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TITLE: A New Paradigm for the Treatment of Ovarian Cancer: The Use of Epigenetic Therapy to Sensitize Patients to Immunotherapy and Chemotherapy

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14. ABSTRACT: Our overall goal remains to bring epigenetic therapy to have major impact for the management of advanced ovarian cancer (OC). This past year, we continue to make exciting advances in our pre-clinical work and interim results from our leveraged clinical trial is pending now for low dose therapy targeting DNA demethylation paired with immune checkpoint therapy. The results of our two relevant, published studies of mouse models have attracted a great deal of attention, including our study of a OC model in which we have identified that the demethylating agent, 5-aza-cytidine (AZA) potently stimulates tumor immune attraction of T-cells to the tumor microenvironment (PNAS, 2017). For components of this treatment paradigm, (Cell, 2017, PNAS, 2017), during the past year we have broadened the degree of immune pathway signaling involved for OC as described in our report below. Studies of how an inhibitor of G9A, an enzyme mediating transcriptional repression, might can augment the above AZA effects for OC continue as well (Cancer Res, 2018). All of the above findings continue to document how epigenetic therapy can potentially improve immune checkpoint therapy for OC.					
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Herein, we transmit our final report for the DOD, Ovarian Cancer TEAL Award. We are beyond grateful that we were awarded the opportunity to pursue this opportunity. We feel we made robust progress for most Specific Aims in the grant and produced findings that do hold promise for true eventual benefit for patients with ovarian cancer. Of course, not every one of our studies worked as well as we would have hoped but this par for the course in every investment of this type. For orientation, we have kept key aspects of the initial proposal in place so this report may be best followed.

1. INTRODUCTION

In this multi-investigator initiative, we explore the potential that “epigenetic” therapy strategies, with relatively low toxicities, might potentially robustly extend the life expectancy of women with advanced ovarian cancer (OC). Over the past six years, we have published a series of studies which continue to suggest how epigenetic therapy can reverse tumor immune evasion states and thereby potentially enhance the efficacy of immune checkpoint therapy. For this report, we will summarize our work and accomplishments for each Specific Aim of the original proposal for which funding was awarded.

A. Specific Aim 1 – *To uncover the mechanisms through which epigenetic therapy may, alone, achieve robust, durable responses in patients with advanced ovarian cancer (OC)*: During the entire period of our award we completed the aims of this work and **Major Task 1**. Since our start of funding, we first completed ongoing studies coming into the program by, first finishing our identification of sensitive cell lines to epigenetic therapy drugs, the DNA demethylating agent, 5-aza- cytosine (5AC) and the histone deacetylase inhibitor (HDACi), entinostat, from among some 30 OC cell lines studied in collaboration with our co-investigator for the TEAL award, Dr. Dennis Slamon at UCLA (Li et al, Oncotarget, 2014). We then made very important progress pursuant to the above over the course of our funding. As reflected in our publications (Chiappinelli et al, Cell 2017; Topper et al, Cell 2017; and the intense focus on Our OC mouse model, (Stone et al, PNAS, 2017) we have played a major role in the field by producing pre-clinical data showing how treatment in-vitro and in-vivo with mouse models induces robust anti-tumor effects for OC and other tumors including blunting of metastatic behavior. These effects have an intense activation of an immune response involving both tumor and key immune cell subsets (**summarized from our Cell and PNAS publications, above**). Key points include: **1)** up-regulation of an interferon signaling triggered by induction of a viral defense response triggered, in part by increased transcripts from endogenous retro-viruses (ERV's) events termed as “viral mimicry”. This all results in the presence of double stranded RNA (dsRNA) in the cell cytosol to trigger the viral mimicry and interferon signaling; **2)** increase in tumor production of key, T-cell attracting cytokines prominently including the interferon responsive, CCL5. This protein has also subsequently been shown by Coukos and colleagues to play such a functional role for OC in pre-clinical studies (Dangai et al, Cancer cell, 2019); **3)** a sharp decrease in the C-Myc oncogene pathway which we show otherwise would suppress the viral mimicry upregulation and diminish the tumor immune attraction signaling; **4)** all the above is accompanied in post-treatment tumors by an increased infiltration of activated T-cells. This series of results now forms the basis of the correlative science we aim to use for tumor samples from the clinical trial leveraged from our TEAL work, but not directly funded from the grant, and which led by Dr. Slamon and colleagues and that has now completed enrollment. We have now just received the samples from Dr. Slamon to begin this work. The clinical results from the whole now completed trial are still pending.

B. Specific Aim 2: *Study how epigenetic therapy may sensitize OC cells to subsequent chemotherapies*: **Major Task 1: Develop the in-vitro and in-vivo pre-clinical systems to outline the sensitivities and derive molecular signatures that track with these.**

We made great strides for this aim and especially as spurred by a new finding over the last two years of funding, about which we are exceedingly excited. The work has been led in great part by Dr. Michael Topper, former Teal trainee and who now just been appointed faculty member at Hopkins. He led efforts in a collaboration with the lab of Dr. Feyruz Rassool at the University of Maryland to directly show how in this work how our epigenetically therapy agents directly involves sensitization of OC cells to a subsequent therapy critical to potential management of

patients with OC, use of PARP inhibitors (PARPi) as outlined below. At the present time, PARPi's have efficacy only in OC and triple negative breast (TNBC) patients whose tumors have BRCA mutations which cause homologous recombination defects (HRD). HRD is the very top signature for sensitivity of tumor cells to PARPi's. We have now found that one key epigenetic drug which is the focus of our grant, a DNA methyl transferase inhibitor (DNMTi) induces HRD, and resultant PARPi sensitivity in BRCA wild type, OC and TNBC cells. The mechanism surprisingly marries the ability of DNMTi's, as extensively outlined in specific Aim 1, to induce viral mimicry to activate tumor immune attraction through interferon signaling with an expansion of the process. This expansion includes not only the viral mimicry but also induction by the DNMTi's of TNF alpha and NF Kappa B signaling plus a response of the STING/CGAS pathway that responds to presence of DNA in the cytosol of cells. This latter STING response is contributed to when we combine the potent PARPi, Talazoparib with the DNMTi. These combined events are all reminiscent of what has recently been termed the inflammasome which is an ancient signaling pathway which triggers death in cells which cannot clear RNA or DNA viral infections or bacterial infections. In turn, this complex signaling then links directly to transcriptional induction of HRD through decreasing expression of Fanconi anemia (FA), DNA repair genes. These series of findings, now summarized in **Fig. 2**, then underlie the drug combination providing a marked tumor cytotoxic sensitivity and in-vivo reduced tumor burden. This work was published (**McLaughlin et al, PNAS 2020**) and ***excitingly our group has, translated these findings into a leveraged clinical trial which is now enrolling and partially funded by Aztex (for their DNMTi) and Pfizer (for their PARPi), with drugs also provided by each company for breast cancer patients with TNBC. An arm will be added for OC if a positive signal for effects is seen in the TNBC patients.***

C. Specific Aim 3: to study how epigenetic therapy may sensitize OC cells to subsequent immunotherapies which targets checkpoints which are driving immune tolerance. Working our team co-investigators, Cindy Zahnow, Dennis Slamon, Drew Pardoll, and Peter Jones we had great progress and final findings for this aim.

Major Task 1: to develop the in-vitro pre-clinical systems to outline the sensitivities and derive molecular signatures that track with these, and **Major Task 2:** to develop in-vivo pre-clinical systems to outline the potential efficacy of epigenetic therapy sensitization to immunotherapy for targeting checkpoints which drive immune tolerance. The progress now includes completed enrollment of the leveraged clinical trial for advanced OC as introduced earlier above headed by Dennis Slamon. The results will determine whether a phase 2 portion of the trial will move forward. Critically, as noted earlier, samples from the trial have now been shipped to Hopkins for performance of a key goal of our performing correlative science biomarker studies to help us learn why we have or have not achieved any promising results and how to predict patient responses and personalize the therapy if warranted.

D. Specific Aim 4: Develop new combinations of epigenetic drugs which may provide for the highest level of efficacy for management of OC: **Major Task 1:** Follow biochemical hypotheses for designing combinations of the epigenetic drugs used in all studies above with new agents targeting additional steps in chromatin control of gene expression – the goal is to improve reversal of abnormal gene silencing in OC

These studies over the entire funding period were under the primary direction of Dr. Peter Jones at the Van Andel Research Institute (VARI), with close collaboration with the Baylin and Zahnow labs and eventually key additional work evolved in the Zahnow lab. Key initial work in the Jones and Baylin labs, published in 2018 (**Liu et al, Cancer Res., 2018**) defines targeting of the enzyme G9A in OC. The G9A gene has become of very major interest for potential cancer management as it has recently been defined as either amplified or mutated in many major cancers

and the mutations are activating ones that increase enzyme activity. Moreover, G9A in development, with evidence emerging for cancer, fosters stem cell maintenance as a key mechanism that may help drive tumorigenesis. Our studies defined that in OC cell lines with high levels of G9A expression, pharmacologic inhibition of G9A, by the G9a inhibitor (G9ai), UNC0638 added to the DNA methylation inhibitor (DNMTi), 5-aza-20- deoxycytidine (5-aza-CdR) AZA, induces synergistic antitumor effects in-vitro. The drug combination, as analyzed by RNA-seq, synergistically enhances the viral defense signaling effects induced by AZA in ovarian cancer cell lines including upregulation of endogenous retroviruses. G9Ai treatment further reduced H3K9me2 levels within the long terminal repeat regions of ERV's, resulting in further increases of ERV expression and enhancing "viral mimicry" effects. In contrast, G9Ai and 5-aza-CdR were not synergistic in cell lines with low basal G9A levels. Taken together, our results began to suggest that the synergistic effects of combination treatment with DNMTi and G9Ai may serve as a novel therapeutic strategy for patients with ovarian cancer with high levels of G9A expression.

We then have greatly extended that through work led in the Zahnow lab with the Baylin lab collaborating. These studies, extrapolating from the data just above, involved work in the in-vivo human model introduced earlier above in Specific Aims 1 and 4 to determine whether the above results can be translated into a therapy model for OC. In this regard, while the G9ai, UNC0638 works specifically to inhibit G9a, it is not soluble for in-vivo administration. However, we received, and began to in mice with the above model, a new drug from the company GBT and have received some funding from the company to do the full studies. We preliminarily have verified that this drug is a potent G9a inhibitor (G9ai) at nM doses from the studies embarked u in the last TEAL funding on the mouse experiments and have some preliminary data that suggests we are going to see some significant anti-tumor effects. Moreover, Dr. Zagnow has received a very specific inhibitor of G9a which has some anti-DNMTi effects from a group in Spain and her early data suggests that combination of this agent with a DNMTi is going to be even more potent. Thus the start provided by the TEAL award to these studies targeting G9A gives us exciting potential to derive a new therapy strategy for management of OC, especially in combination with immune checkpoint therapy, which we may truly be able to extend towards a clinical trial within the next several years.

E. Specific Aim 5: Bring all of the above studies to bear on leveraging clinical trials of epigenetic therapy on OC: Major Task 1: During the course of this entire award, we have worked with TEAL co-investigator, Dr. Dennis Slamon on a leveraged, instituted clinical trial of the DNA demethylating drug, guadecitabine from Astex and the immune checkpoint inhibitor Pembrolizuma (Pembro) from Merck. The goal is to see whether the guadecitabine will help facilitate efficacy of Pembro in patients with advanced OC, a setting where the latter approach alone has proven generally ineffective. While the trial is not funded from this TEAL award but rather from Celgene, it was derived from the paradigm which led to and was further developed in the first 2 years of the TEAL award and especially work performed in Specific Aims 1-3 above. Dr. Slamon received TEAL funding for provision of data from key human OC cell lines, provision of those lines to the Baylin work, and finally through the no cost extension funds to have TEAL award fund the key correlative science for the trial as previously introduced in Aims above. The key trial tumor samples, and serum will subsequently be utilized, for this correlative work have now been provided from UCLA to the Baylin lab for the correlative science and we will now leverage other funds which must be used now that the TEAL award has ended to perform the work. The studies will employ a deep, genome-wide molecular approach for DNA methylation, gene expression and chromatin configurations for not only the tumor cells but also key immune cell populations. The ultimate translational goal will be to derive signatures which may separate the patients who have the best clinical outcomes from those who do not do well and then use the data to derive markers that may facilitate a personalized approach to use of the combinatorial therapy as tested in a subsequent trial. The leveraged clinical trial has been completed, the clinical data have been assembled, and we are now starting the promised deep, genome-wide molecular approach for DNA methylation, gene expression and chromatin configurations for not only the tumor cells but also key immune cell populations.

