

Molecular Pathways: At the Crossroads of Cancer Epigenetics and Immunotherapy

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Abstract

Epigenetic regulation allows heritably modulating gene expression profiles without modifying the primary sequence of gDNA. Under physiologic conditions, epigenetic patterns determine tissue-specific gene expression landscapes, gene imprinting, inactivation of chromosome X, and preservation of genomic stability. The most characterized mediators of epigenetic inheritance are gDNA methylation and histone posttranslational modifications that cooperate to alter chromatin state and genome transcription. According to these notions, it is not surprising that cancer cells invariably deploy epigenetic alterations to achieve gene expression patterns required for neoplastic transformation and tumor progression. In this context, the recently uncovered use of epigenetic alterations by cancer cells to become stealth from the host's

immune recognition has significant immunobiologic relevance in tumor progression, and it appears to have potential clinical usefulness. Indeed, immune evasion is among the major obstacles to further improve the efficacy of cancer immunotherapies and to increase long-lasting disease control. Luckily, different "epigenetic drugs" able to revert these "epimutations" are available, some of which have already been approved for clinical use. Here, we summarize the immunomodulatory activities of epigenetic drugs that lead to improved immune recognition of cancer cells and focus on the potential of this class of agents in improving the anticancer activity of novel immunotherapies through combinatorial epigenetic immunotherapy approaches. *Clin Cancer Res*; 21(18); 4040–7. ©2015 AACR.

Background

The clinical potential of cancer immunotherapy relies on the well-established notion that the immune system can efficiently eradicate cancer cells being recognized as non-self. This anticancer activity results from a series of genetic, epigenetic, and regulatory modifications occurring along with neoplastic transformation that lead to an altered cell membrane molecular repertoire, as well as to a *de novo* or upregulated expression of tumor-associated antigens (TAA). Immune recognition of neoplastic cells is mainly accomplished by innate and adaptive immune responses. The former is not antigen specific and relies mainly on the activity of natural killer (NK) cells to recognize transformed cells on the basis of the fine balance of their surface expression of activating and inhibitory ligands. Among these, the engagement of the NK activatory receptor NKG2D (KLRK1) with a series of stress-induced ligands [e.g., MHC class I-related chain A (MICA), MICB, UL16-binding proteins (ULBP)] expressed on cancer cells appears to have a major role in NK-mediated killing. Target cell destruction is achieved through the activation of death receptors (e.g., FAS and TNFRSF10) on cancer cells engaged by NK-cell ligands

(e.g., FASL and TNFSF10) and/or by the release of cytotoxic granules by NK cells (1). Adaptive immune responses, on the other hand, are antigen specific, involve mainly CD8⁺ and CD4⁺ T lymphocytes, and result in an immunologic memory that allows a more rapid and efficient response at subsequent encounters with the antigen. Within this effector branch of the immune system, CD8⁺ cytotoxic T lymphocytes (CTL) play the main role and, at variance with NK cells, require the engagement of their antigen-specific T-cell receptors (TCR) with TAA presented on neoplastic cell in the form of MHC class I molecule-bound peptides. The generation of the cell surface-bound peptide–MHC complexes is carried out by the antigen processing and presentation machinery (APM) through a multistep process involving (i) generation of peptides through the proteolytic cleavage of TAA by the proteasome [including LMP2 (PSMB9) and LMP7 (PSMB8) subunits]; (ii) translocation of the peptides in the endoplasmic reticulum by the heterodimeric TAP1/TAP2 complex; (iii) loading of MHC class I molecules with peptides, assisted by the chaperones calreticulin (CALR), ERp57 (PDIA3), and tapasin (TAPBP); and (iv) final delivery of MHC class I–peptide complex to the cell surface via the trans-Golgi apparatus. Effective recognition of MHC–peptide complexes by effector T cells further requires tumor cells to express costimulatory/accessory molecules such as CD80, CD86, and ICAM1 that engage their specific counter-receptors on T cells (Fig. 1) (2).

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Epigenetically mediated tumor immune escape and tumor immunomodulation by epigenetic drugs

Tumor outgrowth and peripheral immune tolerance to cancer invariably requires transformed cells to acquire immune evasive phenotypes and the capacity to generate an immunosuppressive microenvironment (3, 4). Among the various molecular tools that

