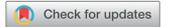


REVIEW



## No patient left behind: The promise of immune priming with epigenetic agents

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### ABSTRACT

Checkpoint inhibitors, monoclonal antibodies that inhibit PD-1 or CTLA-4, have revolutionized the treatment of multiple cancers. Despite the enthusiasm for the clinical successes of checkpoint inhibitors, and immunotherapy, in general, only a minority of patients with specific tumor types actually benefit from treatment. Emerging evidence implicates epigenetic alterations as a mechanism of clinical resistance to immunotherapy. This review presents evidence for that association, summarizes the epi-based mechanisms by which tumors evade immunogenic cell death, discusses epigenetic modulation as a component of an integrated strategy to boost anticancer T cell effector function in relation to a tumor immunosuppression cycle and, finally, makes the case that the success of this no-patient-left-behind strategy critically depends on the toxicity profile of the epigenetic agent(s).

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### Introduction

Strictly speaking, the immunotherapy revolution began, not on the revolutionary date of March 25<sup>th</sup> 2011 with the approval of Ipilimumab (Yervoy) the first checkpoint inhibitor, but in September 1891<sup>1</sup> with Dr. William B. Coley's first published manuscript about the treatment of inoperable sarcomas with bacterial preparations—these preparations, a combination of gram-positive, heat-killed streptococci plus gram-negative, heat-killed *Serratia marcescens*, subsequently referred to as “Coley's toxins”<sup>2</sup> or mixed bacterial vaccine (MBV), led to spontaneous tumor regressions in some patients due to activation of the immune system. Despite a reported cure rate of 10%,<sup>3</sup> Coley's toxin was not widely accepted or used except as a last resort due to the anecdotal and unpredictable nature of the responses, nonspecificity to tumors and the dangerous side effects (e.g. high fever) that the treatment induced.<sup>2</sup> The specific mechanism of action, unknown to Coley at the time, is probably related to a “massive induction of immunoregulatory cytokines,” specifically TNF- $\alpha$ , IFN- $\gamma$ , and IL1- $\beta$ , according to Karbach *et al*, 2012, who conducted a Phase 1 clinical trial with Coley's toxins.<sup>4</sup>

Almost one hundred years later, picking up where Dr. Coley left off, early non-specific immunotherapies such as human IFN $\alpha$ -2b for the treatment of multiple malignancies and interleukin-2 (IL-2) for metastatic renal cell carcinoma were developed to induce an immune response against the tumor.<sup>5</sup> In addition to IL-2 and interferon therapy, a Calmette–Guérin (BCG) vaccine for tuberculosis was repurposed to drive cellular immunity in superficial bladder cancer.<sup>6</sup> However, these early

cytokine-based immunotherapies not only showed disappointing efficacy rates as monotherapies but were also in some cases severely toxic, especially IL-2, which limit their clinical use.<sup>7</sup> Low efficacy is a function of multiple factors: non-selective targeting of tumors, the heterogeneity of host responses,<sup>8</sup> and tumor immune escape<sup>5</sup> notably from induction<sup>9</sup> of tolerance via immunosuppressive cytokines such as TGF- $\beta$  and IL-10 and upregulation of the negative checkpoint molecules, CTLA-4 and PDL-1/PD-1, to limit T cell responses.<sup>10</sup>

With regard to the latter, CTLA-4 and PDL-1/PD-1 checkpoint inhibitors have revolutionized the treatment landscape in advanced melanoma, lung cancer, RCC, bladder cancer, and Hodgkin lymphomas with the demonstration of durable clinical efficacy and improved overall survival. Nevertheless, despite the clear evidence of improved clinical outcomes, the fact remains that the majority of cancer patients do not respond to them. In an expansive clinical trial with the anti-PD-1 mAb, nivolumab, in NSCLC, melanoma, and renal cell cancer, objective responses occurred in 25% of patients,<sup>11</sup> which is notable not only for the patients that did respond but also for the 75% that didn't, supporting a rationale for immunotherapy-based combination treatment to improve on this response rate.

Emerging data places the high rate of epigenetic modifications pathognomonic to cancer cells at the center or, perhaps more appropriately, the *epicenter* of tumor resistance to radiotherapy, chemotherapy and immunotherapy.<sup>12</sup> The term epigenetics, originally coined in the middle of the twentieth century by Conrad Waddington, a British developmental biologist,<sup>13</sup> is used herein to refer to changes in the pattern of gene expression

and activity that are not the result of changes to the primary DNA sequence.<sup>14</sup> Epigenetic modifications include alterations in DNA methylation and chromatin remodeling via acetylation and methylation. In cancer the accumulation of these epigenetic alterations leads to profound changes in the expression of proteins that contribute not only to the development of radio- and chemoresistance but also immunoresistance<sup>15-19</sup> and immune escape through transcriptional silencing of immune-related genes.<sup>20</sup>

Epigenetic agents that increase the expression of these silenced immune-related genes have the potential to overcome cancer cell-induced T cell dysfunction not only in tumors with known susceptibility to checkpoint inhibitors, like melanoma or NSCLC, but also in tumors traditionally considered immune-resistant such as pancreatic or colorectal cancers. On the premise that a combination of nonoverlapping anticancer therapies is necessary to redress the suppressive mechanisms, discussed herein, by which tumors escape immune attack and immunogenic cell death, epigenetic inhibitors currently under clinical evaluation are highlighted in this review as an important part of an integrated strategy to boost anticancer B and T cell effector function and thereby improve therapeutic outcomes, so that fewer and fewer patients are “left behind.”