2. KEYWORDS

1) epigenetic therapy; 2) DNA demethylation; 3) histone deacetylases; 4) immune evasion; 5) immune checkpoint therapy; 6) immune attraction.

3-4. ACCOMPLISHMENTS/IMPACT

Over the 5 years of our TEAL award, we employed several avenues for communication of our findings and advances. First, we have published multiple of our key studies, for original findings and reviews based on our data in top journals (Chiappinelli et al, Cell 2015; Topper et al, Cell 2017; Stone et al, PNAS, 2017; Liu et al, Cancer Research, 2018; Chiappinelli et al, Cancer Res, 2016; Topper et al, Nat Rev Oncol, 2019). Also, as enumerated below in the Presentations section, our faculty investigators, trainees and fellows have presented consistently on work directly evolving from TEAL studies in a robust, and continuing number of top research forums.

Second, over the course of our studies throughout our funding period, the PI, his collaborators, and all trainees have continued to ever deeper into studying biology of serous OC and studies to develop new means to treat this disease. These efforts are poised to continue far beyond just the years we received our funding from the TEAL award. The award allowed us to delve deeply in this field, and as above one key consequence is the fact that each of our trainees are pursuing careers which continue to focus upon extending what they done in the TEAL award time. The ever expanding requests for all the faculty and trainees to lecture in, and attend national and international meetings specifically regarding the potential for epigenetic therapy to increase the efficacy of immune checkpoint therapy with almost always some focus on OC, and for providing insight into the mechanisms that may be involved, again attests to what award has meant to the participants and also to the wider research community. Finally, the paradigms we have developed for potential treatment strategies for OC have generated several ongoing clinical trials based on our work, is further testimony to the high translational value for what we have, and are accomplishing. As has been true throughout the period we were funded, our great hope is that, at the end of the day, our studies will have helped lead to new therapy regimens for OC and other major cancer types to the benefit of a great number of patients.

Third, we truly believe our basic, pre-clinical studies of OC have, and are having widespread impact, perhaps more than we might have originally predicted. Our derived combination regimen of AZA plus HDACi's, given with immune checkpoint therapy, as defined in the in our Cell and PNAS papers have generated lab studies and clinical trials not only for OC but lung, colon, pancreatic, breast and other cancers. These trials have largely all been conducted in our Stand up to Cancer (SU2C) epigenetic therapy team co-led by Drs. Baylin and Jones. The numbers of such trials in this country, over the past several years have risen to surprising levels and our studies in the TEAL and elsewhere can certainly take some credit for that. That said, it remains to be documented whether our approaches, and scenarios for these from others can change the management for patients with OC and other cancers. The promise is still definitely alive and eminently worthy of deep pursuit in the lab and translation of the most poignant findings to clinical trials for patients with OC and other major cancers. Without question, however, refining the approaches and adding novel twists to these are going to be critical to the promise for success at the ultimate level – our patients.

Fourth, our studies conducted during the TEAL award and especially concerning derivation of our viral defense signatures, inclusive of upregulating ERV transcripts and most recently the context of all this signaling in the broader context of the inflammasome have resulted in patent applications for therapy consequences of employing epigenetic therapy to potentially enhance efficacy of immune checkpoint therapies and use of the findings to develop biomarker systems

to predict and monitor the efficacy of applying epigenetic therapy to sensitize patients with advanced OC and all cancer types, to immune checkpoint therapy. The role of inflammasome signaling to create homologous recombination defects, and thus potentially sensitive patients with OC and other cancers has resulted in a joint patent filing from Johns Hopkins and the University of Maryland. It remains to be seen how these applications may result in licensing within the industrial sector. In this regard, our ongoing, leveraged OC and other clinical trials, and the final analyses of patient outcomes with a range of DNA demethylating agents, and histone deacetylase inhibitors plus immune checkpoint therapy will prove the final arbiter of where our work in the TEAL award actually impacts patient care.

Finally, we dearly hope that the most important impact of our studies under the TEAL award will be for patients. As mentioned above, new trials are now testing our therapy paradigms for sensitizing to immune checkpoint therapy in patients with advanced NSCLC, bladder colon and other cancers and any therapy efficacies observed could provide the greatest impact we could seek for our work including for technologies to pursue the biomarkers discussed above. We can add to this by saying that increasingly, for example Dr. Baylin spoke in large meetings three times to a Maryland based society for women's health and also traveled to Florida in 2020, under the auspices of a Johns Hopkins program in the above arena give such public talks to women's health groups. The efforts of our TEAL team and leveraged collaborations with other colleagues are bringing potential for contributions from the standpoint of technology. Thus our genomics approaches for analyses of both laboratory data and also as being applied directly to clinical trial samples has potential for providing new signatures for predicting and monitoring the paradigms as outline earlier above. Hopefully, all of the above activities will have even more impact if our therapy approaches actually come to influence formal clinical management.

Address/explain the lack of specific data in report regarding Specific Aim 2 subtasks in OC mouse model. With regards to this question about specific aim 2, we are still working to complete the tasks. There have been delays because the clinical trial, which was indeed done, has just been completed within the past year. We are now starting to do the molecular, correlative science to match with the clinical data to identify biomarker signatures for those patients who achieved some benefit. Our attempts in subtasks 1 to identify drug sensitivities in a mouse model of OC are producing some exciting results correlative to the data done with Dr. Jones in specific aim 4. We anticipate a paper within the next 6 months which will describe this, and on which this grant will definitely be cited. The data suggest that we have found a new, epigenetic, drug combination which we may be able to take forward to clinical trials.

5. CHANGES/PROBLEMS

None

6. PRODUCTS

Publications, conference papers and presentations

Publications

Chiappinelli KB, Strissel PL, Desrichard A, Li H, Henke C, Akman B, Hein A, Rote NS, Cope LM, Snyder A, Makarov V, Buhu S, Slamon DJ, Wolchok JD, Pardoll DM, Beckmann MW, Zahnow CA, Mergoub T, Chan TA, Baylin SB and Strick R. Inhibiting DNA Methylation Causes an Interferon Response in Cancer via dsRNA including Endogenous Retroviruses. Cell. 162(5):974-86, 2015.

Chiappinelli KB, Zahnow CA, Ahuja N, Baylin SB. Combining Epigenetic and Immunotherapy to Combat Cancer. *Cancer Res.* 2016 Apr 1;76(7):1683-9. doi: 10.1158/0008-5472.CAN-15-2125. Epub 2016 Mar 17.

Strick R, Strissel PL, Baylin SB, Chiappinelli KB. Unraveling the molecular pathways of DNA-methylation inhibitors: human endogenous retroviruses induce the innate immune response in tumors. *Oncoimmunology.* 2015 Dec 29;5(5):e1122160. doi: 10.1080/2162402X.2015.1122160. eCollection 2016.

Stone ML, Chiappinelli KB, Li H, Murphy LM, Travers ME, Topper M, Mathios D, Lim M, Shih I-M, Wang T-L, Hung C-F, Bhargava V, Wiehagen KR, Cowley GS, Bachman KE, Strick R, Strissel PL, Baylin SB, Zahnow CA. Epigenetic therapy activates type I interferon signaling in murine ovarian cancer to reduce immunosuppression and tumor burden. *PNAS*, 114(51):E10981-10990, 2017.

Topper MJ, Vaz M, Chiappinelli KB, DeStefano Shields C, Wenzel A, Hicks J, Ballew M, Stone M, Tran PT, Zahnow CA, Hellmann MD, Strissel PL, Strick R, Baylin SB. Epigenetic Therapy Ties MYC Depletion to Reversing Immune Evasion and Treating Lung Cancer. *Cell*, 171(6):1284-1300, 2017.

Liu M, Thomas SL, DeWitt AK, Zhou W, Madaj ZB, Ohtani H, Baylin SB, Liang G and Jones PA. Dual inhibition of DNA and histone methyltransferases increases viral mimicry in ovarian cancer cells. *Cancer Research* 78(20): 5754-5766, 2018.

Topper M, Vaz M, Marrone K, Brahmer J, Baylin SB. The emerging role of epigenetic therapeutics in Immuno-Oncology. *Nature Reviews Oncology.* *Nat Rev Clin Oncol.* 2020 Feb; 17(2):75-90.

Travers M, Brown SM, Dunworth M, Holbert CE, Wiehagen KR, Bachman KE, Foley JR, Stone ML, Baylin SB, Casero RA Jr, Zahnow CA. DFMO and 5-Azacytidine Increase M1 Macrophages in the Tumor Microenvironment of Murine Ovarian Cancer. *Cancer Res.* 2019 Jul 1;79(13):3445-3454. doi: 10.1158/0008-5472.CAN-18-4018. Epub 2019 May 14. Note: This was not in the specific aims, but research was leveraged from the grant

US Patent

Title: PREDICTING RESPONSE TO EPIGENETIC DRUG THERAPY

Provisional Patent Application 61/937,149; 61/940,488

PCT Patent Application PCT/US2015/015017

Non-Provisional Patent Application 15/115,702

Patent Number 10,619,210

Presentations

Stephen B. Baylin, M.D.