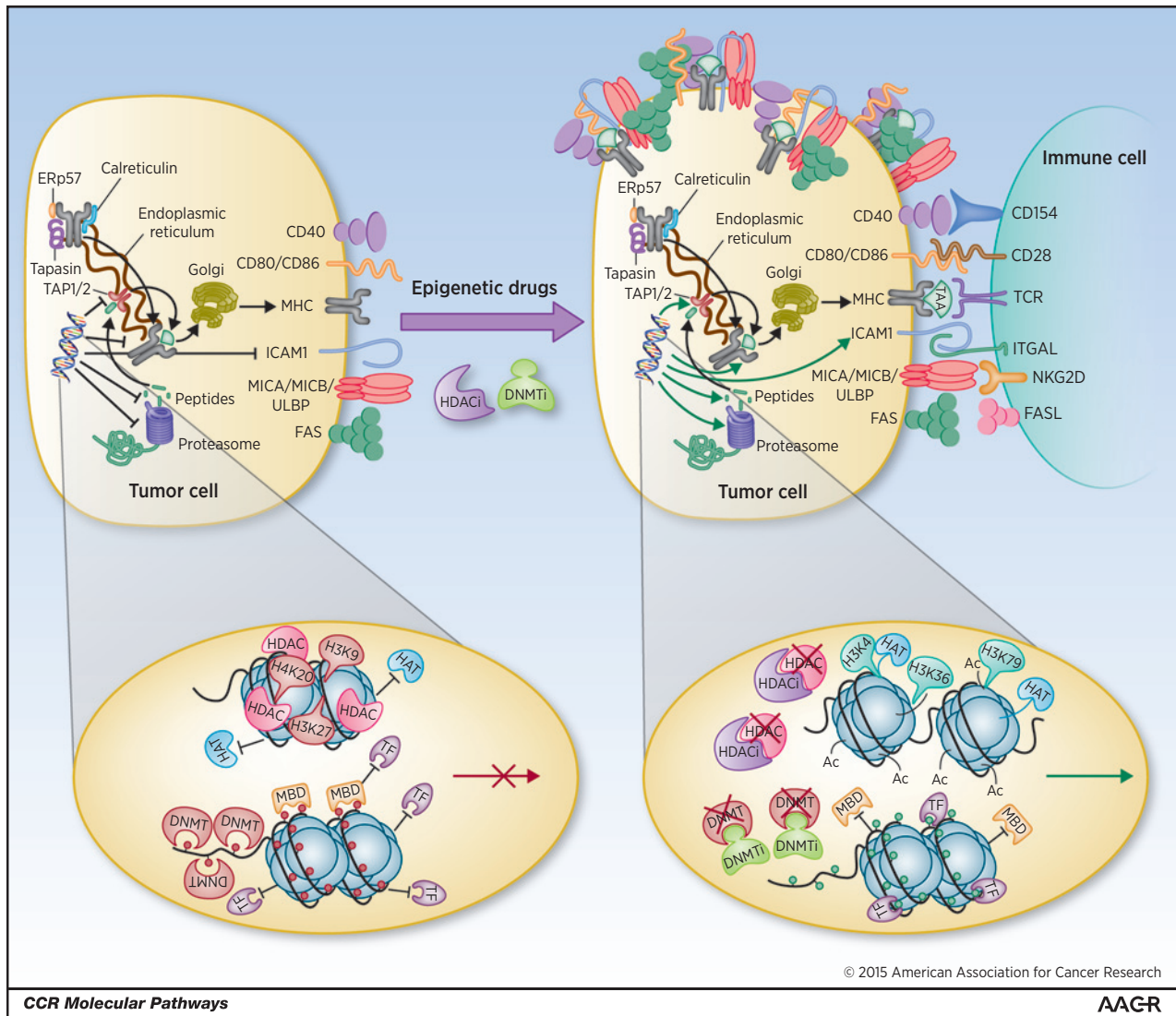


Figure 1.

Epigenetics and its pharmacologic editing in immune recognition of cancer cells. Histone posttranslational modifications and DNA methylation cooperate in the epigenetic control of gene expression. Transcriptionally inactive chromatin (left side of the figure and detailed in spotlight) is characterized by (i) deacetylated histones; (ii) trimethylation of histone H3 lysines 9 and 27 (H3K9, H3K27) and of histone H4 lysine 20 (H4K20); and (iii) methylated cytosines (red circles) within CpG dinucleotides. Inhibition of transcription (red cross arrow) may directly derive from methylated sequence preventing the binding of transcription factors (TF) or may be a consequence of the binding of methyl CpG-binding domain proteins (MBD). CTLA requires engagement of the antigen-specific TCR on their cell membranes with the TAA presented at the surface of the neoplastic cell as of peptides associated to MHC class I molecules. Peptides are generated through proteolytic cleavage of TAA by the proteasome and translocated in the endoplasmic reticulum by the heterodimeric TAP1/TAP2 complex. MHC class I molecules are then loaded with peptide, assisted by the chaperone molecules ERp57, tapasin, and calreticulin, and finally delivered to the cell surface via the trans-Golgi apparatus. Effective recognition of MHC-peptide complexes by effector T cells further requires tumor cells to express costimulatory/accessory molecules such as CD80, CD86, and ICAM1 that engage their receptors on T cells. By downregulating the expression of the above described molecules, the final effect of epigenetic alterations occurring in cancer is to impair the recognition of neoplastic cells by the immune cells. On the other hand, methylation of histone H3 lysines 4, 36, and 79 (H3K4, H3K36, and H3K79), histone acetylation (Ac), and demethylated promoters (green circles) prevent the binding of MBD and recruit activating complexes, including transcription factors, finally resulting in a transcriptionally active state of chromatin (green arrow, right side of the figure and detailed in spotlight; refs. 7, 8). Epigenetic drugs, such as DNMT inhibitors (DNMTi) and HDAC inhibitors (HDACi), can be effectively used to revert the aberrant silencing of genes, improving the immune phenotype of neoplastic cells by affecting one or more of the key requirements for the immune recognition of cancer cells. These include upregulation on neoplastic cells of (i) TAA; (ii) TAP1/2; (iii) chaperones; (iv) MHC class I and class II molecules; (v) the CD40, CD80, CD86, and ICAM1 costimulatory/accessory molecules; (vi) the NKG2D ligands MICA, MICB and ULBP; and (vii) death receptors (e.g., FAS).

tumor cells use to achieve immune escape, triggering of altered epigenetic statuses is a key factor. Indeed, epigenetic modulation through DNA methylation and/or histone posttranslational modifications allows cancer cells to establish heritable, and sometimes

extensive, changes in gene expression profiles without altering the gDNA sequence. Epigenetic marks require the activity of specific cellular enzymes to be generated and maintained (Fig. 1): DNA methyl transferases (DNMT) for DNA methylation, and the

opposite activities of histone acetyl transferases (HAT)/histone deacetylases (HDAC) and histone methyltransferases (HMT)/histone demethylases for determining the status of histone acetylation and methylation, respectively. Epigenetic gene regulation is finally delivered by the cooperation of promoter DNA methylation, histone deacetylation, and by specific patterns of histone methylation that trigger chromatin condensation leading to gene silencing (Fig. 1; refs. 5–10).

Although limited data are available on their role in establishing an immunosuppressive microenvironment, epigenetic alterations are well-acknowledged to be used by tumor cells to impair their immunogenicity and immune recognition. The latter occurs through the downregulation, either direct or indirect, of the expression of key molecules required for the efficient interaction of cancer cells with the host's immune system. Epigenetic silencing affects essentially all steps of antigen processing and presentation, including suppression of TAA expression, generation of intratumor TAA heterogeneity, downregulation of TAP1/2 and chaperone molecules, reduced MHC expression, as well as reduced levels of accessory/costimulatory molecules and of surface exposed stress-induced ligands (Fig. 1; reviewed in ref. 11).