### Epigenetic modifications

Epigenetic changes are a ubiquitous feature of carcinogenesis<sup>21</sup> and progression. Among the most intensively studied epigenetic aberrations are DNA methylation of CpG dinucleotides and post-translational histone modification,<sup>22</sup> both of which work in concert to establish and maintain patterns of gene expression conducive to tumor proliferation.<sup>23</sup> The intrinsic reversibility and plasticity of epigenetic alterations as compared with the relative immutability of genetic events renders cancer a suitable disease for treatment with epigenetic and chromatin modifiers. A brief summary of DNA methylation and histone modification is provided below. For more in-depth reviews on epigenetic alterations the reader is referred to other cited literature.<sup>24,25</sup>

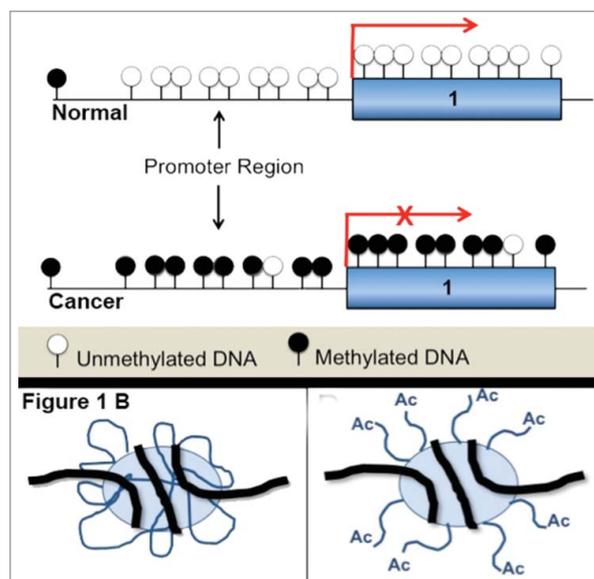
#### DNA methylation

DNA methylation refers to the covalent attachment of a methyl group to the carbon-5 position of cytosine within clusters or islands of CpG dinucleotides<sup>26</sup> (cytosine and guanine separated by a phosphate) found in or near promoter regions of genes where transcription is initiated<sup>27</sup> via a reaction catalyzed by DNA methyltransferases (Fig. 1A).

These methyl groups project into the major groove of DNA and sterically hinder the binding of RNA polymerase, which effectively silences transcription (Fig. 1A).<sup>28,29</sup>

#### Histone covalent modification

Space constraints in the micron-sized nucleus requires coiling of the 2 m<sup>30</sup> long negatively-charged DNA molecule around an octamer of positively-charged histone proteins, which are, in turn, further compacted into chromatin fibers,<sup>31,32</sup> rendering DNA largely inaccessible to transcriptional regulators.



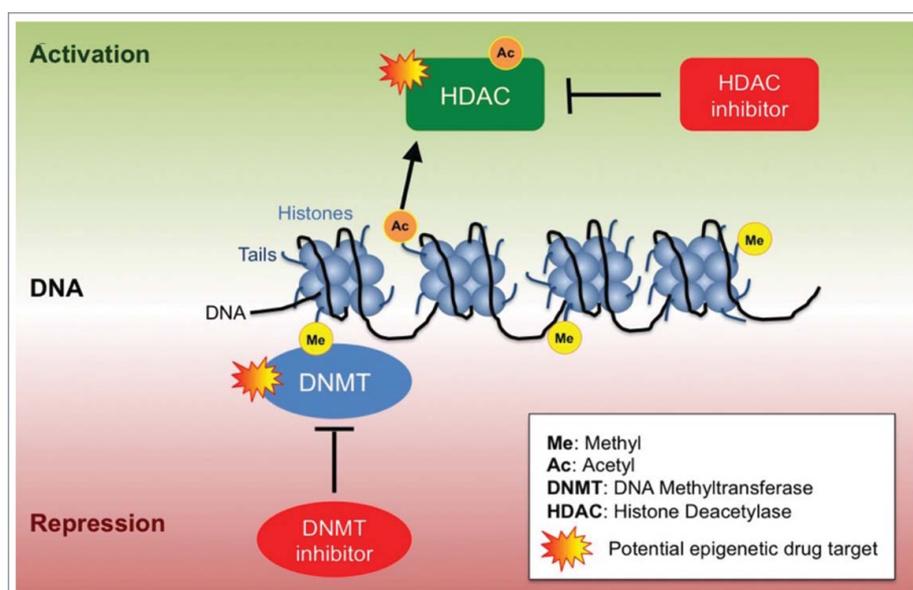
**Figure 1.** (A) Hypermethylation and transcriptional silencing. A hallmark of cancer is hypermethylation of DNA sequences in the promoter region leading to transcriptional silencing. (B) Histone acetylation leads to a switch between transcriptionally repressive and permissive chromatin. Histone acetylation induces a change from compacted to a more ‘open’ chromatin state and increases the accessibility of transcription complexes to genomic DNA. Acetylation is an important factor for the regulation of gene expression.

Acetylation, which occurs at the positively charged lysine residues on the amino-terminal histone tails, neutralizes the positive charge, decreasing the affinity of histones for DNA and thereby resulting in increased accessibility to the transcriptional machinery.<sup>33</sup> Histone acetylation thus maintains chromatin in an open state conducive to active transcription.<sup>34</sup> By contrast, histone deacetylases or HDACs deacetylate chromatin and reinstate the positive charge on the histone tails, resulting in high-affinity binding between histones and DNA,<sup>35</sup> which, in turn, promotes a condensed chromatin state and transcriptional silencing (Fig. 1B and Fig. 2).<sup>27</sup>

#### Epigenetic inhibitors as radio/chemosensitizers

Since DNA methyltransferases (DNMTs) and HDAC are multifunctional enzymes<sup>36</sup> that impact a broad spectrum of pathways and cellular processes, including cell cycle regulation, DNA damage response, cell adhesion, angiogenesis, and immunity, it is not surprising that the gene expression changes wrought by epigenetic agents influence multiple facets of tumor progression and the tumor microenvironment.<sup>37</sup>

Epigenetic agents have demonstrated clinical efficacy not only in hematologic malignancies like acute myeloid leukemia and myelodysplastic syndrome where the DNMT inhibitors azacitidine (Vidaza, AZA, or 5-aza) and decitabine (Dacogen or 5AZA2) are approved, but also in solid tumors,<sup>38</sup> most notably as radio- and chemosensitizers in patients with highly resistant tumors. In a Phase I/II study of 5-azacitidine and carboplatin 46% of patients with platinum-resistant or refractory ovarian cancer demonstrated durable responses and stable disease (median duration of therapy 7.5 months).<sup>39</sup> In addition, an ongoing randomized Phase II trial (NCT02096354) acronymed ROCKET with the experimental systemically nontoxic



**Figure 2.** Epigenetic regulation of transcription. DNA is compressed in the nucleus as chromatin. The building block of chromatin is the nucleosome, which consists of 8 DNA wrapped histones. Epigenetic regulation involves DNA methylation, histone acetylation and histone methylation that depending on the combination of modifications lead to transcriptional repression or transcriptional activation.

epigenetic and tumor-associated myeloid repolarizing agent, RRx-001, followed by irinotecan rechallenge on progression of RRx-001 has resulted in tumor resensitization to irinotecan in multiple patients.<sup>15</sup> Similar<sup>40</sup> preliminary responses have been seen with RRx-001 in notoriously radioresistant melanoma brain metastases in combination with whole brain radiotherapy (WBRT) in a Phase I trial acronymed BRAINSTORM (NCT02215512).