2017 SU2C Summit – Santa Monica CA

2017 AACR Annual Meeting – Washington DC

University of California San Diego – San Diego CA

Baylor College of Medicine – Waco TX

Gordon Research Conference – Tuscany Italy

George Washington University – Washington DC

Institute of Molecular Biology - Mainz Germany

2nd European Cancer Epigenetics Conference 2017 – Heidelberg Germany

VARI Summer Retreat – Grand Rapids, MI
 8th Mildred Scheel Cancer Conference - Bonn Germany
 2017 TRIST Symposium - Northwestern University – Chicago IL
 Ludwig Oxford 10th Anniversary Symposium – Oxford London
 6th Annual Mayo Clinic Individualizing Medicine Conference – Rochester MN
 Translational Genomics and Epigenomics Symposium - Roswell Park Cancer Institute- Buffalo NY
 AbbVie Celebration of Science – Chicago IL
 10/2017 2nd BNMC Translational Genomics and Epigenomics Symposium, Roswell Park
 Cancer Institute, Buffalo, NY
 10/2017 Mayo Civic Center, Rochester, MN
 10/2017 Abbvie Celebration of Science, Chicago, IL
 11/2017 Miriam and Sheldon G. Adelson Medical Research Foundation meeting, Las Vegas,
 NV
 11/2017 Mirati SAB, Boston, MA
 12/2017 ASH Annual Mtg, Atlanta, GA
 12/2017 DOD Oral Presentation, Washington, DC
 1/2018 Joint RSM and Pathological Society Winter Mtg., London, UK
 1/2018 SU2C Scientific Summit, Santa Monica, CA
 2/2018 Keystone, Denver, CO
 3/2018 AACR, Atlanta
 3/2018 ADELIH Congress 3rd Annual Mtg., Paris, France
 4/2018 AACR Annual Mtg., Chicago, IL
 4/2018 National Academy of Science Induction, Washington, DC
 5/2018 Cancer Epigenetic Distinguished Lecture Series, MD Anderson Cancer Center,
 Houston, TX
 5/2018 Miriam and Sheldon G. Adelson Medical Research Foundation meeting, Las Vegas,
 NV
 7/2018 Cancer Epigenetic Therapies, Naples, Italy
 7/2018 VARI Summer Retreat, Grand Rapids, MI
 9/2018 ISREC-SCCL Symposium, Lausaune
 9/2018 Forbeck Focus Mtg., Geneva National Resort, Lake Geneva, WI
 9/2018 SU2C Telecast, Santa Monica, CA
 1/2019 T-cell Forum, LaJolla, CA
 1 2019 Keystone Conference – Cancer Vaccines, Vancouver, Canada
 1/2019 Stand up to Cancer Summit, Los Angeles, CA
 2/2019 St. Jude, Danny Thomas Lecture Series, Memphis Tennessee
 2/ 2019 Ohio State Cancer Center, Visiting Professor, Columbus OH
 3/2019 Baylor, Distinguished Lecture, Department of Molecular and Cellular Biology,
 Houston
 3/2019 MD Anderson, Cancer Epigenetics Distinguished Lecture Series, Houston, Texas
 3/2019 AACR National Meeting, Atlanta GA
 4/2019 Gordon Research Conference, Cancer Epigenetics and Genetics, Tuscany, Italy
 5/2019 A Woman's Journey, Baltimore, MD
 6/2019 European Hematology Association, Amsterdam, Netherlands
 9/2019 Victoria Comprehensive Cancer Center Consortium Annual Mtg., Melbourne,
 Australia
 10/2019 Plenary Lecture, Genetics Branch Retreat, Bethesda, MD
 10/2019 Freiburg Comprehensive Cancer Center 50th Anniversary, Freiburg, Germany
 11/2019 Plenary Lecture, Adelson Medical Research Foundation, Las Vegas, Nevada
 11/2019 Chromatin Symposium, NIH, Bethesda, MD
 11/2019 American Assoc. Cancer Res., Tumor Immunology and Immunotherapy, Boston,
 MA

11/2019 A Woman's Journey, Baltimore, MD
 03/2020 A Woman's Journey Talks, St. Petersburg, FL
 09/2020 DoD/AACR Teal presentation, Virtual Meeting

Cynthia A. Zahnow, Ph.D.

Baylor College of Medicine - Houston TX

Fels Institute for Cancer Research and Molecular Biology - Temple University - Philadelphia PA

Biology Research Seminar Series, Baylor University - Waco TX

In Vivo Cellular & Molecular Imaging Center, Division of Radiology, Johns Hopkins School of Medicine - Baltimore MD

12/2017 Invited Speaker, "Epigenetic Regulation of the Immune Microenvironment" San Antonio Breast Cancer Symposium, San Antonio Texas
 9/2018 Invited Speaker, AACR International Conference on Translational Cancer Medicine being held in cooperation with the Latin American Cooperative Oncology Group (LACOG)
 9/2018 Session Chair, Epigenetics and Metabolism. AACR International Conference on Translational Cancer Medicine being held in cooperation with the Latin American Cooperative Oncology Group (LACOG) in São Paulo, Brazil
 3/2019 Invited Speaker, 19th Annual NCI-CCR-FYI Colloquium. When the Scientist Becomes the Cancer Patient NCI Shady Grove campus in Rockville, MD
 6/2019 *Meghan Travers (CMM graduate student in the Zahnow lab) was invited to speak at the GRS meeting, Polyamines in Cellular Metabolism, Cancer Biology, Plants and Pathogens. "DFMO and 5-Azacytidine increase M1 macrophages in the tumor microenvironment of an ovarian cancer murine model." Waterville Valley, NH.
 11/2019 Invited Speaker, DFMO and 5-Azacytidine increase M1 macrophages in the tumor microenvironment of ovarian cancer. Josep Carreras Leukaemia Research Institute (IJC) Ctra de Can Ruti, Camí de les Escoles, s/n 08916 Badalona, Barcelona, Spain
 11/2019 Invited Speaker, "Targeting the Tumor Immune Microenvironment with Epigenetic Therapy". Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX
 11/2019 Invited Speaker, "The targeting of macrophage in the tumor microenvironment to inhibit tumor development". Baylor College of Medicine, Houston, TX

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on the project

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Name: Ray-Whay Yen 2014-2020
 Project Role: Research Associate

Name: Katherine Chiappinelli, Ph.D. 2014-2017
Project Role: Postdoctoral Fellow / Teal Junior Scientist

Name: Meredith Stone 2014-2017
Project Role: Graduate Student

Name: Michael Topper, Ph.D. 2018-2020
Project Role: Instructor / Teal Junior Scientist

The Regents of the University of California

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Project Role: PI (Senior/Key Personnel)

Name: Judy Dering, Ph.D. 2014-2019
Project Role: Sr Public Analyst

Van Andel Research Institute

Name: Peter Jones, Ph.D. 2014-2019
Project Role: PI (Senior/Key Personnel)

8. SPECIAL REPORTING REQUIREMENTS

Progress of the Teal Junior Scientists

Scientific training – The progress of all our TEAL trainees has been well outlined above and key points are re-summarized here.

As mentioned in sections above, Dr. Chiappinelli benefitted enormously from her time with us. For her studies in the Cell, 2015 paper, she worked with collaborators in Germany, Drs. Reiner Strick and Pamela Strissel for learning how to profile, knockdown, and overexpress several of the endogenous retroviruses (ERVs). She was also mentored extensively, with Dr. Cindy Zahnow's group for the mouse model work for which she served as a key co-author on Meredith's Stone's PNAS paper in 2017. Kate has now been a faculty member at George Washington University in the Cancer Center for three years or so and she is thriving from all interactions we have had with her. She continues some collaborative work with us and particular with our current trainee, Dr. Topper with much concentration on ovarian cancer research.

Scientific training – Dr. Meredith Stone: For her year as a mentee, Meredith Stone worked with Dr. Cindy Zahnow for her dissertation studies and was co-mentored by Dr. Baylin. She benefitted from all of the activities listed above for Dr. Chiappinelli and, as fully outlined in sections above, her work resulted in her paper resulted in a key first author in a 2017 PNAS paper on the mouse OC model. All of the educational activities and mentoring activities outlined for Dr. Chiappinelli were engaged in by Dr. Stone. As reported, Dr. Stone has now been a postdoctoral fellow at University of Pennsylvania in the Cancer Center where she has continued working on aspects of immunology related to OC.

Scientific training – Dr. Michael Topper: Dr. Topper completed a year of postdoctoral fellowship studies with Dr. Baylin after receiving his Ph.D. in 2018. Because of his tremendous talent and contributions starting with his first author Cell paper in 2017, and his invaluable collaboration with Drs. Stone and Zahnow on their 2017 PNAS paper, we quickly promoted him to faculty status in our Cancer Center as an Instructor in Oncology beginning in 2020. He is now working with his oversight of several trainees to start his laboratory and extend the implications of his epigenetic therapy development for OC, lung and other cancer types. As above, Dr. Topper continues his collaborations with Dr. Chiappinelli on a deep molecular analysis of multiple OC cell lines treated with the epigenetic therapy paradigms he has developed. The goal of these studies remains to outline in novel ways, the upstream mechanisms by which the epigenetic therapy helps reverse tumor evasion including defining upstream signals that drive the entire induced viral defense response. Finally, he has deeply extended his studies to learn how our epigenetic therapy alters the immune status of key subsets of immune cells to improve their recognition of tumor and exert increased anti-tumor effects. This is an emerging area which is imperative for taking our therapy approaches forward for OC and other major cancer types.

Presentations for the above trainees:

Participation in Hopkins groups: All three mentees participated in the following meeting formats and Dr. Topper, in his current faculty position continues to do so.

- 1) Methylation Data Group: attended these weekly meetings, and presented several times per year.
- 2) Methylation Journal Club: attended these weekly meetings, and presented many times.
- 3) Tumor Biology Lab Meeting: attended these weekly meetings, and presented at least twice per year.
- 4) Dr. Topper continues his collaborative work with co-investigator Peter Jones and his group at the Van Andel Research Institute in Grand Rapids, Michigan.

Professional development:

As above all three mentees had great development of their professional careers directly dependent on their training time as completely outlined just above.

Teal Innovator's Ovarian Cancer ambassadorship activities

As over the entire course of this TEAL award, as again introduced earlier, Dr. Baylin has been requested to discuss, nationally and internationally the exciting results which are outlined in this final report and in the latest listing of meetings attended etc. Just during this past year, in addition to his many invited lectures (see updated Presentations on pg. 12), he has lectured twice in Baltimore to the major Women's Health Society (A Woman's Journey) which is fully affiliated with the Johns Hopkins and University of Maryland Schools of Medicine and again in this Society participated in a 3 day lecture trip to Florida in March, 2020. Finally, in the fall of 2020, he gave an hour presentation about what happened over the years of the TEAL award in a DOD, AACR session principally aimed at young trainees.

9. APPENDICES

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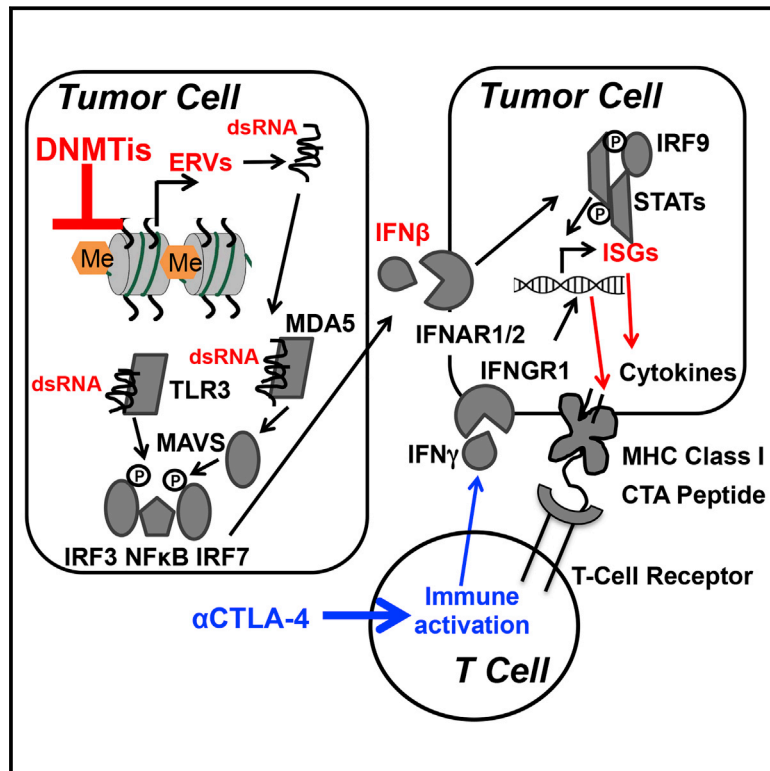
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Inhibiting DNA Methylation Causes an Interferon Response in Cancer via dsRNA Including Endogenous Retroviruses

Graphical Abstract



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In Brief

DNA methyltransferase inhibitors upregulate endogenous retroviruses in tumor cells to induce an growth-inhibiting immune response. High expression of the genes associated with the anti-viral response seems to potentiate a response to immune checkpoint therapy.

