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Immunological effects of hypomethylating agents

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Abstract

Introduction: Epigenetic changes resulting from aberrant methylation patterns are a recurrent observation in hematologic malignancies. Hypomethylating agents have a well-established role in the management of patients with high-risk myelodysplastic syndrome or acute myeloid leukemia. In addition to the direct effects of hypomethylating agents on cancer cells, there are several lines of evidence indicating a role for immune-mediated anti-tumor benefits from hypomethylating therapy.

Areas Covered: We reviewed the clinical and basic science literature for the effects of hypomethylating agents, including the most commonly utilized therapeutics azacitidine and decitabine, on immune cell subsets. We summarized the effects of hypomethylating agents on the frequency and function of natural killer cells, T cells, and dendritic cells. In particular, we highlight the effects of hypomethylating agents on expression of immune checkpoint inhibitors, leukemia-associated antigens, and endogenous retroviral elements.

Expert Commentary: *In vitro* and *ex vivo* studies indicate mixed effects on the function of natural killer, dendritic cells and T cells following treatment with hypomethylating agents. Clinical correlates of immune function have suggested that hypomethylating agents have immunomodulatory functions with the potential to synergize with immune checkpoint therapy for the treatment of hematologic malignancy, and has become an active area of clinical research.

Keywords

Acute myeloid leukemia; azacitidine; decitabine; hypomethylating agents; immune effects; myelodysplastic syndrome; NK cells; T-cells

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1. Introduction

The association between aberrant DNA methylation and cancer has been well established in the hematologic malignancies. Mutations disrupting epigenetic regulation of gene expression are identified in 45-50% of cases of myelodysplastic syndromes (MDS) [1] and acute myeloid leukemia (AML) [2]. Specifically, mutations in *DNMT3A*, *TET2*, *ASXL1*, *IDH1*, and *IDH2* are recurrently found in AML or MDS and contribute directly or indirectly to alterations in DNA methylation. The effects of these mutations are summarized here briefly, and the reader is referred elswhere for a more comprehensive discussion of each mutation[3]. The DNA methyltransferase encoded by *DNMT3A* is responsible for *de novo* DNA methylation and is mutated in approximately 22% of AML cases[4] and 8% of MDS cases[5], primarily altering the catalytic domain at the DNMT3A R882 residue which disrupts homotetramer formation and causes focal hypomethylation[6]. Mutations in *TET2* disrupt conversion of 5-methylcytosine to 5-hydroxymethylcytosine and result in aberrant methylation of genes that regulate hematopoietic development of myeloid and monocytic lineages [7] as well as effector T-cells[8]. *IDH1* and *IDH2* mutations affect the cycle of α-ketoglutarate synthesis, which is required for TET2 catalytic function, and instead increase production of 2-hydroxyglutarate, an inhibitor of TET2 function, resulting in a methylation profile similar to *TET2*-mutated malignancies[9]. In patients with MDS, *TET2* mutation status has been shown to be predictive of response to hypomethylating agents [10]. Mutations in *DNMT3A* or *TET2* are associated with poor prognosis in both AML[11,12] and MDS [13]. Overall, classification of AML patients by DNA methylation profile is reported as an independent predictor of clinical outcome, and identified a signature of recurrent hypermethylation of 16 genes across AML genetic subtypes[14].

Epigenetic changes due to aberrant methylation patterns may lead to either local or global effects on gene expression. Excessive methylation of cytosine bases within CpG-rich sequences of DNA remodels the surrounding chromatin structure to suppress the function of local promoters. In malignancies, a common mechanism of aberrant methylation results in hypermethylation of a promoter leading to loss of tumor suppressor expression. However, the mutations associated AML and MDS described above may influence the global or focal patterns of methylation and consequently gene expression. Hypomethylating agents are cytidine analogs that incorporate during DNA synthesis. Substitution for nitrogen at position 5 of the pyrimidine ring in place of carbon impedes methylation of the base by DNA methyltransferases (DNMTs). The detailed studies elucidating this mechanism have been reviewed previously [15]. Hypomethylation of CpG islands resulting from treatment with hypomethylating agents is considered to be a primary mechanism of these agents and is posited to allow for re-expression of suppressed genes (e.g. silenced tumor suppressor genes) however they are also known to induce direct cytotoxicity at higher doses. Currently, decitabine (5-aza-2'-deoxycitidine) and azacitidine (5-azacitidine) are FDA-approved for treatment of patients with MDS[16,17] and are frequently used for older AML patients unlikely to tolerate induction chemotherapy [18] or for those that have relapsed following stem cell transplantation. Ongoing clinical trials are evaluating additional hypomethylating agents, including CC-486 [19], an oral formulation of azacitidine, and SGI-110 [20], an oral prodrug formulation of decitabine.