One general mechanism of radio/chemoresensitization is related to epigenetic restoration of silenced tumor suppressor genes such as p53.<sup>25</sup> Another mechanism of radio/chemoresensitization is through blood vessel normalization, which equilibrates oxygen levels within the tumor and enhances drug delivery.<sup>41</sup> The endogenous vasodilator, nitric oxide (NO), has been reported as possessing epigenetic activity, modifying global histone methylation through inhibition of Jumonji C (JmjC) domain-containing demethylases.<sup>42</sup> Consequently, NO-mediated radio- or chemo-sensitizing agents may exert their activity through a multiple mechanisms of action that include both vascular modification and NO or Reactive Oxygen Species and Reactive Nitrogen Species (ROS/RNS)-induced epigenetic modifications.<sup>41,43</sup>

A normalized tumor vasculature also makes it possible for immune cells to infiltrate the tumor and exert an anticancer effect, discussed below.

### Epigenetic inhibitors as immunosensitizers

In addition to sensitization of chemotherapy and radiation, epigenetic agents (e.g., 5-AZA) have been used as part of a strategy<sup>44</sup> to prime immunotherapy responses. In 5 patients with NSCLC who received 5-azacytidine and entinostat before treatment with either anti-PD-1 or anti-PD-L1 antibodies, durable objective responses were observed in 3 patients while stable disease for more than 6 months was observed in the other 2 patients.<sup>45</sup> Based on these clinical responses, a study of

azacitidine and entinostat or azacitidine alone before the PD-1 inhibitor nivolumab (NCT01928576) was initiated with the primary end point of overall response rate; however the inherent toxicity of nucleoside DNA methyltransferase inhibitors (DNMTs) such as azacitidine, which are poorly tolerated, is likely to significantly limit the efficacy of this strategy. While HDAC inhibitors such as entinostat are generally well tolerated, they, too, may induce dose-limiting cytopenias.<sup>46</sup>

In contrast to these myelosuppression-associated complications, preliminary results from the first cohort of patients treated with the combination of the experimental systemically non-toxic pan-epigenetic inhibitor and tumor-associated myeloid repolarizing agent, RRx-001, and the PD-1 inhibitor, nivolumab, in a Phase I dose escalation study called PRIMETIME (NCT02518958) indicate promising safety and activity.<sup>47</sup> A low side effect profile is particularly important in the context of an immune priming strategy, due to the potential for a reverse ‘anti-priming’ effect in the event that bone marrow and other host toxicities from the epigenetic agent either reduce tolerance to or prevent subsequent treatment with immunotherapy.

### Mechanisms of tumor-induced immune escape

In 1957 2 prominent immunologists, Burnett and Thomas,<sup>48</sup> put forward the controversial immunosurveillance hypothesis, now largely accepted with revisions, that the ever-vigilant immune system constantly detects and eliminates malignant threats before they clinically present<sup>49</sup> as full-blown tumors. However, immunosurveillance is not 100% effective even in immunocompetent patients; and, in fact, under the selective pressure of this active surveillance it is hypothesized that resistant tumor cells evolve a repertoire of epigenetically mediated escape mechanisms to resist immunogenic cell death (ICD) in a process called immunoediting.<sup>50</sup>

The term immunogenic cell death,<sup>51</sup> refers to a multi-step cycle of immune activation initiated by the release of cell-

associated antigens<sup>52</sup> and danger signals (step 1 in the cycle) from dying tumor cells. These released tumor antigens (TAs) are captured by antigen presenting cells (APCs) such as macrophages and DCs, which process and load TA-derived peptides on major histocompatibility complex (MHC) class I or class II (step 2); the loaded MHCs then activate MHC-restricted T cells (i.e., cytotoxic T-lymphocytes or helper T cells) in lymph nodes (step 3). In particular, cytotoxic T-lymphocytes traffic to (step 4) and infiltrate the tumor bed (step 5), specifically recognizing antigen on cancer cells through interactions between T-cell receptor (TCR) and MHC class I (step 6), and eliminate the targeted cells (Step 7), releasing additional antigens in the process that, in turn, further stimulate the immune response.<sup>53</sup>

This 7-step cycle is interrupted in tumors, which rely on stealth tactics and deploy multiple countermeasures including reduced expression of tumor associated antigens, deficient antigen presentation, reduced or absent costimulation, resistance to apoptosis and even counterattack to escape immune elimination. These countermeasures are, to a greater or lesser degree, regulated by epigenetic processes, and, therefore, potentially susceptible to reprogramming with epigenetic therapies.

The flip side or mirror image of the antitumor immunity cycle is an immunoescape paradigm, depicted schematically as a series of countermeasures to intercept immunosurveillance at each of the 7 steps. (Fig. 3). Each immunoescape countermeasure, described in more detail below, has been matched in boxes with a corresponding potential antitumor strategy.

### Immunoescape mechanism 1: Reduced expression of tumor associated antigens

Despite the failure of effector cells to lyse tumors in patients with cancer, malignant cells are, in fact, immunogenic due, in part, to the variable expression of tumor associated antigens (TAAs). These immunogenic TAAs include cancer testis antigens (CTAs), such as MAGE-A3 and NY-ESO-1, normally restricted to the germ cells of the gonads and silenced in