Highlights

- DNMTi induce an interferon response in cancer cells by activating dsRNA sensors
- DNMTi induce ERV demethylation and expression helping trigger the dsRNA response
- DNMTi viral defense genes in melanoma track with patient response to immune therapy
- DNMTi treatment sensitizes to anti-CTLA-4 immunotherapy in a melanoma mouse model



Inhibiting DNA Methylation Causes an Interferon Response in Cancer via dsRNA Including Endogenous Retroviruses

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SUMMARY

We show that DNA methyltransferase inhibitors (DNMTis) upregulate immune signaling in cancer through the viral defense pathway. In ovarian cancer (OC), DNMTis trigger cytosolic sensing of double-stranded RNA (dsRNA) causing a type I interferon response and apoptosis. Knocking down dsRNA sensors TLR3 and MAVS reduces this response 2-fold and blocking interferon beta or its receptor abrogates it. Upregulation of hypermethylated endogenous retrovirus (ERV) genes accompanies the response and ERV overexpression activates the response. Basal levels of ERV and viral defense gene expression significantly correlate in primary OC and the latter signature separates primary samples for multiple tumor types from The Cancer Genome Atlas into low versus high expression groups. In melanoma patients treated with an immune checkpoint therapy, high viral defense signature expression in tumors significantly associates with durable clinical response and DNMTi treatment sensitizes to anti-CTLA4 therapy in a pre-clinical melanoma model.

INTRODUCTION

DNA methyltransferase inhibitors (DNMTis) such as 5-azacytidine (Aza) and 5-aza-2'-deoxycytidine (Dac) are effective cancer therapies in hematologic neoplasms (Issa, 2005; Matei et al., 2012) and are Food and Drug Administration (FDA)-approved for the pre-leukemic disorder myelodysplasia (MDS) (Kaminskas et al., 2005). These cytidine analogs incorporate into DNA, block catalytic actions of DNA methyltransferases (DNMTs), and trigger their degradation (Stresemann et al., 2006). Preclinical,

low doses avoid early cytotoxicity and DNA damage, allowing cells to exhibit apparent reprogramming and blunting of tumorigenicity (Tsai et al., 2012). Mechanisms can include reversal of abnormal promoter DNA methylation, re-expression of silenced genes including tumor suppressors (Baylin and Jones, 2011), and changes to cancer signaling pathways including apoptosis, cell-cycle activity, and stem cell functions (Tsai et al., 2012).

A long recognized activity of DNMTis described by others (Karpf et al., 1999, 2004) and our group (Li et al., 2014; Wrangle et al., 2013), is induction of immune responses in cancer cells. In recent clinical trials for non-small cell lung cancer (NSCLC) (Jurgens et al., 2011; Wrangle et al., 2013) a small number of patients had remarkably robust and durable responses to immune checkpoint blockade therapy after first receiving Aza (Wrangle et al., 2013). This immune therapy alone also has activity against NSCLC (Brahmer et al., 2010, 2012; Topalian et al., 2012). A larger trial is now ongoing to determine if Aza can indeed prime patients for sensitization to checkpoint inhibition (Brahmer, 2015). For NSCLC and other tumor types, Aza induces interferon signaling and concordant upregulation of surface antigens and their assembly proteins, viral defense pathways, and transcript and surface protein levels of PD-L1, the key checkpoint ligand targeted in the above immunotherapy (Li et al., 2014; Wrangle et al., 2013). Indeed, we have defined a 300-gene expression signature we termed Aza-induced immune genes or AIM (Li et al., 2014) for which activation is greatest for epithelial ovarian cancer (EOC) and NSCLC (Li et al., 2014). Genome-wide expression of AIM separates primary EOC, NSCLC, and other cancers into high and low expression groups (Li et al., 2014). We hypothesize the low group may represent an "immune evasion/immune editing" pattern (Drake et al., 2006; Schreiber et al., 2011) that Aza could reverse to sensitize patients to subsequent immune therapy (Li et al., 2014).

We now show that a major mechanism underlying the Aza-triggered immune response is induction of a cytosolic

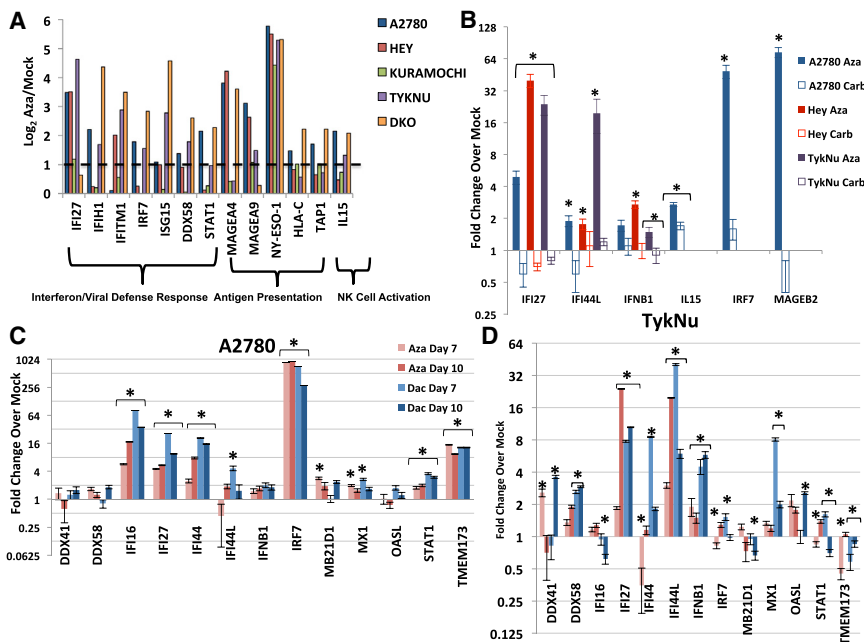


Figure 1. DNMT Inhibitors Upregulate Immune Genes in Ovarian Cancer Cell Lines

(A) Levels of immune genes in four EOC cell lines and DKO colon cancer cell line (DNMT1^{-/-}, DNMT3B^{-/-}) relative to its parental HCT116 line. y axis, log₂ Aza/Mock fold change from microarray data. Dotted line denotes 2-fold change.

(B) qRT-PCR validation of immune genes in EOC cells treated for 72 hr: Mock, 500 nM Aza, or 500 nM- 3 μ M carboplatin and rested for 7 days before assaying (day 10). IC₅₀s were A2780 (Aza, 848 nM; Carb, 457 nM), Hey (Aza, 4.1 μ M; Carb, 12.2 μ M), TykNu (Aza, 491 nM; Carb, 986.2 nM).

(C and D) qRT-PCR validation of interferon response genes in the A2780 (C) and TykNu (D) EOC lines treated with no drug (Mock), 500 nM Aza (Aza), or 100 nM Decitabine (Dac) for 3 days, and rested for 4 (day 7) or 7 (day 10) days before assaying. Data in (B)–(D) are represented as mean $\pm$ SEM of three biological replicates, y axis = fold change over mock. *p $\leq$ 0.05.

See also Figure S1.

double-stranded RNA (dsRNA) sensing pathway used by epithelial and other cell types as a viral defense mechanism that triggers a type I interferon response (Kulaeva et al., 2003; Sistigu et al., 2014). A key contributor is induction of increased expression of multiple DNA hypermethylated endogenous retroviruses (ERVs). In The Cancer Genome Atlas (TCGA), the viral defense gene expression separates primary EOC and other cancers into high and low expression and high tumor expression strongly associates with clinical benefit in a trial of immune checkpoint therapy for advanced melanoma. Aza sensitizes to immune checkpoint blockade in a pre-clinical model of melanoma. We thus define a potential approach in which an epigenetic therapy may sensitize cancer cells to various immunotherapies.

RESULTS

DNMTs Trigger Viral Defense and Type I Interferon Signaling

Induction of AIM in a previous study of 23 EOC cell lines (Li et al., 2014) included, in addition to previously reported DNA hypermethylated cancer testis antigens (*MAGEA4*, *MAGEA9*, *NY-ESO-1*) (James et al., 2013; Karpf et al., 2004, 2009; Odunsi et al., 2014), interferon/viral defense, antigen processing and presentation, and host immune cell attraction genes (Figure 1A). Direct Aza targeting of DNMTs for these changes is suggested by similar findings in DKO colon cancer cells genetically disrupted for two major DNMTs (DNMT1^{-/-}, DNMT3B^{-/-}) versus parental, wild-type HCT116 cells (Figure 1A). The induced responses may not be a general stress phenomenon as they did not occur with carboplatin, a cytotoxic agent commonly used in EOC treatment (Figure 1B). Aza and Dac incorporate into DNA, inhibiting three DNA methyltransferases, but Aza also incorporates into RNA, inhibiting the RNA methyltransferase DNMT2 (Schaefer et al., 2009). Aza thus can demethylate RNA, and unmethylated

RNA may activate TLR3 and the interferon response (Karikó et al., 2005). However, Dac and Aza both mimicked the DKO cell line results (Figures 1A, 1C, 1D, S1A, and S1B) strongly suggesting that the drugs directly target DNA methylation to trigger the interferon response.

Aza and Dac similarly triggered an interferon response that includes interferon beta (*IFN β 1*) and a panel of interferon-stimulated genes (ISGs) (*IFI16*, *IFI27*, *IFI44*, *IFI44L*, *MX1*, and *OASL*) (Figures 1C, 1D, S1A, and S1B). Each ISG functions predominantly in anti-viral and anti-proliferative signaling (Figure 2A; Ivashkiv and Donlin, 2014). In four EOC cell lines, key upstream genes in the type I Interferon pathway (*IFN β 1*, *IRF7*, and *STAT1*) were generally upregulated at the fourth day following the end of Aza treatment (“day 7”) and further increased by day 10 (Figures 1C, 1D, S1A, and S1B). Importantly, cytosolic sensors for DNA (*MB21D1*/CGAS and *TMEM173*/STING) and RNA (*DDX41*, *DDX58*/RIG-I, and *IFIH1*/MDA5) were also variably upregulated (Figures 1A, 1C, 1D, S1A, and S1B). In A2780 and TykNu, but not Hey or Kuramochi, cell lines, variable increases occurred in type III interferon signaling genes, also involved in response to viruses (Ding and Robek, 2014). These included *IFNL1* (IL28A) and *IFNL3* (IL29) ligands (Figure S1C) and especially the IFN III receptor *IFNLR1* (Figure S1C), known to be methylated and activated by epigenetic therapy (Ding et al., 2014).

Key viral RNA sensing proteins include TLR3 on the endosomal membrane and MDA5, PKR, and RIG-I in the cytoplasm (Figure 2A). These induce IRF3, IRF7, and NF- κ B to translocate to the nucleus and activate transcription of *IFN β 1* (Ivashkiv and Donlin, 2014). *IRF7* is frequently promoter DNA hypermethylated in cancer and the associated low basal expression can be reversed by Aza in squamous NSCLC (Wrangle et al., 2013). Among 23 EOC lines examined, *IRF7* was hypermethylated in only one, A2780 (Li et al., 2014) (Figure S3A), potentially not a classic high-grade EOC (Anglesio et al., 2013;

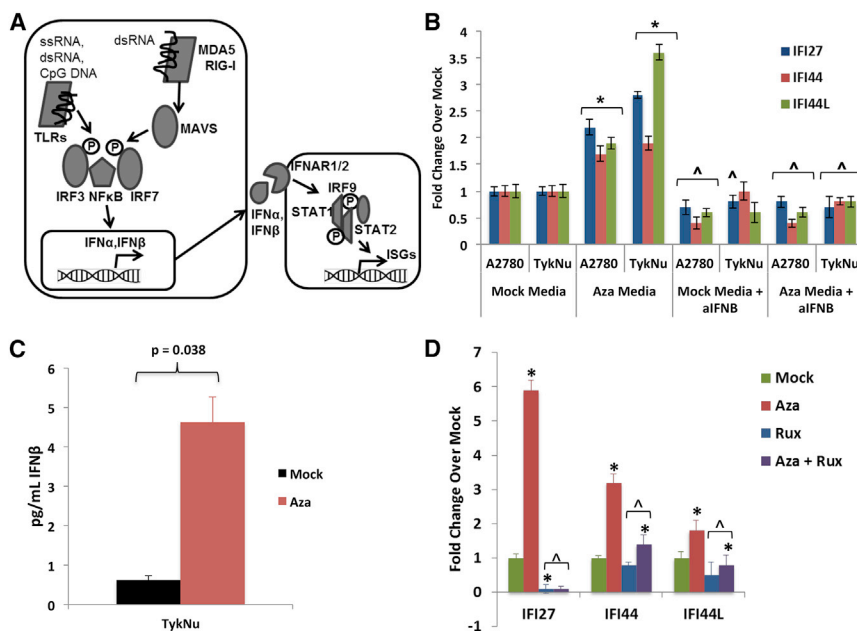


Figure 2. DNMTis Upregulate Immune Signaling through Secreted Interferon

(A) Schematic of interferon pathway. Protein symbols outlined in text.