In addition to past studies that form the basis for the clinical use of hypomethylating agents, additional benefit for hypomethylating agents was most recently identified in patients with AML or MDS who had mutations in the tumor suppressor gene *TP53*. This subset of patients typically has unfavorable cytogenetics, historically poor response rates (i.e. less than 30%), and an overall survival of less than six months when treated with conventional induction chemotherapy. However, treatment of patients with *TP53* mutations using decitabine resulted in morphologic complete remission in every patient; although none of these patients were negative for minimal residual disease and morphologic remissions were short-lived [21]. Attempts to identify methylation or genomic signatures in patient samples to predict potential responders to hypomethylating therapy have been unrevealing to date [22,23] or remain unvalidated.

Several groups have hypothesized that the clinical benefits of treatment with hypomethylating agents may be the result of a combination of effects, with contributions from both direct effects on malignant cells and indirectly by facilitating immune anti-tumor responses. Within a small cohort of patients previously treated with hypomethylating agents on a phase I clinical trial for classical Hodgkin lymphoma, there was observed benefit in later response to subsequent treatment with immune checkpoint inhibitors [24], although the trial was not powered to determine effacacy. All patients within the cohort had been heavily treated for relapsed/refractory Hodgkin lymphoma, including brentuximab vedotin and/or autologous stem cell transplantation, prior to receiving immune checkpoint therapy. The use of immunotherapy for treatment of patients with hematologic malignancies relapsed from, or refractory to, conventional cytotoxic chemotherapy is an area of great interest [25]. The ability to potentially augment any anti-tumor immune response using hypomethylating treatment could usher in a new era of combination therapies for these patients who currently have a very poor prognosis; it might also extend the use of hypomethylating agents beyond hematologic malignancies. Here we provide an overview of the effects of hypomethylating agents on immune cell subsets, with additional discussion of cytokine responses and antigen presentation by malignant cells as a foundation for extending the use of hypomethylating agents in combination with other immune-modulating agents.

2. Modulation of immune cell subsets by hypomethylating agents

2.1 Natural Killer Cells

DNA methylation has been shown to effect innate immunity through the alteration of gene expression profiles in natural killer (NK) cells. NK cells play a pivotal role in both the recognition and regulation of malignant cells [26-28]. Interestingly, NK cell functionality is profoundly affected by the DNA methylation status of various receptors. Unlike B and T-cells, NK cells lack a vast repertoire of antigen-specific receptors and, instead must rely on a balance of inhibitory and activating signals from their comparably promiscuous receptors to differentiate healthy from diseased cells [27,28]. Killer immunoglobulin-like receptors (KIRs) are an important family of both inhibitory and activating, though primarily inhibitory, receptors on NK cells that serve an important role in immune tolerance [26-28]. KIR ligands include major histocompatibility complex (MHC) class I molecules expressed on the cell-surface of all nucleated cells [26-28]. NK cells undergo an educational process in

the bone marrow by which inhibitory KIRs expressed on their cell surface must interact with self-MHC class I molecules expressed on other cells in order for the NK cell to gain functional potential [27,28]. It is through this process that a baseline of KIR expression on healthy cells is established. Malignant cells often express aberrantly low levels of MHC class I molecules (e.g. those adapted to evade CD8+ T-cell recognition); upon encounter with NK cells, the inhibitory signals from those KIRs normally expressed on healthy cells are diminished [28,29]. Furthermore, malignantly transformed cells will often upregulate stress ligands (e.g. MICA/B stress ligands) for which NK cells have activating receptors (e.g. NKG2D) [30,31]. NK cell recognition and activation against malignant cells is induced upon two conditions: the lack of recognition of MHC class I molecules by KIRs and the recognition of stress ligands by their respective activating receptors [27,30]. It is a combination of these mechanisms that enable the downstream effects of NK cell recognition of, and cytotoxicity against, malignant or infected cells (i.e. perforin release, degranulation, and interferon gamma [INF-γ] release).