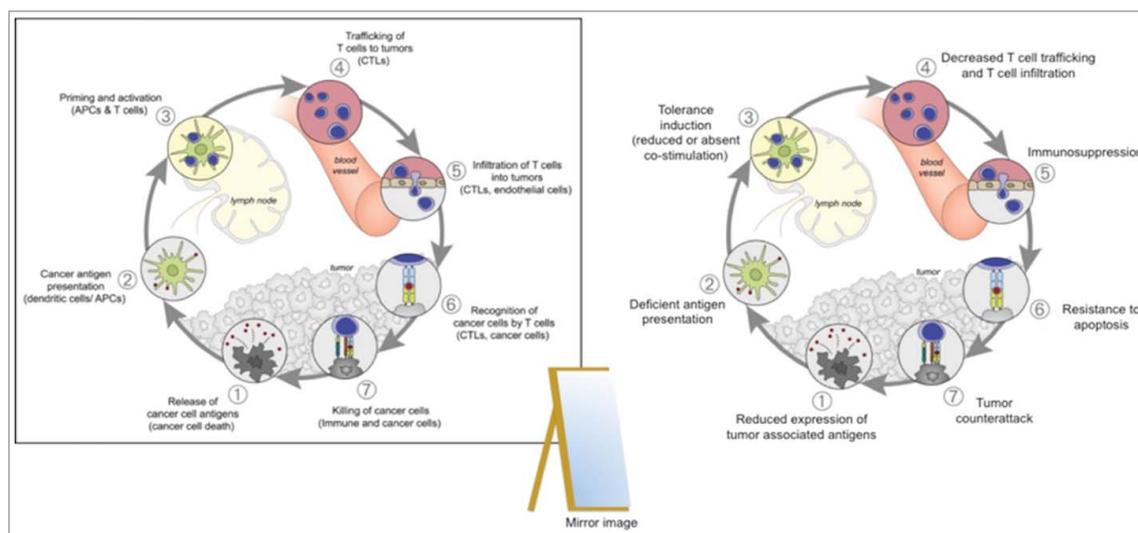
somatic cells, but aberrantly expressed in up to 40% of hematologic and solid tumor types.<sup>54</sup>

Increased CTA levels in both normal and tumor tissue are associated preclinically with epigenetic modification<sup>55</sup> and re-expression of these hypermethylated antigens following exposure to epigenetic agents has been demonstrated in clinical trials. For example, in a Phase II study (NCT01050790), post-transplant multiple myeloma patients receiving sequential azacitidine (Aza) and lenalidomide (Rev) demonstrated a significant increase in MAGEA4, MAGEA6, SPA17 and AKAP4 in bone marrow compared with pre-therapy samples. This induction was associated with a subsequent sustained T-cell-mediated immune response.<sup>56</sup> Similarly, the phase I/II study (NCT02332889) in relapsed neuroblastoma and sarcoma tested the efficiency of decitabine to upregulate cancer testis antigen expression, followed by a dendritic cell MAGE-A1, MAGE-A3, and NY-ESO1 peptide vaccine. A complete response was documented in 1/10 patients and of the 2 patients who were disease-free at start of DC vaccine therapy one was disease-free 2 y post therapy.<sup>57</sup>

These results suggest that the mutational burden of tumors directly correlates with increased neoantigen presentation, immunogenicity and response to checkpoint inhibitors. Accordingly, mutagenic tumors like melanoma and NSCLC are more responsive to immunotherapy than AML or ALL, where the rate of mutations is correspondingly lower (Table 1).<sup>58</sup>

### Mismatch repair deficit and microsatellite instability

In similar way, tumors with mismatch-repair deficits and microsatellite instability status, which correlate with a higher mutational rate and neoantigen load, are more susceptible to treatment with PD-1 blockade.<sup>59</sup> For example, patients with microsatellite instable (MSI) colorectal cancer (CRC) (approximately 15% of the CRC population) are candidates for PD-1 therapy while microsatellite stable (MSS) CRC patients, who comprise the remaining 85%, are not.<sup>60</sup> Microsatellites are



**Figure 3.** The Immunoescape Cycle. To the left, the Antitumor Immunity Cycle illustrating the 7 main steps to stimulate an effective antitumor response. In cancer this cycle is interrupted at one or more steps to prevent specific T-cell immunity. Adapted from *Oncology Meets Immunology: The Cancer-Immunity Cycle* Daniel S. Chen and Ira Mellman *Immunity* 39, July 25, 2013. In a mirror image of the beneficial immunity cycle pictured to the left, which is responsible for the elimination of tumor cells, the harmful Immunoescape Cycle pictured to the right illustrates 7 different mechanisms by which the tumor corrupts or evades the 7 steps of the immunity cycle.

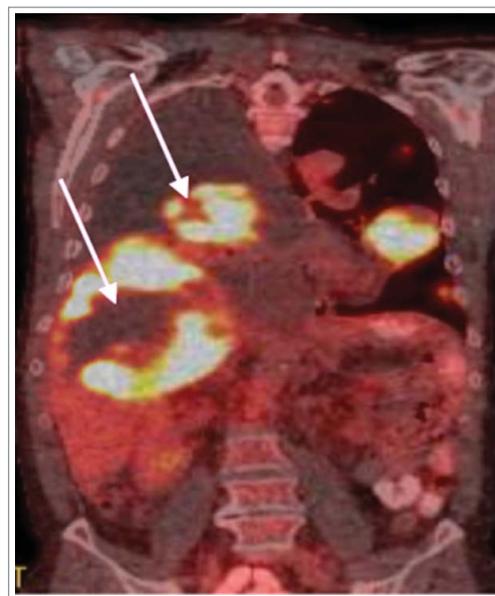
**Table 1.** Somatic mutational burden per tumor type. In theory, tumor-specific neoantigen generation and presentation is directly proportional to mutational burden.

Disease	Somatic mutation prevalence (average number mutations per megabase)	Formation of Neoantigens
Melanoma	~20	Frequently
Lung Squamous	~9.5	Regularly
Lung Adeno	~9.0	Regularly
Stomach	~8.5	Regularly
Esophagus	~8.0	Regularly
Lung Small Cell	~8.0	Regularly
Colorectal	~8.0	Regularly
Bladder	~7.5	Regularly
Uterus	~7.0	Regularly
Cervix	~6.5	Regularly
Liver	~6.0	Regularly
Head and Neck	~5.0	Regularly
Kidney Clear Cell	~2.0	Regularly
Lymphoma B-Cell	~1.5	Regularly
Kidney Papillary	~1.1	Regularly
Breast	~1.0	Occasionally
Pancreas	~1.0	Occasionally
Myeloma	~1.0	Occasionally
Ovary	~1.0	Occasionally
Prostate	~1.0	Occasionally
Glioblastoma	~0.9	Occasionally
Glioma Low Grade	~0.85	Occasionally
Neuroblastoma	~0.8	Occasionally
Medulloblastoma	~0.7	Occasionally
CLL	~0.65	Occasionally
Kidney Chromophobe	~0.6	Occasionally
Thyroid	~0.5	Occasionally
ALL	~0.3	Occasionally
AML	~0.2	Occasionally
Pilocytic Astrocytoma	~0.05	Occasionally

short sequences repeated throughout the genome that are vulnerable to mutation due to a defect in DNA mismatch repair.<sup>61</sup> Despite the higher expression of neoantigens in MSI<sup>+</sup> CRC tumors, the presence of commensal bacteria in the intestines may facilitate upregulation of checkpoint ligands, thereby preventing immune eradication.<sup>62</sup>