(B) Treatment of recipient A2780 or TykNu cells with media from cells treated with Mock or Aza, +/- addition of anti-IFNβ. y axis, qRT-PCR fold Aza/Mock of ISGs. *p ≤ 0.05 and p ≤ 0.05, respectively, for Mock or Aza media with versus without anti-IFNβ versus Mock or Aza media plus anti-IFNβ.

(C) ELISA of IFNβ in media from TykNu cells at day 10 from studies in (B). y axis, pg/mL IFNβ.

(D) Treatment of EOC cells with Aza as in Figures 1C and 1D, but in the presence of 2 μM Ruxolitinib (Rux). y axis, qRT-PCR fold changes for ISGs. *p ≤ 0.05 for fold Aza over Mock and p ≤ 0.05, Mock or Aza versus Mock + Rux or Aza + Rux. Data, mean ± SEM of three (B and D) or four (C) biological replicates.

See also Figure S2.

Domcke et al., 2013). Aza induced partial *IRF7* demethylation and increased expression in this cell line at days 7 and 10 while carboplatin did not (Figures 1B, 1C, and S3A), and *IRF7* knockdown significantly reduced the Aza interferon response (Figures S3B and S3C). Such *IRF7* induction did not occur in two EOC lines or the HCT116 colon cancer cell line where the gene is not hypermethylated (Figures 1D, S1A, S1B, and S3A).

When *IRF7* is not silenced, other mechanisms must then be operative for Aza to trigger viral defense signaling. Secreted IFNβ is critical to this signaling and, through interaction with surface receptors IFNAR1/2, activates JAK/STAT signaling, transcription of ISGs, and resultant translation inhibition and apoptosis (Platanias, 2005; Ivashkin and Donlin, 2014) (Figure 2A). Indeed, media transferred to untreated cells from Aza-treated cells 7 days after drug withdrawal caused an interferon response with increased expression of ISGs *IFI27*, *IFI44*, and *IFI44L* (Figure 2B). Moreover, Aza treatment induced secreted IFNβ in media (Figures 2C and S2A) and an IFNβ blocking antibody significantly blocked the Aza-induced ISG media response (Figure 2B). Like type I IFN signaling, type III IFN signaling can be activated by viral infection (Robek et al., 2005; Ding and Robek, 2014). However, even though we observed upregulation of type III ligand transcripts *IFNL1* (IL28A) and *IFNL3* (IL29) (Figure S1C), secreted type III interferon proteins were undetectable by ELISA (Figure S2B).

Aza appears to activate type I, IFNβ-mediated signaling through JAK/STAT, as the JAK/STAT inhibitor ruxolitinib strongly reduced ISG responses (Figures 2D and S2C). Further, antibody blocking of IFNAR2, the IFNβ receptor, abrogated Aza induction of *IFI27*, *IFI44L*, and *IFI6* transcription (Figures 3A and S2D), as did inhibition of IFNβ itself (Figures 3B and S2E). In contrast, blocking the type III interferon, IL10RB receptor, gave only a modest block of *IFI27* increase (Figure S2F). IFNβ binding

to IFNAR2 also may contribute to late, Aza-induced apoptosis that peaks at 4–7 days after Aza withdrawal, since anti-IFNAR2 led to a lower ratio of cleaved/total PARP (Figures 3A, 3C, and S2D and S2G).

DNMTis Trigger Viral Defense through Induction of dsRNA

Aza-induced viral defense genes and *IFNβ1* are not generally DNA methylated at promoter regions (Li et al., 2014), thus Aza may activate the pathway upstream of these genes. We considered increases in dsRNA, viral single-stranded RNA (ssRNA), and unmethylated CpG DNA that might trigger cytosolic sensors (Sun et al., 2013). Indeed, 3 days after ending Aza treatment of A2780 and TykNu ovarian cancer cells and subsequent transfection into HT29 colon cancer cells, known to have a robust interferon response (Chiappinelli et al., 2012), cytoplasmic total RNA (without rRNA) and PolyA⁺ RNA, but not PolyA[−] RNA or DNA, increased *IFNβ1* transcripts (Figure 3D) and downstream ISGs (Figures 3E and S3D). Further, RNaseH treatment of the cytosolic nucleic acids, which specifically digests dsRNA, eliminated the *IFNβ1* upregulation (Figure 4A), but this was not seen with RNaseH treatment that digests DNA-RNA hybrids (Figure S4A).

If dsRNA is required for the above Aza effects, then the key cytosolic sensors, TLR3, MDA5 (*IFIH1*), and RIG-I (*DDX58*), the latter two signaling through the mitochondrial protein, MAVS, should be involved in subsequent *IFNβ1* induction (Figure 2A). Aza increased transcript (Figure 1A) and protein levels for these (Figure 4B). However, RIG-I, which requires a 5' triphosphate group on RNA for activation, is likely not a key player since alkaline phosphatase treatment of cytosolic nucleic acid fractions did not abolish *IFNβ1* upregulation (Figure S4D). In contrast, knockdown of TLR3 and MAVS in A2780 cells (Figure 2A) decreased Aza upregulation of interferon genes *IFNB1*, *IFI44*, *IFI44L*, and *IFI27* by 2-fold as did MAVS knockdown (Figures 4C, 4D, and S4B). In TykNu, knockdown of TLR3 and MAVS significantly blunted Aza induction of these gene responses

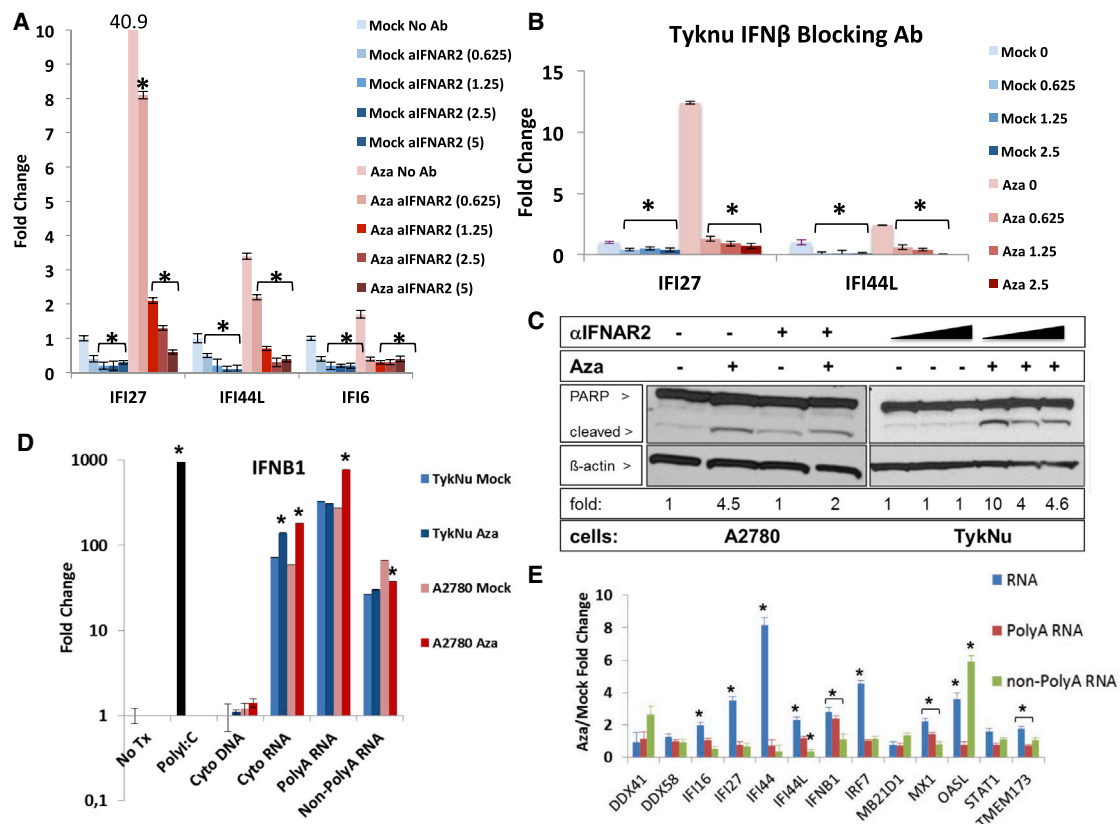


Figure 3. Aza Induces Immune Signaling through dsRNA Activation of Secreted Interferon

(A and B) Blocking (A) IFNAR2 (αIFNAR2) or (B) IFNβ in TykNu cells treated versus non-treated with Aza as in Figures 1C and 1D; parentheses, U/ml of antibody. y axis, qRT-PCR for ISGs. *p ≤ 0.05 for (A), Mock or Aza –anti-IFNAR2 versus +anti-IFNAR2, (B) Mock or Aza with no anti-IFNβ versus with anti-IFNβ.

(C) Immunoblotting for cleaved PARP with β-actin loading control. Fold change shown for cleaved/total PARP ratio, normalized to β-actin for each dose of anti-IFNAR2 (triangles, 0–1.25 U/ml). Aza compared to Mock = 1. The A2780 and TykNu cell lysates were run on two separate gels to perform western blots. The space in between the PARP and B-Actin proteins was cropped out to conserve space.

(D and E) Indicated nucleic acids from the cytoplasm of A2780- or TykNu-treated cells with no drug (Mock) or 500 nM Aza (Aza) for 3 days and rested without drug for 4 days before transfection into recipient HT29 cells. y axis, fold change, Aza/Mock, for IFNB1 transcript (D) or ISG transcripts (E) induced in HT29s. No Tx, no transfection; Cyto DNA, cytoplasmic DNA; Cyto RNA, cytoplasmic RNA excluding rRNA. *p ≤ 0.05 for (D) fold change Aza versus Mock, (E) Aza/Mock. Data in (A), (B), (D), and (E) are mean ± SEM of three biological replicates.

See also Figure S3.

(Figure S4C). Importantly, knock down of STING, the cytosolic DNA sensor (Mankan et al., 2014) did not blunt Aza-induced interferon signaling (Figures 4C and 4D). A previous report had implicated STING in viral cytosolic sensing in B cells, but this was dependent upon viral reverse transcriptase activity, likely to be low in our cells (Mankan et al., 2014). We thus conclude that MAVS and TLR3 are centrally involved in Aza triggering cytosolic sensors to induce an interferon response.