Promotor methylation of KIR genes has been shown to consistently suppress KIR expression on NK cells, whereas hypomethylation enables KIR expression [29,30,32]. Bisulfite sequencing of a region around the translation initiation site of the NKG2D gene in cells expressing various levels of the protein product revealed a significant correlation between methylation at this site and protein expression[31]. Moreover, in the investigation of the genome-wide methylation status of activated NK cells, Wiencke *et al.* found that the activated NK cell phenotype was associated with hypomethylation of CpG sites at 81% of significant loci; whereas naive NK cells, on the other hand, demonstrated a lower degree of demethylation [33].

The direct effects of hypomethylating agents on NK cell functionality have been examined in great detail both *in vitro* and *ex vivo*. A few studies have investigated the direct effects of hypomethylating agents on NK cell gene expression and ultimate functionality; however, there does not appear to be a consistent consensus across sources. To begin, two studies conducted by Gang *et al.* and Gao *et al.* investigating the effects of azacitidine exposure on NK cell functionality yielded similar conclusions – *in vitro* treatment of NK cells with azacitidine hinders NK cell-mediated cytotoxicity against the K562 leukemic target cell line in a dose-dependent manner [29,34]. Gao *et al.* attributed this to the induced overexpression of inhibitory KIRs as well as diminished perforin and granzyme B production [29]. Interestingly, Gang *et al.* did not observe the same strong inhibitory effect on NK cell functionality when examined *in vivo* in MDS patients receiving comparable *in vitro* doses of azacitidine [34]. They did however find a small decrease of an NK cell subpopulation expressing CD158b, an inhibitory receptor [34]. Sohlberg *et al.* also demonstrated that low-dose *in vitro* administration of azacitidine upregulates KIR expression on NK cells from both healthy donors and MDS patients [30]. However, in contrast to the other studies, Sohlberg *et al.* observed an amplification of NK cell-mediated cytotoxicity against K562 cells, defined by increased INF-γ production, degranulation, and killing frequency [30]. These results pertained only to Ki-67+ NK cells, an indicator of NK cells that have undergone recent activation or proliferation [30]. A study conducted by Kopp *et al.* established that NK cells exposed to increasing doses of a hypomethylating agent, this time decitabine, upregulated KIR expression in a linear dose-dependent manner [35].

Furthermore, Kopp *et al.* investigated expression patterns of other NK receptors including the activating receptors NKp44 and NKG2D, which demonstrated a linear increase and decrease, respectively, with increasing decitabine exposure [35]. However, this study revealed that decitabine induces a biphasic effect on the cytotoxicity of human peripheral blood mononuclear (PBMC)-derived NK cells, where inhibition was observed only at concentrations between 0.3uM-2.5uM [35]. Rather than reflecting NK cell receptor expression, this effect mirrored the global hypomethylation pattern in which the percentage of methylation decreased linearly until 0.3uM concentrations, then began to rise to baseline with further increasing doses of decitabine [35]. Finally, using 5uM concentrations of both azanucleosides, Schmiedel *et al.* found that azacitidine inhibits NK cell IFN-γ production and cytotoxicity whereas decitabine significantly augments these NK cell functions [36]. Currently there is not a clear consensus on the effects of hypomethylating agents on NK cell functionality. This is likely due to difficulties in consistent *in vitro* and *ex vivo* conditions, variable concentrations of azanucleoside administration, consistency in functional assays across groups, as well as the different sources of NK cells used in each study.