The premise that tumors with microsatellite instability that lead to a higher level of somatic mutations are more susceptible to immune therapy, once the immune checkpoint 'brakes' have been released is intriguing and suggests potential ways of extending the activity of immune therapy to tumors that are currently not susceptible to this therapeutic approach. It has been well known that exposing tumors to ionizing radiation induces cellular changes that result in the upregulation and cellular expression of neoantigens,<sup>63</sup> often as a result of ROS-induced central necrosis,<sup>64</sup> that can manifest as pseudoprogression.<sup>65</sup> Indeed, ROS induced necrosis results in pro-inflammatory and immuno-stimulatory responses<sup>66</sup> such that the effect may be more accurately described as immunogenic cell-death, mediated through important signaling to the immune system.<sup>67,68</sup> HDAC and DNMT inhibitors both exert an immunostimulatory effect: DNMTs through the induction of an interferon response by viral mimicry,<sup>69,70</sup> and HDAC inhibitors through induction of immunogenic cell death.<sup>71</sup>

ROS-mediated epigenetic agents, such as RRx-001, has shown effects on global methylation and histone acetylation,<sup>72</sup> may therefore act through a dual mechanism of action – inducing an immune response through HDAC and DNMT inhibition, while amplifying that response by neoantigen



**Figure 4.** PET-CT showing tumor central necrosis and pseudoprogression in a patient recruited in the Phase 1 clinical trial. The pathology report indicated that the rim was >90% necrotic suggesting an influx of lymphocytes.

presentation as a consequence of central necrosis. As a ROS-mediated epigenetic agent, extensive tumor central necrosis has been observed in the phase 1 clinical trial and subsequently in ongoing Phase 2 trials. Fig. 4 shows a typical PET-CT image of liver lesion with extensive tumor necrosis. Necrosis was surgically confirmed in both the central region and rim (> 90% necrotic) suggesting extensive lymphocyte recruitment to the tumor.<sup>73</sup>

#### Therapeutic strategy in MSI high tumors e.g., colon, gastric and endometrial tumors as well as patients with Lynch Syndrome:

Combine an epigenetic agent with radiation, CAR-T, oncolytic viruses, bispecific T-cell engagers (BiTE) and DNA repair inhibitors to increase the complement of targetable T-cell antigens

#### Immunoescape mechanism 2: Deficient antigen presentation

The major histocompatibility complex (MHC) is a cluster of genes that function to bind and present antigens to the adaptive immune system. In humans, MHC, known as human leukocyte antigen (HLA), includes coding regions for different co-receptors on the T cells, that is, CD8 and CD4, hence allowing for identification of CD4+ T helper (Th) cells and CD8+ cytotoxic T lymphocytes (CTLs), which recognize antigen presented by MHC class I-derived molecules and execute anti-tumor responses.<sup>74</sup> MHC class I antigens also regulate the lytic activity of NK immune cells, which are programmed to kill any cells lacking MHC class I expression.<sup>74</sup>

As reviewed elsewhere,<sup>75</sup> malignant transformation is associated with the epigenetic loss and/or downregulation of MHC class I antigens via hypermethylation of the HLA promoter, which impairs the recognition of tumor cells by CTLs, leading to escape from immune destruction; treatment with epigenetic agents<sup>76</sup> restores expression of MHC class I and/or components of the class I antigen processing and presentation machinery

(TAP1, TAP2, LMP7, Tapasin),<sup>77,78</sup> which enhances tumor specific T-cell killing.<sup>79</sup> MHC class I and antigen presentation machinery are also regulated by DNA methylation.<sup>80</sup>; likewise, treatment with demethylating agents restores their expression in cancer cells,<sup>81</sup> which increases susceptibility to destruction by immune effector cells.

#### Therapeutic strategy for tumors with low MHC class I expression:

Combine an epigenetic agent with IFN-gamma, GM-CSF or high dose IL-2 to induce the upregulation of MHC class I expression.

### Immunescape mechanism 3: Tolerance induction (Reduced or absent costimulation)

In accordance with the so-called “2 signal model,” T-cell activation requires at least a double signal.<sup>82</sup> The first signal<sup>83</sup> results from the interaction of the T-cell receptor (TCR) with its cognate antigen plus major histocompatibility complex on the surface of antigen-presenting cells (APCs) or target cells.<sup>84</sup> The second set of antigen-independent signals termed costimulation are expressed on the surface of the APCs (Fig. 5). In the absence of this double signal, these anti-tumor T cells enter a state of unresponsiveness termed anergy.<sup>85</sup> The major costimulatory signals implicated in T cell activation is the glycoprotein CD28, which interacts on the APC with CD80 and CD86 (also called B7.1 and B7.2) and the OX40-OX40 ligand interaction.<sup>86</sup> The co-inhibitory molecules, CTLA-4, and PD-1, also interact with CD80 and CD86.<sup>87</sup> The third signal is the production of cytokines (e.g., IL-2, which, in turn, induces IFN- $\gamma$  production) required for differentiation of lytic CD8+ T cell lytic effectors.<sup>88</sup> Epigenetic mechanisms are linked with the expression of costimulatory and coinhibitory molecules<sup>89</sup> as<sup>90</sup> well as the repression of several pro-inflammatory cytokines (IL-1, IL-2, IL-8, and IL-12).<sup>91</sup> Chiappinelli *et al*<sup>70</sup> recently demonstrated that 5-azacytidine directly induces the expression of interferon. The mechanism, which overlaps with the targeted inflammation induced during oncolytic virus therapy, is related to derepression of endogenous retroviral (ERV) and other viral gene sequences in the tumor, triggering a type I interferon response.