The significant role of epigenetics in cancer immune escape provides a strong rationale for the use of epigenetic modifiers to improve immunologic targeting of neoplastic cells and to eventually design more effective cancer immunotherapies (11). Along this line, different epigenetic drugs that can revert epigenetic modifications are available, being currently used in the daily practice or in clinical trials (12–15). Among these, the best characterized are DNMT inhibitors (DNMTi) and HDAC inhibitors (HDACi). DNMTi lead to passive demethylation of gDNA following DNA replication and, among others, comprise 5-azacytidine (Vidaza; Baxter Oncology GmbH/Celgene), 5-aza-2'-deoxycytidine [5-aza-CdR, decitabine, Dacogen (Otsuka America)], zebularine, and RG108. However, second-generation DNMTi [e.g., guadecitabine (SGI-110)] have been recently developed to improve their *in vivo* stability and to reduce toxicity to normal tissues. On the other hand, HDACi lead to the accumulation of hyperacetylated histones into the chromatin, by shifting the balance of HAT/HDAC activity toward HAT (16). HDACi include sodium butyrate, valproic acid, trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA, vorinostat), LBH589 (panobinostat), FK288 (depsipeptide/romidepsin), and MS-275 (entinostat). Both DNMTi and HDACi have proved effective in inducing/upregulating gene expression *in vitro* and *in vivo*, apparently displaying a preferential activity on transformed versus normal cells (17). Furthermore, concomitant use of DNMTi and HDACi can show synergism in gene reexpression/upregulation as expected from the physiologic cooperation of DNA methylation and histone posttranslational modifications in regulating gene expression (18).

Epigenetic drugs have been consistently demonstrated to be able to modulate the immune phenotype of neoplastic cells by affecting one or more key requirements for their efficient immune recognition, independently from tumor histotype. Although DNMTi are undoubtedly the best characterized immunomodulatory epigenetic agents, consistent data are emerging also for HDACi, and to a lesser extent for modulators of histone methylation (recently reviewed in ref. 11). Using mouse and human neoplastic cells, epigenetic drugs have been shown to upregulate essentially all the components of the APM, including (i) TAA [e.g., PMEL, cancer testis antigens (CTA)], for presence, levels, and intratumoral distribution; (ii) the proteasome subunits LMP2

and LMP7; (iii) the TAP1 and TAP2 transporters; (iv) the chaperones ERp57 and calreticulin; and (v) MHC class I and class II molecules. Besides APM, epigenetic agents improved the costimulatory properties of cancer cells by upregulating surface expression of CD40, CD80, CD86, and ICAM1 and restored their sensitivity to immune cell triggered apoptosis by enhancing the expression of death-inducing receptors (e.g., FAS; Fig. 1; refs. 19–22). Although the immunomodulatory activities of DNMTi and of HDACi are frequently overlapping, some preferential tropism of the 2 drug classes can be observed, essentially depending on the prevalent molecular mechanism regulating the specific target molecules. Along this line, induction/upregulation of CTA and ICAM1 is mostly affected by DNMTi, whereas HDACi mainly affect the expression of costimulatory molecules. Of relevance, long-lasting phenotypic immunomodulation appears to be essentially restricted to treatments with DNMTi through their ability to generate heritable changes in gene expression (23). The clinical appeal of these findings relays on the ability of the phenotypic modulations induced by epigenetic compounds to translate into an upregulated functional recognition and killing of tumor cells by antigen-specific CTL, both *in vitro* and *in vivo* (11, 20). In addition, HDACi were found particularly effective in inducing a stress response on tumor cells, which led to the upregulated expression on their surface of the NKG2D ligands (MICA, MICB, and ULBPs) and to an increased killing of tumor cells by NK cells (Fig. 1; ref. 24). These observations are undoubtedly not exhaustive of the full immunomodulatory potential of epigenetic drugs as demonstrated by high-throughput gene expression analyses substantiating their broader tumor immunomodulatory activity both *in vitro* and *in vivo* (17, 25).

In line with these preclinical data, initial clinical evidence, mainly derived from hematologic malignancies, confirmed the ability of DNMTi to modulate the immune phenotype of cancer cells, inducing long-lasting expression of TAA (26, 27), that can elicit antigen-specific humoral and T-cell immune responses (26, 28).

Immunogenic cell death

Another means by which epigenetic drugs can improve immune rejection of cancer stems from their ability to induce immunogenic cell death (ICD). ICD is a newly described form of cell death caused by selected chemotherapeutic drugs. In this context, drug-induced cell death leads to the release of a series of danger-associated molecular signals that label tumor cells as a pathogen, thus rendering them immunogenic. These molecular events finally lead to an increased uptake and immunogenic presentation of tumor antigens by professional antigen-presenting cells, which is mandatory for the induction of antitumor T-cell immune responses (29).

Among epigenetic drugs, the ability to induce ICD is mostly a feature of HDACi. Indeed, the pan HDACi vorinostat was proven effective in improving the phagocytosis of colon carcinoma cells by dendritic cells (DC) *in vitro* and in inducing the release of different ICD mediators, including ATP and HMGB1, and an early surface exposure of the "eat me signal" calreticulin by neoplastic cells of different histology (30, 31). In line with these notions, the anticancer activity of vorinostat and panobinostat was significantly reduced in immunocompromised versus immunocompetent mice, highlighting the requirement of an intact immune system for the full therapeutic activity of the drugs (31). Similarly, when mice were vaccinated with pancreatic carcinoma cells either

untreated or pretreated with vorinostat, only the latter elicited antitumor immune responses that increased CD8⁺ T-cell infiltration of tumor tissues and protected mice from tumor growth (32).