As a notable example from a recent clinical trial, patients treated with decitabine for a 10-day course demonstrated significantly higher antibody-dependent cell-mediated cytotoxicity (ADCC) in response to an investigational anti-CD33 monoclonal antibody[37]. Expression of NKG2D ligand was significantly increased at 28 days following decitabine treatment compared to pre-treatment control samples. Additionally, *in vitro* use of a blocking antibody to the corresponding activating receptor negated the increase in ADCC. The more complicated interactions observed between NK cells and leukemic blasts in AML patients reported to date [38] as well as the potential contributions of NK cells to graft-versus-leukemia effects following allogeneic stem cell transplantation [39] both emphasize the importance of further studies on *in vivo* mechanisms of the effect of hypomethylating agents on NK cells.

2.2 Dendritic Cells

As the bridge between innate and adaptive immunity, dendritic cells (DCs) serve a critical role in their ability to recognize pathogens and activate B and T-cells. Genome-wide base-resolution mapping of 5-methylcytosine and locus-specific bisulfite sequencing revealed a significant loss of DNA methylation associated with DC development and maturation [40]. This loss could be attributed to the noted down-regulation of three DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) [40]. Since DNA methylation appears to significantly affect DC development, it would be interesting to further investigate whether hypomethylating agents directly modulate DC phenotype and function.

While the effect of hypomethylating agents on DC function has not been extensively studied, there is some evidence indicating that azacitidine may impact various features of DCs. Using *in vitro* studies on PBMC-derived DCs, Frikeche *et al.* determined that azacitidine upregulates CD40 and CD86 expression on mature DCs [41]. CD40 expression on DCs is acquired during their maturation process, in which binding of CD40 on DCs to CD40L on antigen-specific CD4+ T helper 1 (Th1) cells permits future priming of cytotoxic lymphocytes [42]. CD86 is a costimulatory molecule on DCs that mediates polarization of

T-cells upon interaction with peptide:MHC II complexes on DCs [41]. Without this second co-stimulatory signal, T-cells would become anergic rather than activated. Additionally, these mature DCs secreted significantly less interleukin (IL)-27 and IL-10 compared to the control [41]. Furthermore, *in vivo* studies conducted by Frikeche *et al.* revealed a significant decrease in IL-4-secreting CD4+ T-cells in the peripheral blood of advanced MDS and AML patients but a significant increase in IL-17A- and IL-21-secreting CD4+ T-cells [41]. Indicative of a Th17 response, azacitidine may affect DCs in a manner that alters T-cell polarization.

2.3 T-cells

T-cell phenotype and function are intensively modulated through epigenetic changes, as illustrated by the following examples. CD28, a costimulatory molecule, is important in activation, proliferation, and survival of CD4+ T-cells. Reduced expression of CD28 on CD4+ T-cells is associated with aging and is associated with impaired CD4+ T-cell responses in the elderly [43]. Upon comparison to CD4+CD28+ T-cell counterparts, these CD4+CD28- T-cells exhibit an overall increase in gene expression involved in inflammasome and T-cell receptor (TCR) signaling [43] and an associated distinctive DNA methylation landscape [43]. A second example is found in colonic regulatory T-cells; Obata *et al.* found that inoculation of gut microbiota in germ-free mice induced upregulation of Uhfr1 in this cell type [44]. Uhrf1 is a DNA methylation adapter that forms gene-repression complexes through the binding of hemi-methylated DNA and recruitment of DNMT1 [44]. Expression of this Uhrf1 resulted in the silencing of the gene encoding cyclin-dependent kinase inhibitor p21, whose expression promotes cell cycle arrest [44]. Mice engineered to have a T-cell-specific deficiency in Uhfr1 developed severe colitis due to an inability of colonic regulatory T-cells to mature and repress excessive immune responses to commensal gut microbiota [44]. Furthermore, global DNA methylation profiling of regulatory T-cells has revealed that CpG sites within known FOXP3 binding regions throughout the genome are hypomethylated [45]. Regulatory T-cells are distinguished from CD4+ T-cells by hypomethylation of the FOXP3 locus and subsequent expression of this transcription factor. Zhang *et al.* demonstrated that expression of TIGIT, an inhibitory receptor, in regulatory T-cells was associated with a hypomethylated locus and subsequent FOXP3 binding [45]. Expression of programmed death-1 (PD-1), an inhibitory receptor on the cell-surface of T-cells, is also epigenetically regulated [46]. PD-1 regulates the activation of both antigen-specific CD8+ T-cells and regulatory CD4+ T-cells in normal circumstances. While the degree of methylation at the PD-1 locus varies across immune cells, T-cells are relatively hypomethylated compared to other immune subsets, and interestingly the PD-1 locus is hypomethylated in exhausted CD8+ T-cells during chronic viral infections [47]. During chronic infections and cancer, there is persistent expression and engagement of the PD-1 receptor on tumor-infiltrating CD8+ T-cells, leading to T-cell exhaustion and subsequent immune evasion by tumor cells [48,49]. Tumor-infiltrating CD8+ lymphocytes with high PD-1 expression have demonstrated specificity against autologous tumors and are potentially capable of antigen-specific responses targeting the tumor [50]. The effects of DNA methylation on T-cell subsets become an important consideration in patients being treated with hypomethylating agents or with immune checkpoint inhibitors.