#### Therapeutic strategy for tumors with low-to-intermediate expression of B7 and OX40 costimulatory molecules:

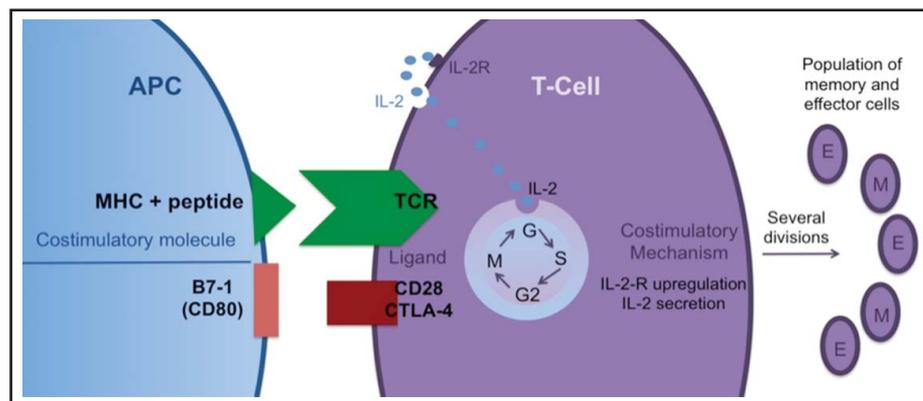
Combine an epigenetic agent with a viral vector for transduction of IL-2 and B7.1 genes, antagonistic antibodies directed against the checkpoint inhibitors (e.g. CTLA-4, PD-1) and agonist antibodies such as OX40 to improve costimulatory signaling to T cells.

### Immunescape mechanism 4: Decreased T-cell trafficking and T-cell infiltration

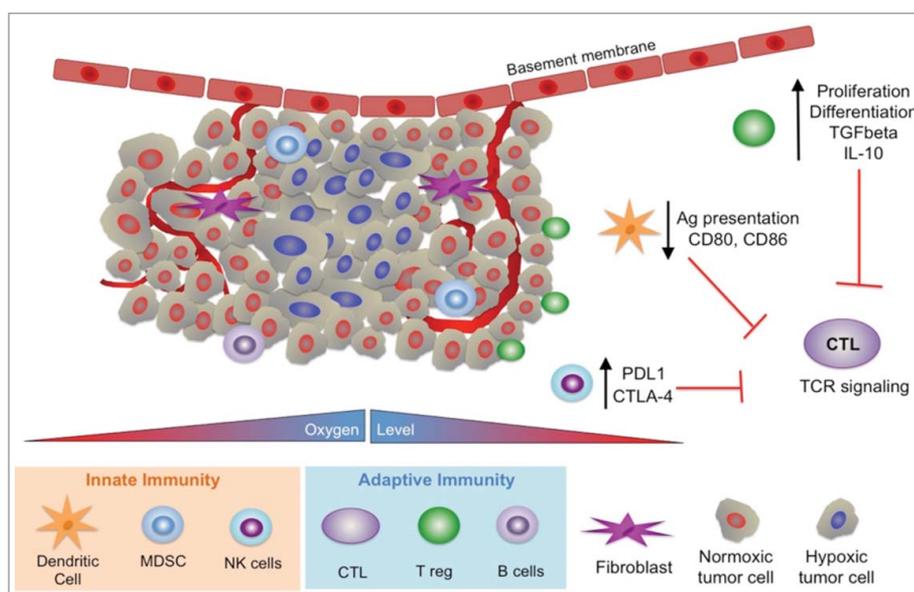
Tumor vessels differ from normal counterparts<sup>92</sup> by virtue of their tortuosity, immaturity and hyperpermeability. In particular, the leakiness of the vessels due to the prevalence of pro-angiogenic signals results in raised interstitial pressure with compression of the vasculature, which further impedes blood flow and exacerbates pre-existing hypoxia.

Hypoxia not only increases resistance to chemotherapy and radiation but it also contributes to immune escape through different mechanisms including amplification of levels and activity of regulatory T cells (T<sub>regs</sub>), myeloid suppressor cells (MDSC) and tumor associated macrophages (TAMs).<sup>93</sup> In addition, hypoxia influences the cell adhesion molecule profile of the endothelium leading to preferential T<sub>reg</sub> adhesion, infiltration and immune suppression.<sup>94</sup> The mechanisms of T cell adhesion and transmigration across the tumor endothelium have been covered in other reviews,<sup>95,96</sup> and therefore due to space limitations will not be discussed here. Finally, hypoxia leads to global epigenetic changes and silencing of tumor suppressor genes like BRCA1 and DNA repair enzymes such as RAD51,<sup>97</sup> which favors a proliferative phenotype. Examples of hypoxic tumor types include head and neck, cervix, sarcomas and glioblastomas.<sup>98</sup> (Fig. 6)

Treatment with anti-VEGF agents such as Bevacizumab or receptor tyrosine kinase (RTK) inhibitors like sunitinib prunes or disrupts the morphologically aberrant blood vessels that compromise blood flow in the tumor, leading to a more homogeneous distribution of functionally mature tumor vessels and a transient window of increased blood perfusion, assessable with magnetic resonance imaging, termed “vascular normalization”.<sup>99</sup> Several preclinical studies have demonstrated that antiangiogenic therapy not only



**Figure 5.** Naïve (T)cells require 2 signals to become activated: TCR/MHC and co-stimulation. The specific signal alone or the co-stimulatory signal alone leads to T cell anergy. The co-stimulatory signal is necessary for the synthesis and secretion of IL-2, which stimulates the T cell to divide and generate a larger population of memory and effector cells.



**Figure 6.** Influence of hypoxia in the tumor on the innate and adaptive immune response. In general hypoxia suppresses the response of the immune system. The adaptive immune system includes cytotoxic T lymphocytes (CTL), T regulatory cells (T reg) and B cells. The innate immune system includes dendritic cells, myeloid derived suppressor cells (MDSC) and natural killer cells (NK).

facilitates T-cell infiltration<sup>100</sup> but also reverses the hypoxia-induced recruitment of immunosuppressive T<sub>regs</sub> and the M2-like skewing of tumor associated macrophages,<sup>101</sup> thereby reprogramming the microenvironment for tumor inhibition. Like bevacizumab and the RTK inhibitors, the experimental, pan-epigenetic and tumor-associated myeloid repolarizing agent, RRx-001<sup>102</sup> has also been associated with normalization of the tumor vasculature, although, admittedly, this non-VEGF-related property may be attributable to the unique mechanism of RRx-001 rather than a general class effect of epigenetic agents.<sup>103</sup> However, as a class, epigenetic therapies have the potential to reverse hypoxia-induced silencing of tumor suppressor and other beneficial genes with or without vascular normalizing agents.

#### Therapeutic strategy for tumors with low blood flow and high vascular permeability as determined by DCE-MRI:

Combine an epigenetic agent with a VEGF inhibitor to increase penetration of T-lymphocytes and amplify the response of the adaptive immune system.