Clinical-Translational Advances

The well-established immunomodulatory potential of epigenetic drugs provides a strong scientific rationale to translate it into the clinic by designing novel combination regimens with emerging immunotherapies. This process can be swiftly implemented taking advantage from the recent availability of highly effective immunotherapeutic agents that are being extensively investigated in most solid tumors (33) and by the consolidated clinical use of the DNMTi 5-azacytidine and 5-aza-CdR and the HDACi vorinostat and romidepsin in patients with myelodysplastic syndrome and cutaneous T-cell lymphoma, respectively (12, 34).

Combined epigenetic immunotherapies in the mouse

Preclinical *in vivo* evidence in different tumor types has begun to show an improved antitumor activity by the concomitant immunomodulation of the tumor and of the host's immune system by epigenetic and immunotherapeutic drugs, respectively.

Initial approaches investigated combinations with cytokine-based immunotherapy, vaccination with tumor cells either producing cytokines or administered with adjuvants, as well as with peptide vaccines. In these models, the antitumor activity of the different combinations accounted for reductions from 50% to 90% in tumor volumes as compared with untreated mice. The efficacy of treatment was found to be immune mediated as it required CD4⁺ and CD8⁺ T and/or NK cells (11, 35–38). Interestingly, in the murine renal cell carcinoma RENCA model, HDACi combined with IL2 was demonstrated to impair the function of the T-regulatory (Treg) immunosuppressive cells, by downregulating the expression of their master transcription factor *Foxp3*. The effect on Treg cooperated with the increased activation of effector T cells by IL2, leading to about 80% reduction in tumor growth in mice treated with the combination as compared to controls. On the basis of this evidence, it is tempting to speculate that HDACi could further contribute to a more efficient immune clearance of tumor cells in immunotherapies by counteracting the immunosuppressive environment that sustains cancer progression (37). A similar reduction in the number of Treg cells, both at the periphery and at the tumor site, was achieved by the HDACi panobinostat when combined with the adoptive transfer of Pmel-specific T cells. The enhanced effect on adoptive therapy appeared to depend on the ability of the HDACi to deplete Treg, to create a highly proinflammatory environment, and to enhance Pmel-specific T-cell survival (39). Overall, boosting of adoptive cell therapy could take advantage of the concomitant activity of epigenetic drugs on tumor cells, effector cells, and recipient (37, 40, 41). Supporting this hypothesis, the epigenetic induction of the murine CTA TRAP1a, and its proper presentation by tumor cells, accounted for a dramatic reduction in the average number of lung metastases observed in the 4T1 syngeneic mammary carcinoma model when an adoptive transfer of anti-TRAP1a CTL was combined with systemic administration of 5-aza-CdR: 72, 72, 32, and 3 lung metastases for mice untreated or treated with anti-TRAP1a CTL, 5-aza-CdR, or their combination were found, respectively (41). On the other hand, the HDACi LAQ824 upregulated MHC class I antigens and Pmel TAA on B16 mouse melanoma grafts, simultaneously improving the activity of adop-

tively transferred anti-Pmel CTL also through a selective reduction of endogenous competing lymphocytes in the recipient mice. These activities collectively contributed to the synergistic antitumor activity of the HDACi once combined with immunotherapy consisting of anti-Pmel T-cell transfer plus Pmel peptide-pulsed DC vaccine, leading to a significant 70% reduction in tumor volume versus untreated mice, as compared with reductions in tumor volumes of 49% and 21% achieved with immunotherapy or HDACi alone, respectively (40).

The improved understanding of the fine mechanism(s) regulating the physiologic cross-talk among immune cells has recently led to the development of a new therapeutic approach in the clinic: rather than directly targeting tumor cells, monoclonal antibodies (mAb) have been developed to target well-defined inhibitory or activating immune checkpoint molecules expressed on immune cells, thus boosting effective antitumor immune responses (33). This paradigm shift in immunotherapy allowed us to circumvent, at least in part, the immunosuppressive features of neoplastic cells, leading to notable clinical responses also in human malignancies characterized by therapeutic resistance (42). Owing to these results, mAbs blocking the immune checkpoint inhibitors cytotoxic T-lymphocyte antigen 4 (CTLA-4; i.e., ipilimumab) and programmed cell death protein 1 (PD-1, PDCD1; i.e., pembrolizumab and nivolumab) were recently approved for the treatment of advanced cutaneous melanoma (33), as most recently has been nivolumab for chemotherapy-resistant lung cancer. Besides representing the most promising cancer immunotherapeutic tools presently available, mAb targeting immune checkpoints appear to be ideal candidates to explore novel therapeutic combinations with epigenetic drugs. Indeed, an improved antitumor activity could be expected by concomitantly acting on the host's immune system using an immunomodulatory mAb and at the tumor site using DNMTi and/or HDACi as immunomodulators. Providing experimental support to this hypothesis, combined treatment with the CTLA-4-blocking mAb 9H10 and either 5-aza-CdR (43) or the second-generation DNMTi guadecitabine (44) significantly reduced the growth of poorly immunogenic syngeneic grafts of murine mammary carcinoma TS/A and of mesothelioma AB1 with respect to treatment with the single agents. Compared with control mice, mice treated with 5-aza-CdR, mAb 9H10, or their combination showed tumor growth that was reduced by 54% ($P < 0.01$), 33% ($P = 0.2$), and 77% ($P < 0.01$) in the TS/A model and by 33% ($P = 0.10$), 0% and 81% ($P < 0.05$) in the AB1 model, respectively (43). Results consistent with those obtained with 5-aza-CdR were subsequently obtained with SGI-110 in the TS/A model (44). The role of immune mechanism(s) in delivering the improved antitumor effectiveness of the therapeutic combination was supported both by its absence in immunocompromised mice and by the highest degree of infiltrating CD3⁺ T cells in the tumors of immunocompetent mice undergoing combination therapy (43, 44). The fine molecular and cellular mechanism(s) underlying the improved anticancer activity of the combined treatment remain to be further explored but can be explained, at least in part, by the upregulated expression of TAA and MHC class I antigens induced in tumor grafts by DNMTi (43, 44). In line with these data, combined treatment with 5-azacytidine and entinostat and with checkpoint inhibitors [anti-PD-1 (PDCD1) and anti-CTLA-4 mAb] markedly improved therapeutic outcomes in syngeneic mammary (i.e., 4T1) and colorectal (i.e., CT26) carcinoma mouse models. Combination therapy eradicated primary tumors in 91% of CT26- and

in 100% of 4T1-bearing mice after 3 weeks and 2 weeks, respectively. Moreover, 4T1-bearing mice that received combination therapy did not develop metastases as compared with mice treated with single agents. In this setting, the improved antitumor activity of the combination was found to be mainly dependent on the elimination of myeloid-derived suppressor cells by the epigenetic drug (45).