Aza-Induced Human Endogenous Retrovirus Transcripts Can Activate Viral Defense Responses in EOC

The above data suggests Aza might activate endogenous retroviral sequences (ERVs) that constitute more than 8% of the human genome, can activate cytosolic RNA sensors, and are silenced in normal somatic cells by promoter DNA methylation (Bannert and Kurth, 2004; Tristem, 2000; Hurst and Magiorkinis,

2014; Mankan et al., 2014). Some cancers lose ERV DNA methylation and aberrantly overexpress ERVs (Larsen et al., 2009; Rycaj et al., 2015; Strick et al., 2007; Strissel et al., 2012; Wang-Johanning et al., 2001, 2007) while others maintain silencing. Aza can induce specific ERV transcripts in melanoma, choriocarcinoma, and endometrial cancer cells (Laska et al., 2013; Ruebner et al., 2013; Stengel et al., 2010; Strissel et al., 2012). Indeed, in initial testing, the ERVK subfamily (Wang-Johanning et al., 2003) transcripts increased 2.5-fold in the A2780 cell line upon Aza treatment (data not shown). Upregulation of individual ERVs (22 full-length env, 6 partial coding env, 1 full-length gag, and 2 partial coding pols) (Tables S1 and S2), in PCR assays for non-repeat sequences, occurred especially at day 7 (coinciding with ISG expression) in three EOC lines following Aza and Dac treatment (Figures 5A, 5B, and S5A–S5C). These included several known ERV env genes like Syncytin-1, ERV-3, env-K, and env-H (Blond et al., 1999; Löwer

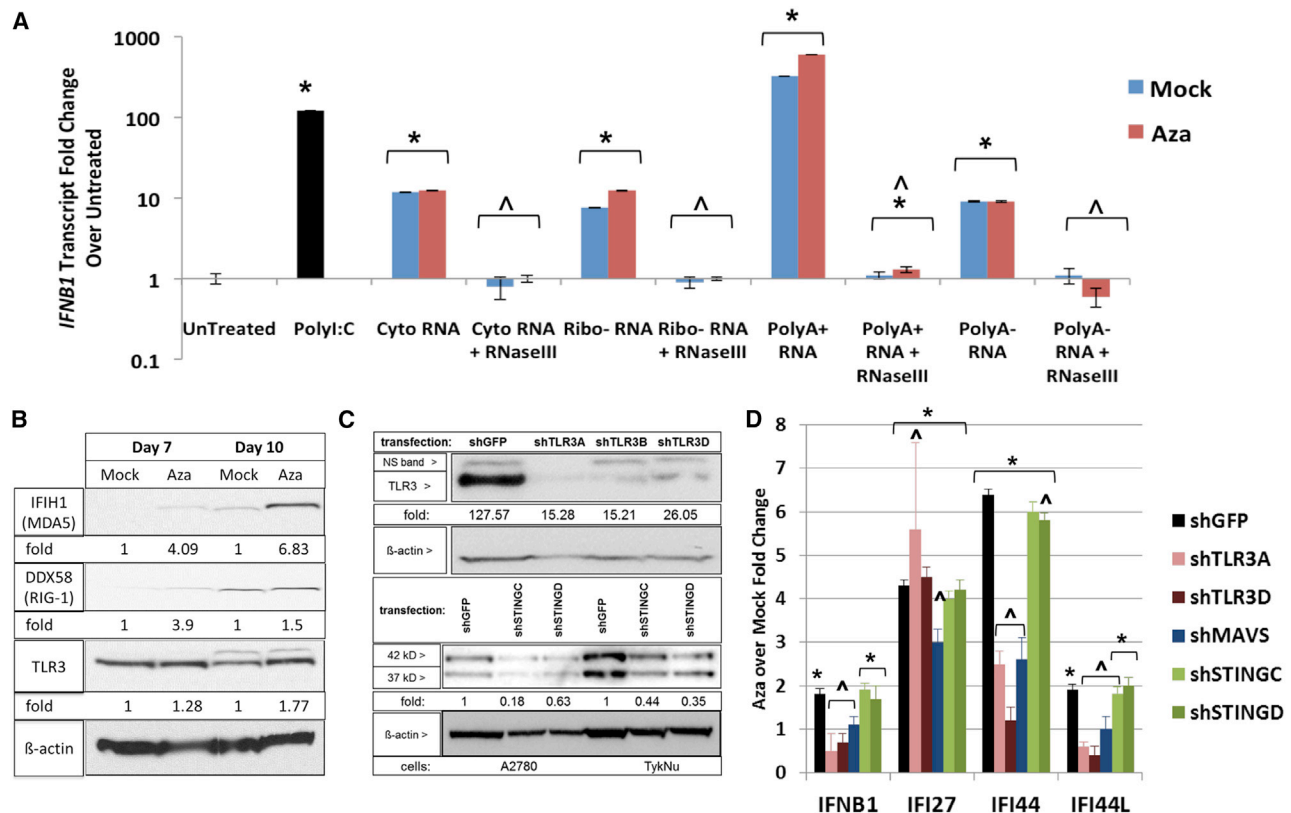


Figure 4. Aza Activates dsRNA Sensors to Induce Interferon Signaling

(A) Effects on *IFNβ1* transcripts, at 24 hr, in HT29 recipient cells transfected with nucleic acid fractions, treated with RNaseIII, from A2780 as in Figures 3D and 3E. *IFNβ1* transcripts were measured at 24 hr. y axis = *IFNβ1* fold change, * $p \leq 0.05$ for fold change over untreated; * $p \leq 0.05$ for Mock or Aza + versus – RNaseIII. (B) Western blots for MDA5, RIG-I, and TLR3 in A2780 cells at four (day 7) and seven (day 10) days after Mock versus 500 nM Aza (Aza) for 3 days. The same A2780 lysate gel was blotted for MDA5 and β-actin, then stripped and reprobed for RIG-I and TLR3. The space between the MDA5, RIG-I, and TLR3 and β-actin proteins was cropped out to conserve space.

(C) Knockdown upon lentiviral infection, with puromycin selection, of A2780 and TykNu with shGFP, shTLR3, and shSTING hairpins. Immunoblotting with anti-TLR3, anti-STING, and anti-β-actin. Densitometry fold change, normalized to mock or shGFP, shown at the bottom of the gels. The shTLR3 and shSTING cell lysates were run on two separate gels to perform western blots. The space in between the TLR3 and β-actin proteins was cropped out to conserve space. The STING western blot was stripped and reprobed with β-actin as a loading control.

(D) qRT-PCR for ISGs from (B) and (C). * $p \leq 0.05$ Aza over Mock; * $p \leq 0.05$ shGFP versus each shRNA sensor with mean fold change $\pm$ SEM of three biological replicates.

See also Figure S4.

et al., 1993; Mi et al., 2000; Rote et al., 2004) and at especially high levels, *env-Fc2*, a less well-characterized gene (Béni et al., 2003). Finally, in DKO as well as Aza-treated A2780 and TykNu cells, loss of *env-Fc2* promoter methylation correlated with increased *Fc2* expression (Figures 5B, 6A–6C, and S6A) but not in Hey cells (Figure S6A).

Further linking ERVs with a dsRNA-triggered IFN response, bidirectional transcription producing sense and anti-sense transcripts occurred for *Syngytin-1* and five *env-Fc2* gene loci, but not β-actin, in three EOC lines and HCT 116 and DKO cells (Figure 5C; Table S2), analyzed by the TAG-aided sense/antisense transcript detection (TASA-TD) technique (Henke et al., 2015). Such sense and antisense transcripts can form dsRNA (Faghihi et al., 2008; Su et al., 2012). Interestingly for TykNu, there was a 6.69-fold increase of *env-Fc2* antisense transcript levels compared to the sense transcript (Figure 5C) but substantially

lower antisense transcripts were seen in both HCT116 and DKO cells (Figures 5C and 6A). Disrupting DNMTs seems integral to the above ERV upregulation since increases of *env-Fc2* and *env9-1* occurred in DKO versus wild-type HCT116 cells (Figure 6A).

ERV transcripts seem directly involved in the Aza responses that, first, although drug-induced upregulation of ERV transcripts began early after Aza, both ERVs and viral defense gene increases generally peaked by day 7 (Figures S6D and S6J). Second, ERV env proteins such as Syn1 and ERV-3 were not increased after Aza treatment, supporting a dominant role for viral defense signaling via RNA transcripts (Figures 6D, S6B, and S6C). Third, overexpression of *ERV-3*, *EnvW2*, and *Syngytin-1* in TykNu (Figures 6E and 6J), A2780 (Figure S6D), and Hey cells (Figures S6E and S6J), as compared to control genes, increased the same interferon genes induced by Aza (*IFNβ1*,

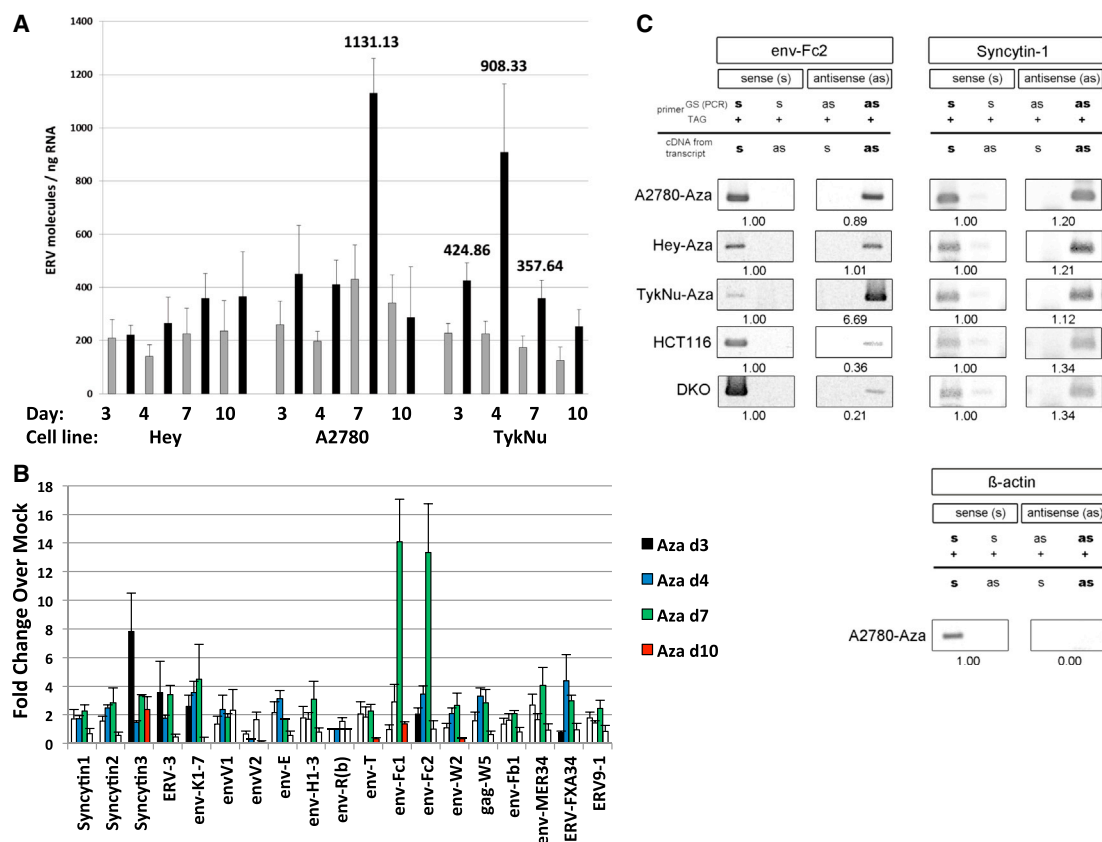


Figure 5. Aza Upregulates Sense and Antisense ERV Transcripts

RNA was isolated from cells at last (day 3), one (day 4), three (day 7), and seven (day 10) days after Mock or 500 nM Aza (Aza) for 3 days.

(A) Total number of molecules for all ERV genes (y axis = ERV molecules/ng RNA). Error bars, SEM for four independent experiments; numbers above bars, significant data for indicated days; gray, Mock; black, Aza.

(B) qRT-PCR of ERV genes in A2780 cells for four independent experiments. y axis, fold increases for Aza/Mock $\pm$ SEM and normalized to Mock = 1. White bars, non-significant; colored bars, significant ERV gene induction ($p < 0.05$).

(C) TASA-TD PCR amplified sense and antisense transcripts of the *env-Fc2* (731 bp) and *Syncytin-1* (202 bp) genes from first strand cDNA. Aza-treated A2780, Hey, and TykNu, and HCT116 and DKO cells are indicated. Ratios of sense (s) and antisense (as) determined by ImageJ. PCR primers, gene-specific (GS); TAG. β -actin sense 399 bp amplification product = negative control for as transcripts (Chen et al., 2004). The products from TASA-TD PCR were run on the same gel, then cropped and presented.