#### Immunoescape mechanism 5: Immunosuppression

Even if all the biochemical and physical barriers that might inhibit immune infiltration of tumors are overcome, it is by no means ensured that the TILs will manage to mount an effective anti-cancer response due to the induction of an immunosuppressive microenvironment. This section, which provides a basic—but far from exhaustive—overview of key immunosuppressive factors under epigenetic control, including secretion of cytokines, expression of negative regulatory checkpoint ligands that bind to cognate receptors on T cells, the induction of indoleamine 2,3-dioxygenase (IDO) and the recruitment of regulatory T cells.

#### Cytokines

The immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ), secreted from the

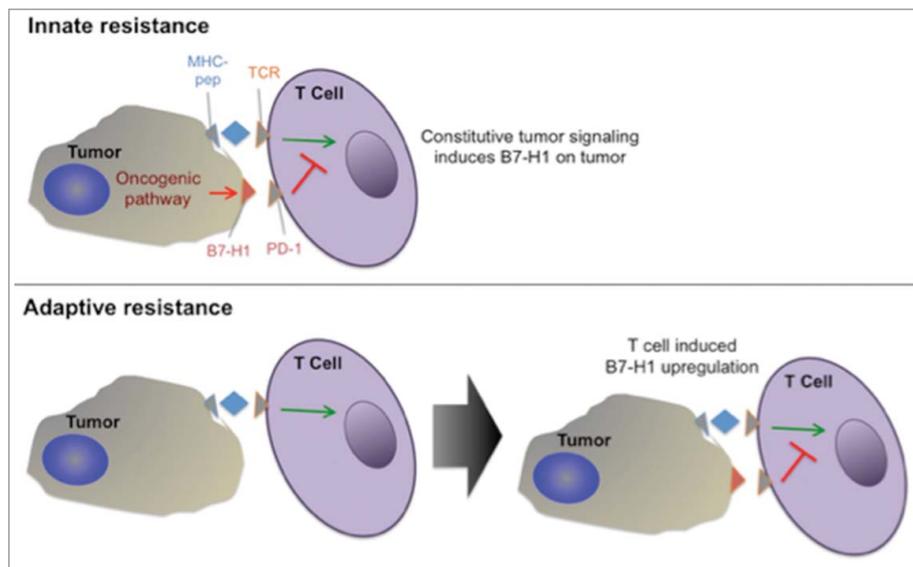
malignant cells themselves as well as noncancerous cells present at the tumor site, such as immune, epithelial, or stromal cells inhibit the maturation and antigen-presenting function of dendritic cells (DC) and T cell effector responses through regulatory T cells.<sup>104</sup> In addition to the epigenetically regulated expression of these tumor-derived cytokines,<sup>105</sup> the small molecule prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), VEGF and macrophage-colony stimulating factor change the repertoire of immune cells and inhibit T cell proliferation, activation, and differentiation. Neutralizing antibodies that bind or “trap” these cytokines, in particular TGF- $\beta$ , is a strategy to reverse local immunosuppression. As an example, oncolytic viruses may be armed with transgenes that express cytokine traps locally within the tumor microenvironment.<sup>106</sup>

#### Checkpoint inhibition

Checkpoint proteins such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death 1 (PD-1) constitute a part of the normal suppressive mechanism, which serves in a physiologic setting to dampen inflammatory responses of autoimmune or infectious origin or in pathologic situations to downregulate anti-tumor responses<sup>107</sup> (Fig. 7). PD-1, in particular, has 2 coinhibitory ligands: PD-L1 (also named B7-H1; CD274) and PD-L2 (B7-DC; CD273).<sup>108</sup> PD-L1 is expressed in multiple tumor types especially NSCLC, melanoma, and renal cell carcinoma while PD-L2 expression is far less prevalent.<sup>109</sup> The checkpoint inhibition pathways are co-opted and epigenetically upregulated by the tumor to direct the pattern of host immune responses in its own favor.<sup>44</sup> Approved PD-1 inhibitors are nivolumab (Opdivo) and pembrolizumab (Keytruda).

#### Induction of indoleamine 2,3-dioxygenase (IDO)

In what has been termed immunosuppression by starvation,<sup>110</sup> expression of IDO,<sup>111</sup> a ubiquitous enzyme that converts tryptophan, the essential amino acid required by T



**Figure 7.** Innate vs adaptive resistance. PD-L1 can be constitutively expressed or induced adaptively if the right inflammatory cytokines are present in the immune environment.

cells for proliferation and survival, to the immunotoxic metabolite, kynurenine, mediates the immunosuppression of pregnancy. Moreover, as a component of the compensatory anti-inflammatory response syndrome (CARS), it is epigenetically inducible by pro-inflammatory cytokines,<sup>112,113</sup> including interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Similar to the checkpoint pathway, cancer cells co-opt the physiologic mechanism of IDO-mediated tryptophan degradation to turn off antitumor responses.<sup>114</sup> Inhibitors of IDO are currently under investigation in clinical trials.<sup>115</sup>

### Recruitment of Regulatory T cells

One of the primary physiologic mechanisms to limit excessive and harmful immune responses is through the induction of a specialized subset of CD4+ T cells, CD4+CD25+ regulatory T cells (T<sub>regs</sub>),<sup>116</sup> which are conscripted to enhance tumor cell proliferation. As “major players” in the immune microenvironment,<sup>117</sup> the presence of regulatory T cells, which correlates with poor survival in a variety of tumors, including prostatic, lung, hepatocellular and renal cell carcinomas,<sup>118</sup> leads to the suppression of T<sub>helper</sub>1 (Th1)/CD8<sup>+</sup> T cell responses that mediate tumor cytotoxicity. Under the influence of cytokine CCL22,<sup>119</sup> T<sub>regs</sub> migrate to tumors where multiple factors including IL-2, TGF- $\beta$ <sup>120</sup> and, in particular, the epigenetically regulated transcription factor, Foxp3,<sup>121</sup> are responsible for their expansion. Strategies to deplete T<sub>regs</sub> include the administration of metronomic low dose therapy, cyclophosphamide and daclizumab, a US FDA-approved humanized anti-CD25 antibody. VEGF-A blockade may also reduce the numbers of tumor-infiltrating T<sub>regs</sub>.<sup>122</sup>

### Therapeutic strategy to relieve immunosuppression:

Combine an epigenetic agent with oncolytic viruses that encode cytokine traps, checkpoint inhibitors, the anti-CD25 antibody, daclizumab and a VEGF inhibitor to increase penetration of T-lymphocytes and amplify the response of the adaptive immune system.