The broader potential of mAb targeting immune checkpoints as useful therapeutic partners for epigenetic drugs is further supported by the demonstration that treatment with agonistic mAb against the activating immune checkpoint targets CD40 and CD137 (*Tnfrsf9*) in combination with vorinostat and panobinostat promoted DC activation, CTL proliferation and survival, being capable of eradicating tumors by CD8⁺ CTL and NK cells (46). Indeed, anti-CD40 plus anti-CD137 immunotherapy combined with vorinostat delayed the outgrowth of mammary (i.e., 4T1.2), colorectal (i.e., MC38), and renal (i.e., Renca) carcinomas, leading to the eradication of tumors in 25%, 56%, and 25% of mice, respectively. Similar antitumor responses were observed when panobinostat was used instead of vorinostat for the treatment of mice bearing mammary (i.e., 4T1.2), prostate (i.e., MR1), and colon (i.e., CT26) carcinomas.

Although generated in mouse models, these data provide support to the use of novel therapeutic combination(s) with epigenetic drugs to improve the antitumor activity of mAbs against different immune checkpoints, particularly in the context of poorly immunogenic tumors that still represent a major therapeutic challenge.

Clinical perspectives

On the basis of the *in vitro* and *in vivo* preclinical data substantiating the immunomodulatory potential of epigenetic drugs and their ability to improve antitumor activity when combined with immunotherapeutic agent(s), several clinical studies are under way to investigate the safety and effectiveness of these novel combinations in patients with cancer (Table 1). Although limited, the clinical results so far available offer valuable insights that could help with the design of epigenetic/immunotherapy combinations. Combining 5-aza-CdR and PEGylated IFN α -2b in patients with advanced melanoma demonstrated the efficacy of 5-aza-CdR in inducing gDNA hypomethylation at all doses investigated; however, the slow accrual, limited therapeutic efficacy, and significant myelosuppression associated with treatment led to the discontinuation of the trial (47). On the other hand, 5-aza-CdR combined with NY-ESO-1 (CTAG1B) whole protein vaccination and doxorubicin chemotherapy in patients with relapsed ovarian cancer showed limited and manageable toxicities, still providing both global and gene-specific demethylation. Increased humoral and T-cell immune responses were observed against NY-ESO-1 and additional TAA and disease stabilization or partial clinical response occurred in 6 of 10 evaluable patients (48). Overall, both studies confirmed the epigenetic editing ability of DNMTi in patients with cancer and also clearly suggest that careful attention must be paid in designing new studies due to the significant myelotoxicity of DNMTi. The latter undoubtedly represents a major side effect of this class of agents to be taken into consideration and highlights that doses, schedules, and timing of their administration must be carefully reasoned in future studies; nevertheless, ongoing studies will hopefully provide some practical insights on this specific aspect (Table 1). Along this line, a preferred strategy will be represented by the use of DNMTi in combination with immunotherapies, including therapeutic can-

cer vaccines and mAbs directed to diverse immune checkpoints, due to their substantial nonoverlapping toxicity with epigenetic drugs.

Sparing patients from the toxicity of epigenetic compounds administered systemically, although still taking advantage of their immunomodulatory activity, represents another intriguing strategy. Along this line, the phase I trial NCT01258868 in patients with resectable thoracic cancers foresees the *ex vivo* treatment of autologous neoplastic cells with 5-aza-CdR to improve their immunogenicity by upregulating a series of molecules, including CTA. *In vitro* demethylated tumor cells are then emulsified in ISCOMATRIX adjuvant and used to vaccinate patients together with systemic administration of the anti-inflammatory drug celecoxib to improve immune responses by targeting immunosuppressor cells. Although this strategy overcomes the systemic toxicity of epigenetic drugs, a significant limitation derives from the need of autologous tumor tissue to be cultured *in vitro* to generate the autologous vaccine and from the finite source of vaccinating neoplastic cells; an additional limit to this approach may derive from the lack of *in vivo* tumor immunomodulation induced by the systemic administration of 5-aza-CdR. These specific issues are not present in other vaccine-based combinations being tested in phase I and phase I/II studies (NCT02332889, NCT01241162, NCT01483274) in different solid tumors and in acute myelogenous leukemias (AML). In these studies, patients are pretreated with 5-aza-CdR to upregulate the expression, processing, and presentation of CTA by the neoplastic cells, aiming to improve their CTA-specific immune recognition boosted by the subsequent vaccination with autologous DC pulsed with CTA peptides (i.e., MAGEA1, MAGEA3, and NY-ESO-1). Although undoubtedly intriguing, a potential drawback of this approach could derive from the limited number of CTA used to pulse DC and against which patients should mount/boost an immune response, thus not taking full advantage of the multiple target CTA that are induced/upregulated on tumor cells *in vivo* by the systemic administration of 5-aza-CdR. This aspect is being addressed in part by a pilot study in metastatic colorectal cancer in which systemic treatment with guadecitabine is combined with an allogeneic colon cancer cell vaccine (GVAX) that provides multiple potential antigenic targets (NCT01966289). Although the safety of the combination is the primary objective, this study will also evaluate the recruitment of CD45RO⁺ T cells at the tumor site as a surrogate marker of potential antitumor activity of treatment. Of note, this phase I trial will likely provide initial hints also on the preferable scheduling of treatments by comparing their concurrent administration versus the administration of guadecitabine followed by patient vaccination, which, however, seems to represent a more desirable approach. More comprehensively, the approach of combining CTA-based vaccinations and the administration of epigenetic drugs will likely take further advantage of the availability of the multivalent, CTA-based, immune cell-derived demethylated autologous vaccine DeMethaVax that some of us will bring shortly in the clinic.