Patients with hematologic malignancies treated with hypomethylating agents have demonstrated changes in methylation at the PD-1 locus, with associated increases in expression of PD-1 transcripts and protein expression on the cell surface[51,52]. For example, the PD-1 promoter in CD8+ T-cells from AML and MDS patients can become further hypomethylated after treatment with azacitidine [51]. Using AML cell lines, the effect appears to be specific to hypomethylating agents, with similar PD-1 demethylation observed in response to decitabine but not other chemotherapeutic classes [52]. In a small group of AML and MDS patients who were treated with azacitidine and a histone deacetylase inhibitor (vorinostat), decreased methylation of PD-1 was observed in both clinical responders and non-responders [52]. These observations raise concern that decreased methylation of PD-1 in response to treatment with hypomethylating agents may contribute to T-cell exhaustion and poor clinical responses.

In addition to antigen presented in the context MHC complexes, the effective priming and activation of T-cells requires a crucial second signal provided by interaction with costimulatory molecules such as CD80 and CD86. Downregulation of CD80 cell-surface expression is a potential route for immune evasion by malignant cells [53,54]. In a T-cell lymphoma mouse model, decitabine appeared to stimulate CD80 expression on the malignant cells and augment subsequent cytolytic activity of IFNγ-producing CD8+ T-cells. Increased CD80 expression due to decitabine treatment was confirmed in CD80-negative cell lines, and the CD80 promoter was shown to be hypomethylated in response to decitabine [55]. Decitabine-induced upregulation of CD80 gene and protein expression has also been observed in colon cancer cell lines [54].

Another molecule within the immune checkpoint axis that regulates T-cell responses is CTLA4, the inhibitory counterpart to CD80. Whereas CD80 and CD86 provide co-stimulatory signals for T-cell priming and activation by binding CD28, ligation of CD80 to CTLA4 receptors on T-cells attenuates T-cell activity and this binding interaction has a higher affinity than that of CD80 and CD28[56]. In an ovarian cancer mouse model, decitabine treatment increased recruitment of activated CD8+ T-cells into tumor-draining lymph nodes and the immune effect was further augmented by the addition of anti-CTLA4 antibody [57]. There is potentially a dual effect of hypomethylating agents on the immune checkpoint axis through increased CD80 expression on tumor cells and recruitment of activated IFNγ-producing CD8+ T-cells into the tumor microenvironment. The availability of CD80 on the surface of tumor cells following decitabine treatment, together with the internalization of CTLA4 observed in T-cells, may contribute to a more sustained immune response through interaction with CD28 [58].