### Immunescape mechanism 6: Resistance to apoptosis

Resistance to apoptosis, a genetically and epigenetically regulated process of cell suicide,<sup>123</sup> which underpins treatment failure, is a hallmark of cancer cells.<sup>124</sup> Initiation of the intrinsic or the extrinsic apoptotic pathways induces the caspases, a family of cysteine proteases that act as common death effector molecules. Aberrant methylation in the promoters of genes coding for proteins implicated in the extrinsic and intrinsic pathways has been implicated as an important mechanism of apoptosis resistance in cancer cells.<sup>125</sup> In addition, the epigenetic mechanism, which is arguably the best understood currently,<sup>126</sup> involves the transcriptional silencing of a growing list of tumor suppressor genes, most notably the key guardian p53,<sup>127</sup> that trigger apoptosis in the presence of cell abnormalities.

### Therapeutic strategy to induce apoptosis:

In this context, an epigenetic agent may be combined with any number of different treatment options since, in general, most chemotherapies irrespective of the mechanism of action or molecular target have the potential to induce apoptosis. However, in particular, agents, which generate reactive oxygen species or deplete reduced glutathione and induce oxidative stress like the platinum, arsenic trioxide, motexafin gadolinium, nitroglycerin, and various flavonoids may activate p53 (and other tumor suppressors) and synergize with an epigenetic agent.

### Immunescape mechanism 7: Tumor counterattack

In addition to the defensive strategies described above, a controversial hypothesis proposes that tumors also “go on the offensive” or on the “counterattack” through upregulation of Fas ligand (FasL/CD95L) expression. FasL is a transmembrane protein that belongs to the Tumor Necrosis Factor (TNF) family.<sup>128</sup> The interaction of FasL with its cell surface receptor, Fas (CD95/APO-1) leads to apoptosis<sup>129</sup> in Fas-positive cells, which is hypothesized to maintain the immune tolerance of the testis, hair follicle, placenta, eye and brain.<sup>130</sup> Evidence suggests that tumors epigenetically<sup>131</sup> upregulate the expression of Fas to

‘counterattack’ Fas-ligand-expressing tumor-infiltrating lymphocytes, which favors immune escape. If this controversial hypothesis is correct—the controversy relates to the fact that some studies support it,<sup>132,133</sup> while others refute it<sup>134</sup>—then the tumor walks a fine line between protection and harm, since Fas expression also predisposes to apoptotic cancer cell death, and further upregulation of the protein may ultimately suppress tumor proliferation.

#### Therapeutic strategy to ‘counter the counterattack’:

Further upregulate Fas expression in tumors with epigenetic therapies. In addition to epigenetic inhibition, other strategies include viral mediated delivery of Fas or fusion proteins connecting FasL to a single chain antibody fragment that specifically recognizes tumor cells or tumor stroma antigens is a promising field of research in therapeutics. Upon binding, the initially inactive protein would convert into a protein with membrane-bound-FasL-like activity.

### Summary and conclusions

According to the double-entendred Chinese proverb, “May you live in interesting times,” the modern era of immunotherapy has been both blessed and cursed. On the one hand, immunotherapy constitutes a revolution in cancer care with the promise of durable benefit and the potential of long-term survival in a subset (approximately 20-30%) of patients and tumor types.<sup>135</sup> On the other hand, success has so far eluded the majority of patients (70-80%), for whom the promise of anti-tumor immune activity remains tantalizingly out of reach.

This review presents evidence for the centrality of epigenetic alterations i.e., histone modifications and DNA methylation in *de novo* and acquired immune resistance. Unlike genetic lesions, epigenetic changes are highly dynamic and potentially reversible with DNA-demethylators and HDAC inhibitors.<sup>136</sup> By analogy with the United States No Child Left Behind Act of 2001 for disadvantaged students, the declarative premise in the title of this review, “No Patient Left Behind” is that the unacceptably high immunotherapy failure rate is conceptually as reversible as the methylation and acetylation modifications which underlie it: epigenetic reprogramming may reactivate silenced immune-related genes, including MHC class I, tumor-associated antigens, and accessory/costimulatory molecules, leading to enhanced immune recognition and tumor clearance, so that patients once stigmatized as “resistant to therapy” with an attendant poor prognosis will now have the opportunity to reap the same benefits as the minority of patients with intrinsically susceptible tumors.

Several approved (e.g., azacitidine and decitabine) and experimental (e.g., RRx-001) epigenetic therapies have demonstrated preclinical and preliminary clinical evidence of immune priming before or in combination with immune therapies in apparent contradiction to the “diminishing returns” usually associated with additional lines of treatment. However, whether or not the law of increasing or diminishing returns ultimately applies in this context depends on the side effect profile of the epigenetic agent(s): Grade 3-4 toxicities increase the risk and the likelihood that patients will not tolerate immunotherapy, which is the antithesis of a priming strategy. Immune checkpoint blockade, in particular, is associated with

its own particular set of serious side effects termed immune-related adverse events or irAEs<sup>137</sup>; consequently, even if the overlap in toxicities between the PD-1 inhibitor and the epigenetic agent(s) is minimal the combination of toxicities may result in a dose and treatment limiting degradation of performance status and quality of life tantamount to an anti-priming effect. This is especially the case with decitabine,<sup>138</sup> which is poorly tolerated as a result of hematologic side effects such as myelosuppression.

In addition, due to the broad spectrum of resistance and the multifactorial escape mechanisms as well as the multiplicity of targets, both known and unknown, in tumors, a therapeutic cocktail or combo, similar to treatment of the HIV virus, that includes an epigenetic agent may optimize immune priming responses, provided that the individual components combine advantageously i.e., that severe toxicities and/or antagonistic interactions do not interfere with activity. To that end, the specific strategies presented in this review are not mutually exclusive or incompatible but dovetail given the multifactorial nature of immune derangement in cancer.

Since the relative toxicities of the epigenetic agents in decreasing order are: DNMTs>HDACs>RRx-001, potential anticancer combinations might include RRx-001 as the epigenetic priming agent. Finally, as the strategy of immune priming with epigenetic agents gathers wider traction and momentum, the hope is that fewer and fewer patients will be left behind.

To paraphrase the Chinese proverb from the beginning of this Discussion section: May all cancer patients, especially those who were formerly resistant to the effects of immunotherapy, live in interesting *prime* times.

### Disclosure of potential conflicts of interest

EpicentRx Inc. funds research of RRx-001. Authors B.O. and J.S. are employees of EpicentRx, Inc. The remaining authors have no conflicts of interest to declare.

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### Author contributions

The authors declare that each author contributed to the writing of this manuscript.

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