The above studies have a clear-cut rationale and promise to improve the effectiveness of therapeutic vaccines a great deal, rejuvenating interest in cancer vaccination. Nevertheless, epigenetic drug combinations with mAb targeting immune checkpoints represents an additional, highly promising, feasible, and intriguing strategy. This notion stems from the convincing preclinical data generated in mouse models, by the significant clinical effectiveness of immunomodulating mAbs as monotherapies,

Table 1. Ongoing clinical trials evaluating the combination use of epigenetic drugs and immunotherapy

Clinical trial identifier	Title	Recruitment status	Epigenetic drug	Immunotherapy	Date
EUDRACT 2015-001329-17	A phase Ib, open-label, dose-escalation study investigating different doses of guadecitabine in combination with ipilimumab in unresectable or metastatic melanoma patients	Not yet recruiting	Guadecitabine (s.c.) ^a	Ipilimumab	Not submitted
NCT01834248	DEC-205/NY-ESO-1 fusion protein CDX-1401 and decitabine in treating patients with myelodysplastic syndrome or AML	Recruiting	Decitabine (i.v.)	DEC-205/NY-ESO-1 fusion protein CDX-1401 (s.c. and i.d.) and PolyICLC (s.c.)	First received: April 12, 2013 Last updated: January 15, 2015 Last verified: January 2015
NCT01928576	Phase II anti-PD-1 epigenetic priming study in NSCLC (NA_00084192)	Recruiting	Azacitidine (s.c.) and entinostat (p.o.) or CC-486 (oral azacitidine) alone (p.o.)	Nivolumab	First received: August 21, 2013 Last updated: August 23, 2013 Last verified: August 2013
NCT01038778	Entinostat in combination with aldesleukin in treating patients with metastatic kidney cancer	Recruiting	Entinostat (p.o.)	Aldesleukin (i.v.)	First received: December 18, 2009 Last updated: February 18, 2015 Last verified: December 2014
NCT02332889	Phase I/II: decitabine/vaccine therapy in relapsed/refractory pediatric high-grade gliomas/medulloblastomas/CNS PNETs	Not yet recruiting	Decitabine (i.v.)	Vaccine (autologous DC pulsed with pooled, overlapping peptide mixes derived from full-length MAGE-A1, MAGE-A3, and NY-ESO-1) and hiltonol (PolyICLC) (i.m.)	First received: October 20, 2014 Last updated: January 5, 2015 Last verified: January 2015
NCT01483274	Decitabine and vaccine therapy for patients with relapsed AML following allogeneic stem cell transplantation	Not yet recruiting	Decitabine	Donor lymphocyte infusion and dendritic cells pulsed with peptides derived from full-length NY-ESO-1, MAGE-A1, and MAGE-A3	First received: November 3, 2011 Last updated: December 1, 2014 Last verified: December 2014
NCT01673217	Decitabine, vaccine therapy, and PEGylated liposomal doxorubicin hydrochloride in treating patients with recurrent ovarian epithelial cancer, fallopian tube cancer, or peritoneal cancer	Completed	Decitabine (i.v.) PEGylated liposomal doxorubicin hydrochloride (i.v.)	NY-ESO-1 peptide vaccine emulsified in incomplete Freund adjuvant and sargramostim (s.c.)	First received: August 22, 2012 Last updated: January 10, 2014 Last verified: January 2014
NCT01241162	Decitabine followed by a cancer antigen vaccine for patients with neuroblastoma and sarcoma	Recruiting	Decitabine (i.v.)	Autologous DC vaccine pulsed with overlapping peptide mixes derived from full-length NY-ESO-1, MAGE-A1, and MAGE-A3 with hiltonol (PolyICLC)	First received: November 15, 2010 Last updated: December 17, 2014 Last verified: December 2014
NCT01258868	Tumor cell vaccines with ISCOMATRIX adjuvant and celecoxib in patients undergoing resection of lung and esophageal cancers and malignant pleural mesotheliomas	Recruiting	Decitabine for preparation of autologous cellular vaccine	Epigenetically modified autologous tumor cell vaccines with ISCOMATRIX (patient tumor cells exposed to decitabine, exposed to radiation and emulsified in ISCOMATRIX adjuvant) (i.m.)	First received: December 10, 2010 Last updated: February 20, 2015 Last verified: February 2015
NCT01966289	Guadecitabine in combination with an allogeneic colon cancer cell vaccine (GVAX) and cyclophosphamide (CY) in metastatic colorectal cancer (mCRC)	Not yet open for participant recruitment	Guadecitabine (s.c.)	Allogeneic colon cancer cell vaccine (GVAX) (i.d.) and cyclophosphamide (i.v.)	First received: October 10, 2013 Last updated: October 16, 2013 Last verified: October 2013

Abbreviations: CNS, central nervous system; PNET, primitive neuroectodermal tumor.

^aAdministration route: i.d., intradermal; i.m., intramuscular; i.v., intravenous; p.o., per os; s.c., subcutaneous.

and by the potential higher reproducibility of results achievable by using already "industrialized" immunotherapeutics. In addition, even though immune checkpoint inhibitors have demonstrated clear clinical activity in subsets of patients with cancer, additional strategies are needed to extend their benefits to the majority of treated patients; thus, combining them with the tumor immunomodulation induced by epigenetic drugs represents an additional highly rational approach. Along this line, the ongoing NIBIT-M4 trial (EUDRACT 2015-001329-17), combining guadecitabine and the anti-CTLA-4 mAb ipilimumab in metastatic melanoma, will test this intriguing concept through a phase Ib dose escalation design aimed to assess the safety of guadecitabine combined with ipilimumab in patients with previously treated or untreated unresectable or metastatic cutaneous melanoma. At the same time, preliminary signs of biologic and clinical activity will be collected. Along this very same track goes the randomized phase II clinical trial NCT01928576, which is seeking the response rate to regimens including an "epigenetic priming" with azacitidine/oral azacytidine, given alone or in association with the HDACi entinostat, followed by anti-PD-1 immunotherapy with nivolumab in patients with non-small cell lung cancer.