See also Figure S5.

IFI27, and *IFI44L*). The increases often exceeded that for the drug likely because total ERV RNA molecules were higher in the overexpression experiments (Figures S6F–S6I). Finally, although siRNA knockdown of individual ERVs (*Syncytin-1*, *ERV-3*) during Aza treatment produced more complex results, targeting two ERVs significantly blunted the Aza-induced gene expression of *IFI27*, *IFI44L*, and *IFI6* in TykNu cells, but not A2780 or Hey cells (Figure S7A).

Importantly, a driving role for ERV transcripts in triggering Aza-induced viral defense gene responses is evidenced by a high correlation of basal levels of both in 19 primary EOC. Total molecules of 22 ERV *env* genes queried were increased ($p < 0.05$) in tumor versus normal ($n = 9$) and divided tumors into lower ($n = 9$) and higher ERV ($n = 10$) expression groups as compared to normal controls. High ERV tumors had significantly higher viral defense response gene expression ($p = 0.000141$) (Figure 7A).

Viral Defense Gene Levels Divide Human Tumors into High and Low Expression Groups that Track with Responses to Immune Checkpoint Therapy

Human cancers can evolve immune evasion to become less responsive to immune modulation (Drake et al., 2006; Schreiber et al., 2011). In this regard, basal transcript levels for the Aza-induced viral defense genes grouped primary EOC, breast, colon, and lung cancers, and melanoma from The Cancer Genome Atlas (TCGA) studies into high and low groups (Figures 7B and S7C–S7F). For EOC, this basal expression divided tumors into high, medium, and low expression groups and the former two encompass virtually all of the TCGA (Verhaak et al., 2013) immune reactive (IMR) good prognosis tumors. The Low group encompasses the PRO (high proliferative), poor prognosis subtype ($p < 0.001$ to 0.0001) (Figures 7B and S7B). Interestingly, virtually all of the right-sided colon cancers with a high DNA hypermethylation frequency phenotype ($p < 0.002$), termed CIMP, which have a high

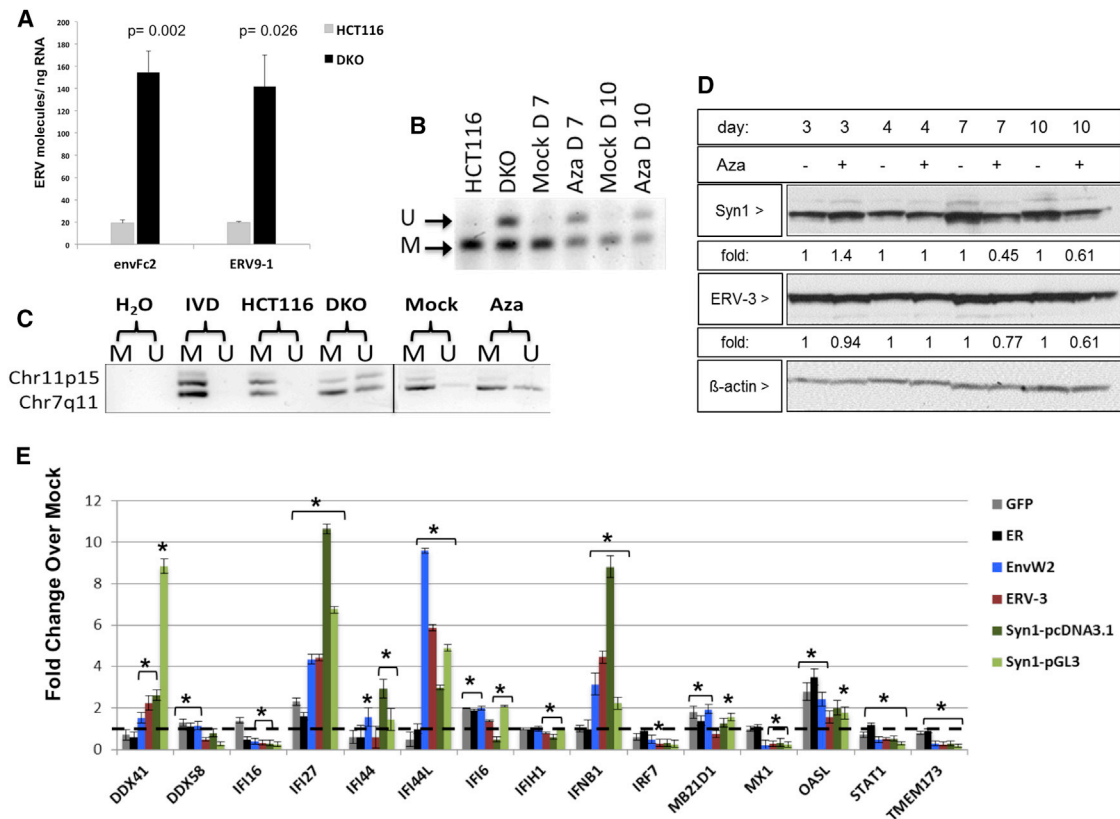


Figure 6. Aza Upregulates ERV Transcripts, but Not Proteins, through DNA Demethylation

(A) *env-Fc2* and *env-9-1* ERV gene total number of molecules, assayed by qRT-PCR, for DKO (DNMT1^{-/-}, DNMT3B^{-/-}) and parental HCT116 cells. y axis, mean $\pm$ SEM for n = 6 biological replicates.

(B) DNA methylation changes in ERVs in A2780 cells treated with Mock or 500 nM Aza for 3 days at post-treatment day 4 (day 7) or 7 (day 10). Bisulfite-treated DNA was amplified and digested with the AclI enzyme producing 155 and 44 bp fragments of methylated DNA while unmethylated DNA does not digest (199 bp fragment). U, unmethylated band; M, methylated band.

(C) DNA from (B) was subjected to methylation-specific PCR for ERV-Fc2 family members on chromosomes 7 and 11. U, unmethylated; M, methylated; IVD, in vitro methylated DNA. The products from the same MSP reaction were run on two different gels and presented together. Vertical line indicates distinction between gels on the left and right.

(D) Syncytin-1 and ERV-3 protein levels in EOC cells treated as in (B). Fold change for densitometry by ImageJ for Aza versus Mock cells normalized to β -actin protein levels. The same gel blotted for Syncytin-1 and β -actin, then stripped and reprobed for ERV3. The space between the Syncytin-1 and ERV3 and the β -actin proteins was cropped out to conserve space.

(E) Transfection of full-length *env* genes from EnvW2, ERV-3, or Syncytin-1 or EGFP and ER controls in TykNu cells. qRT-PCR was performed for ISGs 7 days after transfection. Dotted black line indicates 1. Y axis, mean $\pm$ SEM fold change of three biological replicates for overexpression/Mock. * $p \leq 0.05$.

See also Figure S6.

burden of DNA mutations and respond robustly to immune checkpoint therapy (Le et al., 2015), were in the High and Intermediate groups (Figure S7C). High mutation burden has recently been defined as a key correlate to response to immune checkpoint therapy (Rizvi et al., 2015; Snyder et al., 2014). Similar sharp high versus low clustering is seen for subgroups of breast and lung cancers and melanoma (Guan et al., 2015) (Figures S7D–S7F), the last being very responsive to immune checkpoint therapy (Topalian et al., 2015; Hodi et al., 2010; Weber et al., 2015).

Could the levels of viral defense pathway signaling correlate with improved responses to immune checkpoint therapy? Indeed, for RNA-seq transcriptomes of melanoma patients treated with anti-CTLA-4, high levels of the viral defense signature expression in tumor samples correlated with long

term benefit (disease control [stable disease or better] >6 months as measured radiographically) in patients treated with anti-CTLA-4 therapy (Snyder et al., 2014) (Figures 7C and 7D; Tables S5 and S6). Importantly, high viral defense signature again correlated with high mutational burden (Figure 7C).

Aza Treatment Potentiates Immune Checkpoint Therapy in a Mouse Model of Melanoma

In the B16-F10 mouse melanoma model, multiple combinations of low dose Aza directly enhanced tumor responses to anti-CTLA4 immune checkpoint therapy (Figures 7E, 7F, and S7G). Further, B16 cells treated in vitro with Aza, then injected into mice and treated with anti-CTLA-4, were cleared completely (data not shown). Thus DNMTis can potentiate the anti-tumor effects of immune checkpoint inhibitors.

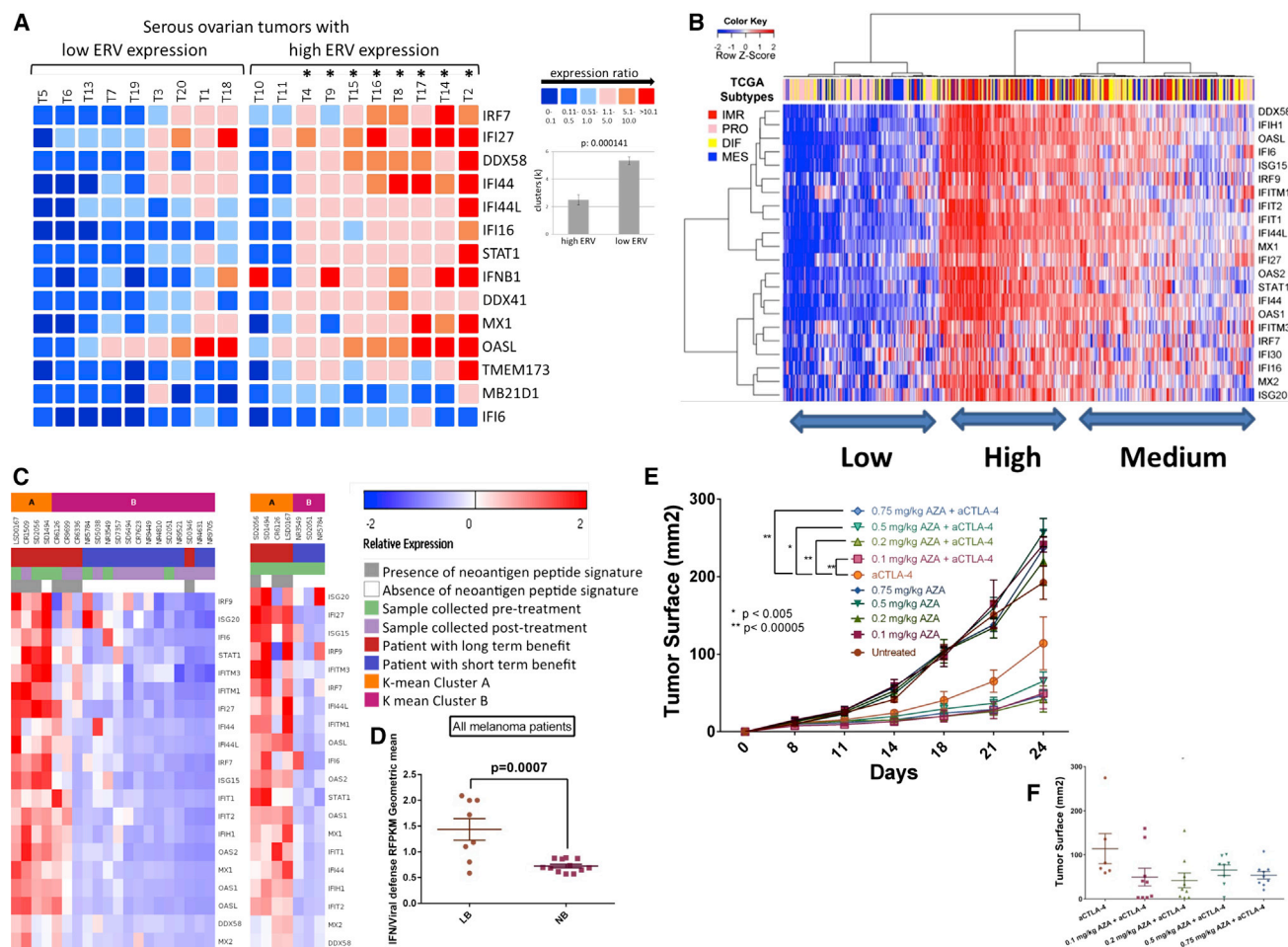


Figure 7. Aza-Upregulated Viral Defense Genes Are Significantly Correlated with ERVs in Primary Tumors and Correlate with Sensitivity to Immune Therapy

(A) Heatmap comparing basal levels of viral defense genes and ERVs in primary EOC. The cut-off for lower or higher ERVs was the mean control tissue value of 237.57 ± 83.05 molecules/ng RNA. Mean ISGs of the high ERV ovarian tumor (T) cohort ($n = 10$) is 12.65-fold higher than the mean of ISGs of the low ERV cohort ($n = 9$). The (*) denotes that eight of ten high ERV tumors had significantly higher ISG expression compared to the low ERV tumors. ISG expression is organized according to low and high ERV expression cohorts in arbitrary units; color code from blue to red shows increasing ISG expression. For clusters ($k = 6$), differences are significant between the high ERV expression (2.5 ± 0.37) and the low ERV expression cohort (5.33 ± 0.28).

