).

121 Wang L, Amoozgar Z, Huang J *et al.* Decitabine enhances lymphocyte migration and function and synergizes with CTLA-4 blockade in a murine ovarian cancer model. *Cancer Immunol. Res.* 3(9), 1030–1041 (2015).

- **Authors show that the synergistic combination of the DNMTi decitabine with anti-CTLA-4 prolonged mouse survival in a murine ovarian cancer model promoting naive T cells differentiation into effector T cells and prolonging cytotoxic lymphocyte responses.**

122 Youngblood B, Oestreich KJ, Ha SJ *et al.* Chronic virus infection enforces demethylation of the locus that encodes PD-1 in antigen-specific CD8(+) T cells. *Immunity* 35(3), 400–412 (2011).

123 Wrangle J, Wang W, Koch A *et al.* Alterations of immune response of non-small cell lung cancer with azacytidine. *Oncotarget* 4(11), 2067–2079 (2013).

124 Roulois D, Loo Yau H, Singhania R *et al.* DNA-demethylating agents target colorectal cancer cells by inducing viral mimicry by endogenous transcripts. *Cell* 162(5), 961–973 (2015).

125 Chiappinelli KB, Strissel PL, Desrichard A *et al.* Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. *Cell* 162(5), 974–986 (2015).

• **Authors report that DNMTi upregulate immune signaling in ovarian cancer through the viral defense pathway, triggering cytosolic sensing of dsRNA and correlate high viral defense signature expression in melanoma patients treated with an immune checkpoint therapy with durable clinical response.**

126 Peng D, Kryczek I, Nagarsheth N *et al.* Epigenetic silencing of TH1-type chemokines shapes tumour immunity and immunotherapy. *Nature* 527(7577), 249–253 (2015).

• **Authors report that H3K27me3 and DNA methylation can independently repress tumor TH1-type chemokine expression and that tumor EZH2 and DNMT1 are negatively associated with tumor-infiltrating CD8$^+$ T cells and patient outcome.**

127 Nagarsheth N, Peng D, Kryczek I *et al.* PRC2 epigenetically silences Th1-type chemokines to suppress effector T-cell trafficking in colon cancer. *Cancer Res.* 76(2), 275–282 (2016).

128 Patel SP, Kurzrock R. PD-L1 expression as a predictive biomarker in cancer immunotherapy. *Mol. Cancer Ther.* 14(4), 847–856 (2015).