The relative proportion of T-cell subsets appear to be affected by treatment with hypomethylating agents, although to varying degrees among *in vivo* and *in vitro* studies. In a group of 68 patients with intermediate-2 or high-risk MDS, those who responded well to hypomethylating treatment with azacitidine were found to have fewer regulatory T-cells in circulation at long-term follow-up than before treatment and appeared more similar to healthy donors in regulatory T-cell frequency [59]. Correlative studies of CD4+ T-cells treated with azacitidine *in vitro* demonstrated reduced proliferative capacity, reduced suppressor function, and increased IL-17 production. Notably, there was demethylation of

the FOXP3 promoter and increased expression of FOXP3 in this cell population, but without regulatory T cell function or cytokine production [59]. In a similar finding using a mouse model treated with azacitidine or decitabine, increased FOXP3 expression was observed in regulatory T-cells, although the suppressor function of these induced regulatory T-cells was independent of FOXP3 expression [60]. Conversely, in a separate cohort of MDS patients the effect of azacitidine appeared to transiently increase FOXP3+ regulatory T-cells and decrease the T helper 17 (Th17) population *in vivo*, and was corroborated with *in vitro* assays [61]. In the post-stem cell transplantation setting, use of hypomethylating agents for relapsed AML has been studied for its effects of T-cell subsets in a small cohort of three patients who were treated for one to two cycles with azacitidine. There were increased populations of CD4+CD25+FOXP3+ regulatory T-cells (also confirmed as CD4+CD25hiCD127lo T-cells), with correspondingly fewer CD8+ T-cells and Th1 cells, as well as decreased CD8+ cytotoxicity, decreased IFN-γ production, and increased transcription of IL-10 and TGF-β cytokines [62]. In a separate study of 28 patients treated with azacitidine for relapsed AML following allogeneic stem cell transplantation, 16 patients were observed to elicit CD8+ T-cell responses to stimulation with at least one tumor-associated antigen; this was correlated with a reduced risk of relapse [63].

In small subsets of patients with MDS or AML, there was also a notable change in the peripheral T-cell repertoire during responses to treatment with hypomethylating agents. Patients with MDS or AML who responded well to treatment with azacitidine demonstrated decreased skewing of the TCR CDR3 profile as assessed by spectratyping [64]. A similar result was observed in four patients with solid tumors treated with decitabine on a clinical trial protocol who demonstrated increased TCR diversity and lower abundancies of expanded clones in comparison to pre-treatment levels [65]. Changes in peripheral TCR repertoire diversity observed during hypomethylating treatment may be a more relevant correlate, than changes in T-cell phenotype subsets, of tumor antigen-specific immune responses.

A wide range of leukemia associated antigens have been described [66], many of which are thought to be epigenetically regulated. The ability to augment expression of such antigenic targets is of great interest. For example, despite finding no changes in the T-cell subsets of patients with AML, MDS, or CMML treated with azacitidine, Gang *et al.* observed augmented T-cell recognition of CD34+ leukemic blasts as well as increased T-cell recognition of nine cancer-testis antigens (CTAs), albeit transiently: SART-3, MAGE-A1, MAGE-A2, TAG-1, NY-ESO-1, NUF3, GnTV, CDCA1 and/or Sp17 [34]. A trial investigating azacitidine-based maintenance treatment for patients with AML who remained in remission following allogeneic stem cell transplantation observed a transient increase in regulatory T-cells followed by antigen-specific CD8+ T-cell responses to MAGE-A1, MAGE-A2, MAGE-A3, BAGE-1, RAGE-1 and/or WT-1 in 15 out of 22 patients after 3 months of maintenance treatment and 14 out of 18 patients after 6 months of maintenance therapy in comparison to 1 out of 21 patients evaluated prior to azacitidine treatment [67].

While induction of cancer antigen expression by hypomethylating agents across a broad range of tumors has been reviewed previously [68], one novel class of antigens will be highlighted here. Increased expression of endogenous retroviral (ERV) elements was

recently reported following treatment with hypomethylating agents. This triggered double-stranded RNA sensing via TLR3, MAVS and, subsequently IFN-β[69]. In melanoma patients, a signature of viral defense gene expression was associated with durable clinical responses to anti-CTLA4, an immune checkpoint inhibitor[69]. There is therefore an intriguing potential for synergism between expression of cancer antigens, activation of innate interferon response pathways to ERV upregulation, and immune checkpoint blockade in those receiving hypomethylating agents [70]. Hypermethylation of *PD-L1* was associated with lower risk for relapse and prolonged overall survival, although this was not an independent risk factor in multivariate analysis with cytogenetics and TP53 mutations [71]. Promoter hypomethylation at the *PD-L1* locus in leukemic blasts was correlated to unfavorable cytogenetic risk and *TP53* mutations [71]. As the use of immune checkpoint inhibitors expands into the hematological malignancies [72], combining immunotherapies targeting the PD-1/PD-L1 axis with hypomethylating agents may offer improved clinical outcomes by counteracting the observed upregulation of PD-1/PD-L1 checkpoints by demethylating agents [52].