Conclusions

A large body of *in vitro* experimental evidence has demonstrated that epigenetic drugs have broad cancer immunomodulatory properties, and upcoming preclinical *in vivo* data are providing sound evidence of their potential to improve the antitumor activity of immunotherapies. Although in its infancy, actively testing this brand new "epigenetic immune-

sequencing approach" in the clinic will hopefully provide cancer patients with more effective and long-lasting therapeutic strategies in the future.

Disclosure of Potential Conflicts of Interest

M. Maio is a consultant/advisory board member for AstraZeneca, Bristol-Myers Squibb, Celgene, GlaxoSmithKline, MedImmune, and Roche. M. Maio, A. Covre, and S. Coral are listed as co-inventors on a patent-pending application (WO2014/128245) for DNA hypomethylating agents for cancer therapy, which is partially based on findings included in this article. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: P.G. Natali

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Di Giacomo, P.G. Natali

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Fratta, A.M. Di Giacomo, P.G. Natali

Writing, review, and/or revision of the manuscript: M. Maio, A. Covre, E. Fratta, A.M. Di Giacomo, P. Taverna, P.G. Natali, S. Coral, L. Sigalotti

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References

- Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. *Oncogene* 2008;27:5932-43.
- Bukur J, Jasinski S, Seliger B. The role of classical and non-classical HLA class I antigens in human tumors. *Semin Cancer Biol* 2012;22:350-8.
- McDermott D, Lebbe C, Hodi FS, Maio M, Weber JS, Wolchok JD, et al. Durable benefit and the potential for long-term survival with immunotherapy in advanced melanoma. *Cancer Treat Rev* 2014;40:1056-64.
- Maio M, Grob JJ, Aamdal S, Bondarenko I, Robert C, Thomas L, et al. Five-year survival rates for treatment-naïve patients with advanced melanoma who received ipilimumab plus dacarbazine in a phase III trial. *J Clin Oncol* 2015;33:1191-6.
- Kulis M, Esteller M. DNA methylation and cancer. *Adv Genet* 2010;70:27-56.
- Turner BM. Histone acetylation and an epigenetic code. *Bioessays* 2000;22:836-45.
- Cheung P, Lau P. Epigenetic regulation by histone methylation and histone variants. *Mol Endocrinol* 2005;19:563-73.
- Vakoc CR, Mandat SA, Olenchok BA, Blobel GA. Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. *Mol Cell* 2005;19:381-91.
- Zhao Q, Rank G, Tan YT, Li H, Moritz RL, Simpson RJ, et al. PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. *Nat Struct Mol Biol* 2009;16:304-11.
- Tachibana M, Matsumura Y, Fukuda M, Kimura H, Shinkai Y. G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. *EMBO J* 2008;27:2681-90.
- Sigalotti L, Fratta E, Coral S, Maio M. Epigenetic drugs as immunomodulators for combination therapies in solid tumors. *Pharmacol Ther* 2014;142:339-50.
- West AC, Johnstone RW. New and emerging HDAC inhibitors for cancer treatment. *J Clin Invest* 2014;124:30-9.
- Campbell RM, Tummino PJ. Cancer epigenetics drug discovery and development: the challenge of hitting the mark. *J Clin Invest* 2014;124:64-9.
- Navada SC, Steinmann J, Lubbert M, Silverman LR. Clinical development of demethylating agents in hematology. *J Clin Invest* 2014;124:40-6.
- Dhanak D, Jackson P. Development and classes of epigenetic drugs for cancer. *Biochem Biophys Res Commun* 2014;455:58-69.
- Richon VM. Targeting histone deacetylases: development of vorinostat for the treatment of cancer. *Epigenomics* 2010;2:457-65.
- Coral S, Covre A, Nicolay HJ, Parisi G, Rizzo A, Colizzi F, et al. Epigenetic remodelling of gene expression profiles of neoplastic and normal tissues: immunotherapeutic implications. *Br J Cancer* 2012;107:1116-24.
- Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 1999;21:103-7.
- Murakami T, Sato A, Chun NA, Hara M, Naito Y, Kobayashi Y, et al. Transcriptional modulation using HDACi desipeptide promotes immune cell-mediated tumor destruction of murine B16 melanoma. *J Invest Dermatol* 2008;128:1506-16.
- Srivastava P, Paluch BE, Matsuzaki J, James SR, Collamat-Lai G, Karbach J, et al. Immunomodulatory action of SGI-110, a hypomethylating agent, in acute myeloid leukemia cells and xenografts. *Leuk Res* 2014;38:1332-41.
- Yang H, Lan P, Hou Z, Guan Y, Zhang J, Xu W, et al. Histone deacetylase inhibitor SAHA epigenetically regulates miR-17-92 cluster and MCM7 to upregulate MICA expression in hepatoma. *Br J Cancer* 2015;112:112-21.
- Coral S, Parisi G, Nicolay HJ, Colizzi F, Danielli R, Fratta E, et al. Immunomodulatory activity of SGI-110, a 5-aza-2'-deoxycytidine-containing demethylating dinucleotide. *Cancer Immunol Immunother* 2013;62:605-14.
- Fratta E, Sigalotti L, Colizzi F, Covre A, Nicolay HJ, Danielli R, et al. Epigenetically regulated clonal heritability of CTA expression profiles in human melanoma. *J Cell Physiol* 2010;223:352-8.

24. Armeanu S, Bitzer M, Lauer UM, Venturelli S, Pathil A, Krusch M, et al. Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. *Cancer Res* 2005;65:6321–9.
25. Li H, Chiappinelli KB, Guzzetta AA, Easwaran H, Yen RW, Vatapalli R, et al. Immune regulation by low doses of the DNA methyltransferase inhibitor 5-azacitidine in common human epithelial cancers. *Oncotarget* 2014;5:587–98.
26. Schrupp DS, Fischette MR, Nguyen DM, Zhao M, Li X, Kunst TF, et al. Phase I study of decitabine-mediated gene expression in patients with cancers involving the lungs, esophagus, or pleura. *Clin Cancer Res* 2006;12:5777–85.
27. Sigalotti L, Altomonte M, Colizzi F, Degan M, Rupolo M, Zagonel V, et al. 5-Aza-2'-deoxycytidine (decitabine) treatment of hematopoietic malignancies: a multimechanism therapeutic approach? *Blood* 2003;101:4644–6; discussion 4645–6.
28. Goodyear O, Agathangelou A, Novitzky-Basso I, Siddique S, McSkeane T, Ryan G, et al. Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. *Blood* 2010;116:1908–18.
29. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol* 2013;31:51–72.
30. Sonnemann J, Gressmann S, Becker S, Wittig S, Schmutte M, Beck JF. The histone deacetylase inhibitor vorinostat induces calreticulin exposure in childhood brain tumour cells in vitro. *Cancer Chemother Pharmacol* 2010;66:611–6.
31. West AC, Mattarollo SR, Shortt J, Cluse LA, Christiansen AJ, Smyth MJ, et al. An intact immune system is required for the anticancer activities of histone deacetylase inhibitors. *Cancer Res* 2013;73:7265–76.
32. Guillot F, Boutin B, Blanquart C, Fonteneau JF, Robard M, Gregoire M, et al. Vaccination with epigenetically treated mesothelioma cells induces immunisation and blocks tumour growth. *Vaccine* 2011;29:5534–43.
33. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. *J Clin Oncol* 2015;33:1974–82.
34. Garcia JS, Jain N, Godley LA. An update on the safety and efficacy of decitabine in the treatment of myelodysplastic syndromes. *Onco Targets Ther* 2010;3:1–13.
35. Kozar K, Kaminski R, Switaj T, Oldak T, Machaj E, Wysocki PJ, et al. Interleukin 12-based immunotherapy improves the antitumor effectiveness of a low-dose 5-Aza-2'-deoxycytidine treatment in L1210 leukemia and B16F10 melanoma models in mice. *Clin Cancer Res* 2003;9:3124–33.
36. Simova J, Pollakova V, Indrova M, Mikyskova R, Bieblova J, Stepanek I, et al. Immunotherapy augments the effect of 5-azacytidine on HPV16-associated tumours with different MHC class I-expression status. *Br J Cancer* 2011;105:1533–41.
37. Shen L, Ciesielski M, Ramakrishnan S, Miles KM, Ellis L, Sotomayor P, et al. Class I histone deacetylase inhibitor entinostat suppresses regulatory T cells and enhances immunotherapies in renal and prostate cancer models. *PLoS One* 2012;7:e30815.
38. Sun JZ, Gao L, Wang W, Du N, Yang J, Wan L, et al. Demethylation of cancer/testis antigens and CpG ODN stimulation enhance dendritic cell and cytotoxic T lymphocyte function in a mouse mammary model. *Biomed Res Int* 2013;2013:196894.
39. Lisiero DN, Soto H, Everson RC, Liao LM, Prins RM. The histone deacetylase inhibitor, LBH589, promotes the systemic cytokine and effector responses of adoptively transferred CD8+ T cells. *J Immunother Cancer* 2014;2:8.
40. Vo DD, Prins RM, Begley JL, Donahue TR, Morris LF, Bruhn KW, et al. Enhanced antitumor activity induced by adoptive T-cell transfer and adjunctive use of the histone deacetylase inhibitor LAQ824. *Cancer Res* 2009;69:8693–9.
41. Guo ZS, Hong JA, Irvine KR, Chen GA, Spiess PJ, Liu Y, et al. *De novo* induction of a cancer/testis antigen by 5-aza-2'-deoxycytidine augments adoptive immunotherapy in a murine tumor model. *Cancer Res* 2006;66:1105–13.
42. Calabro L, Morra A, Fonsatti E, Cutaia O, Fazio C, Annesi D, et al. Efficacy and safety of an intensified schedule of tremelimumab for chemotherapy-resistant malignant mesothelioma: an open-label, single-arm, phase 2 study. *Lancet Respir Med* 2015;3:301–9.
43. Covre A, Coral S, Nicolay H, Parisi G, Fazio C, Colizzi F, et al. Anti-tumor activity of epigenetic immunomodulation combined with CTLA-4 blockade in syngeneic mouse models. *Oncoimmunology* 2015 Apr 2. [Epub ahead of print].
44. Covre A, Coral S, Di Giacomo A, Taverna P, Azab M, Maio M. Epigenetics meets immune checkpoints. *Semin Oncol* 2015;42:506–13.
45. Kim K, Skora AD, Li Z, Liu Q, Tam AJ, Blosser RL, et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc Natl Acad Sci U S A* 2014;111:11774–9.
46. Christiansen AJ, West A, Banks KM, Haynes NM, Teng MW, Smyth MJ, et al. Eradication of solid tumors using histone deacetylase inhibitors combined with immune-stimulating antibodies. *Proc Natl Acad Sci U S A* 2011;108:4141–6.
47. Plimack ER, Desai JR, Issa JP, Jelinek J, Sharma P, Vence LM, et al. A phase I study of decitabine with pegylated interferon alpha-2b in advanced melanoma: impact on DNA methylation and lymphocyte populations. *Invest New Drugs* 2014;32:969–75.
48. Odunsi K, Matsuzaki J, James SR, Mhawech-Fauceglia P, Tsuji T, Miller A, et al. Epigenetic potentiation of NY-ESO-1 vaccine therapy in human ovarian cancer. *Cancer Immunol Res* 2014;2:37–49.