3. Conclusions

Currently, hypomethylating agents are FDA-approved in the treatment of patients with MDS, and have a well-established palliative role in the treatment of patients with AML who are unlikely to benefit from induction chemotherapy due to older age or poor performance status or have disease refractory to intensive salvage chemotherapy. Recent investigations have identified potential direct effects of hypomethylating agents on immune cell subsets including NK cell, DC, and T-cell functions. To summarize, NK cells respond to treatment with hypomethylating agents by modulating the expression of inhibitory and activating receptors on th cell surface, including increased expression of KIR proteins and decreased expression of the NKG2D activating receptor, but the functional effects on cytotoxicity have not been firmly established, Dendritic cells respond to hypomethylating agents by increasing expression of CD40 and CD86 co-stimulatory molecules on the cell surface, permitting interactions with with activated T cells in the microenvironment. Within T cell subsets, methylation is critical to regulate expression of the immune checkpoint inhibitors PD-1, CTLA4 and TIGIT, co-stimulatory molecules including CD28 and CD80, and the developmental pathway of regulatory T cells through FOXP3. Emerging evidence indicates that the direct effects of hypomethylating agents include increased expression of tumor-associated antigens, as well as expression of anti-viral cytokines in response to increased expression of endogenous retroviral elements.

4. Expert Commentary

Hypomethylating therapies are a mainstay of management for patients with MDS or AML but provide only palliative benefits for disease control. Clinical research has primarily focused on exploring optimal dosing regimens, until recently with an emergence of interest in expanding options for immunotherapy. For patients with high-risk MDS who are unable to undergo hematopoietic stem cell transplantation, patients with AML who are unsuitable for intensive chemotherapy, or patients with relapsed or refractory AML, there is a pressing clinical need to develop novel, effective therapies. Treatment of MDS or AML with

immunotherapy has been well established, with hematopoietic stem cell transplantation as the leading example. Despite remarkable improvements in transplant-related mortality, the risks and barriers to transplant remain formidable for many patients. Identifying an immune-mediated treatment that is effective for AML or MDS, but would ideally spare many of the treatment-related risks of stem cell transplantation, has remained a primary objective for many clinicians and scientists in the field. As a deeper understanding of clinically relevant immune mechanisms has grown from the advancement of the field of immunotherapy as a whole, there is eager interest in identifying combinations of treatments to provide optimal immune-mediated anti-tumor effects. Suggestions of immune modulation by hypomethylating agents, based on *in vitro* and *ex vivo* effects on NK cells, DCs and T cells described above, has prompted renewed interest in these therapies. However, the complicated immune subset interactions that likely underpin much of the clinical benefit of hypomethylating agents are inherently difficult to untangle in the laboratory. Ongoing earnest basic research in this area will surely continue to be informative and invaluable.

As a complement to active basic science research into the mechanism of hypomethylating agents, there will be an increasingly important role for translational studies to provide relevant clinical specimens for detailed analysis, as well as context for interpretation of the results. With the increasing depth of data that can potentially be obtained from translational research samples, and with rapid advancements in data analysis for rich data sets including genome methylation mapping and functional immune characterization, there are exciting opportunities to probe research questions that have been elusive in the past. For example, the relative importance of global disruptions in DNA methylation, versus focally aberrant methylation changes or specific changes in patterns of methylation at CpG islands, remains an open question that is beyond the scope of this review. However, attempts to identify a genomic methylation signature to predict treatment response to hypomethylating agents, or to tailor targeted therapeutic agents, have been unsuccessful to date. New technologies that facilitate site-specific and single-cell DNA methylation analysis are evolving to address these questions.

Most importantly, in order to achieve novel therapeutic regimens for patients with AML who currently have no curative options, there are active clinical trials enrolling patients to evaluate treatment combinations of hypomethylating agents with immunotherapy, such as anti-PD1 antibodies. Future studies of novel immunotherapy combinations with hypomethylating agents may also hold promise in certain cases of MDS, but the vast heterogeneity of the disease presents an additional challenge to identifying patients who could potentially benefit. Over the next five years, we anticipate that results from a number of current clinical trials for AML in this area will be able to inform future treatment discussions with these patients, as well as providing experience to extend these combinations to solid malignancies and other hematologic malignancies.

5. Five-Year View

There is still much to uncover regarding the impact of hypomethylating agents on normal immune subsets and the tumor microenvironment. Further studies correlating *in vivo* and *ex vivo* experiments will be needed to unravel the direct effects of hypomethylating agents on

NK cells, DCs, T-cells, malignant cells, and the interactions that potentiate anti-tumor immune responses. The role of DNA methyltransferase inhibition on myeloid derived suppressor cells (MDSCs) and other cell populations and/or immune checkpoint molecules that would potentially be inhibitory to an anti-tumor immune response in humans also remains to be determined [73, 74]. Elucidating the functional consequences of hypomethylating therapies is a first step toward a goal to identify patients who will likely benefit from hypomethylating agents, and from novel combination therapies currently in clinical trials. Evaluating the effects of these agents on the immune system in translational research studies will provide valuable additional insight into this challenging field and further develop therapeutic options for patients in future clinical trials.

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AML	Acute myeloid leukemia
CDR3	Complementary determining region-3
CMML	Chronic myelomonocytic leukemia
CTA	Cancer testis antigen
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNMT	DNA Methyltransferase
ERV	Endogenous retrovirus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
KIR	Killer immunoglobulin-like receptor
MDS	Myelodysplastic syndromes
MHC	Major histocompatibility complex
NK	Natural killer
PBMC	Peripheral blood mononuclear cells

PD-1	Programmed death-1
PDL-1	Programmed death ligand-1
TCR	T-cell receptor
Th	T helper

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6.

Key Issues

- Many AML and MDS patients have abberant epigenetic regulation of gene expression. Hypomethylating agents are FDA-approved in the treatment of MDS and are used in certain cases of AML, such as in patients unable to tolerate induction chemotherapy.
- Hypomethylating agents increase and decrease expression of KIRs and NKG2D, respectively, on NK cells and have controversial effects on NK cell-mediated cytotoxicity against malignant cells and tumor cell lines.
- *In vitro* treatment of PBMC-derived DCs leads to increased expression of CD40 and CD80 on mature DCs. One *in vivo* study found that azacytidine likely induces a Th17 response in the peripheral blood of MDS and AML patients; this points to a potential mechanism by which azacytidine effects DCs in a way that may modify T-cell polarization.
- There is substantial evidence suggesting that hypomethylating agents cause demethylation at the PD-1 promoter region and subsequent upregulation of PD-1 expression on immune cells. Increased PD-1 expression may further induce T-cell exhaustion, leading to poor clinical outcomes. The opportunity to combine these agents with anti PD-1 therapy is an obvious area of interest.
- Upregulation of cancer antigens together with augmented T-cell-mediated recognition has been observed upon treatment with hypomethylating agents.
- Hypomethylating agents appear to induce expression of ERV elements, which initiates double-stranded RNA sensing; a specific expression signature of viral-defense genes has been correlated with good clinical outcomes in patients receiving immune checkpoint inhibitors.
- Currently, very little known about indirect mechanisms of hypomethylating therapy on the immune system. It is possibile the clinical efficacy observed with hypomethylating agents may be due in part to the combinatorial effect on the tumor immune microenvironment and malignant cells directly including immune cell subsets, such as NK cells, DCs, and T-cells as well as upregulated expression of CTAs and ERV elements. Future studies will be required to further unveil exactly how these agents modulate cell subsets within the immune system and induce anti-tumor responses.