(B) Interferon-stimulated viral defense genes upregulated at least 2-fold by Aza in EOC cell lines (right y axis) were used to cluster EOC tumors for RNA-seq data (blue, low; red, high) from The Cancer Genome Atlas (TCGA). EOC TCGA subtypes are shown: DIF (differentiated), IMR (immune reactive), MES (mesenchymal), and PRO (proliferative).

(C and D) Viral defense gene signature is upregulated in tumors from anti-CTLA-4-treated metastatic melanoma patients who derived durable clinical benefit (complete response, partial response, or progression free-survival >6 months as previously described) (Snyder et al., 2014) compared to those without benefit. Tumors collected pre-CTLA-4 treatment and shortly post-treatment are shown.

(D) y axis = RPKM mean of viral defense genes in all melanoma patients.

(E and F) Tumor responses of mice injected with B16-F10 cells and treated with either PBS, anti-CTLA-4, Aza, or both anti-CTLA-4 and Aza. Data represent results from one of two independent experiments with identical results, each with $n = 10$ per arm. Y axis = mean tumor surface, error bars $\pm$ SEM.

See also Figure S7 and Tables S5 and S6.

DISCUSSION

Our present data now provide functional context for our earlier reports that DNMTis induce a complex set of immune pathway responses in tumor cells (Li et al., 2014; Wrangle et al., 2013). DNMTis trigger cytoplasmic dsRNA sensing, central to cellular viral defense responses, and activate interferon in EOC and colon cancer cells by disrupting DNMTs. This activation could

induce tumor attraction of lymphocytes (Ivashkiv and Donlin, 2014). There are some important implications for one of the most exciting new developments in cancer treatment, immune checkpoint therapy (Brahmer et al., 2010, 2012; Berger et al., 2008; Leach et al., 1996; Topalian et al., 2015; Hodi et al., 2010; Weber et al., 2015) and for underlying mechanisms inherent to both tumor and host cells for reversal of immune tolerance in tumor infiltrating T-lymphocytes (Pardoll, 2012).

Indeed, basal levels of our viral defense gene transcripts divide EOC, and other major cancer types in TCGA, into low and high expression subgroups. Perhaps most intriguingly, such high basal expression in tumors tracks with favorable patient responses in a trial of immune checkpoint therapy for advanced melanoma, and strong Aza sensitization to immune checkpoint therapy is seen in a pre-clinical mouse melanoma model.

A major trigger of the Aza-induced viral defense response appears to be bidirectional transcription of ERVs that are known to fold into dsRNA secondary structures. ERVs, representing more than 8% of the human genome (Bannert and Kurth, 2004; Tristem, 2000), integrated into the genome of mammals between 0.1 and 40 million years ago via exogenous retroviral infections of germ cells (Egan et al., 2004; Turner et al., 2001). Most ERV genes are non-functional due to DNA recombination, mutations, and deletions, but some produce functional proteins including group-specific antigen (gag), polymerase (pol) with reverse transcriptase (RT), and the envelope (env) surface unit (SU) with a transmembrane immunosuppressive-like peptide (Mi et al., 2000; Blaise et al., 2005; de Parseval et al., 2003; Villesen et al., 2004). The *env* gene of *ERVW-1* (chromosome 7q21.2) called *Syncytin-1* has an essential role in placentogenesis (Blond et al., 1999; Mi et al., 2000).

Importantly, and key to our findings, a major function of DNA methylation in humans is silencing of ERVs and other viral sequences in the human genome; up to 90% of methylated CpGs are located in 45% of the human genome harboring repetitive elements like ERVs (Walsh et al., 1998; Bestor and Tycko, 1996). However, ERV genes are unmethylated and expressed in embryonic stem cells (Santoni et al., 2012) and especially *Syncytin-1* is epigenetically regulated throughout placentogenesis (Matoušková et al., 2006). Some tumors have ERV demethylation and increased expression such as the ERV-K (HML-2) 5'LTR-UTR in melanoma (Stengel et al., 2010) and the 5'-LTR region of several ERVs in testicular cancer (Gimenez et al., 2010). A 20% overall mean demethylation of single CpGs in the *ERVW-1* 5' LTR regulating *Syncytin-1* correlates with increased expression in endometrial cancer (Strissel et al., 2012). Indeed, ERVs can be targeted as tumor-associated antigens on melanoma cells (Cooper et al., 2015). In contrast, as in our present data and those of others (Maksakova et al., 2008), in some cancers, individual ERVs can maintain full or partial promoter DNA methylation and low expression and DNMTis can induce ERV demethylation and viral defense signaling in human embryonic stem cells (Grow et al., 2015).

In addition to ERVs, other noncoding RNAs could contribute to the Aza-induced immune response, such as repetitive Alu elements (Tarallo et al., 2012). UV light can damage small nucleolar RNA and activate an interferon response via TLR3 (Bernard et al., 2012) and very high dose (10 μ M) Dac can induce an interferon response, apoptosis, increased ERVs and repetitive satellite RNAs in p53 null mouse fibroblasts (Leonova et al., 2013). We suspect, however, that such high Dac doses induce DNA damage rather than simply epigenetic effects. Half of the ovarian cancer lines we studied (Li et al., 2014) have wild-type *P53* but we see no differences in Aza interferon response between these and those with mutant *P53*.

The high translational connotations of our findings, including the small number of patients in clinical trials for NSCLC who may have been sensitized by epigenetic priming to immune therapy (Wrangle et al., 2013), remain to be validated in larger clinical trials. These are ongoing for NSCLC (Brahmer, 2015) and planned for advanced ovarian cancer. Moreover, ERV-K env proteins have been shown to increase immunotherapeutic potential of melanoma, breast, and ovarian cancer patients (Rycaj et al., 2015; Wang-Johanning et al., 2012; Cooper et al., 2015). Also, our hypotheses that drugs like Aza might sensitize patients with multiple cancer types to immune checkpoint blockade and other immunotherapies are further strengthened by the data in our pre-clinical melanoma model. For immune checkpoint therapy, in addition to the functional significance of our data, a potential biomarker strategy is suggested by our findings in a melanoma trial. The high correlation of viral defense signaling with mutational burden suggests that genetic changes, increases in ERVs, and viral defense genes could predict response to immune checkpoint and other immunomodulatory approaches. Finally, our drug approach to upregulate viral defense signaling might be compared to the use of oncolytic viruses to induce inflammatory immune infiltrates at tumor sites to sensitize to immunomodulation (Zamarin et al., 2014).

EXPERIMENTAL PROCEDURES

Detailed materials and methods can be found in the [Supplemental Experimental Procedures](#).

Cell Line Treatments

Cell lines were treated with 500 nM Aza, 100 nM Dac, or 500 nM-3 μ M carboplatin (Sigma) for 72 hr, and DNA and RNA were isolated using standard methods at 1, 3, or 7 days following removal of drug. Ruxolitinib (2 μ M) (Innvogen tlr-rux), 0.625–5 U/ml of anti-IFNAR2 antibody (PBL Interferon Source 21385-1), 0.625–2.5 U/ml of anti-IFNB antibody (PBL Interferon Source 31400-1), or 1.25–5 U/ml of anti-IL10RB antibody (Abcam ab89884) were added during DNMTi treatment. Preparation of nuclear and cytoplasmic fractions of cultured cells was performed as described (O'Hagan et al., 2011). Ribosomal RNA was depleted using the Ribominus kit (Invitrogen), and PolyA⁺ and PolyA[−] RNA were isolated using the Oligotex Direct mRNA Mini Kit (Invitrogen). Nucleic acids were treated with 1 U/ μ g of RNase III (Ambion), 10 U/ μ g of RNaseH (Invitrogen), or 3 U/1 μ g calf intestine alkaline phosphatase (New England Biolabs) according to manufacturer's instructions, and 400 ng of each nucleic acid was transfected into HT29 cells.

DNA Methylation Analysis

DNA was bisulfite converted and subjected to methylation-specific PCR (Herman et al., 1996) for *IRF7* and *ERV-Fc2* and COBRA (Xiong and Laird, 1997) for the *ERV-Fc2* locus on chromosome 11.

Transcript Abundance

Real-time RT-PCR was performed with an Applied Biosystems 7500 Fast Real-Time PCR machine by the $2^{-\Delta\Delta CT}$ method and TASA-TD strand-specific PCR by the method of Henke et al. (2015).

Protein Analysis

Western blot analyses employed antibodies against ERV-3 (1:1,000, Everest), B-actin (1:5,000, Sigma), MDA5 (1:1,000, Cell Signaling 5321), PARP (9542, 1:1,000; Cell Signaling Technology), RIG-I (1:1,000, Cell Signaling 4200), STING (1:1,000, Abcam ab82960), Syncytin-1 (1:350, Dr. Hervé Perron, Gen-euro, Geneva Switzerland), and TLR3 (1:1,000, Cell Signaling 6961). IFNB

ELISA utilized the *Verikine-HS* Human Interferon Beta Serum ELISA kit (PBL Interferon Source) and IFNL ELISA the DuoSet ELISA for Human IL-29/IL28-B (IFNL 1/3) kit (R&D Systems).

Knockdown and Overexpression Experiments

Syncytin-1, *ERV-3* and *ERV-W2 env*, *Estrogen receptor alpha (ER)*, and E-GFP vectors and siRNAs targeting *Syncytin-1*, *ERV-3*, or a scrambled control, were transfected using the JetPei or Hyperfect transfection reagents, respectively. TLR3, MAVS, and STING shRNA were performed according to established methods (Stewart et al., 2003).

RNA-Seq Expression Analysis of Tumors from Anti-CTLA-4-Treated Patients

Patients were described previously (Snyder et al., 2014) and samples were obtained with written informed consent per approved institutional review board (IRB) protocols. Expression data were obtained using RNA-seq with all data deposited at The cBio portal under the study name Metastatic Melanoma (MSKCC Cell, 2015).

B16-F10 Melanoma Mouse Model

C57BL/6J mice were subcutaneously injected with 1×10^5 B16-F10 tumor cells. On days 4, 8, 11, 14, and 18, mice were treated intraperitoneally with 100 μ g anti-ctla-4. Mice received two cycles of intraperitoneal injection of 0.1 to 0.75 mg/kg Aza in PBS for 5 consecutive days followed by 7 days off treatment, starting at day 8 after developing palpable tumors, with control groups receiving corresponding doses of non-specific isotype antibody control and PBS intraperitoneally. Tumor surface was measured with a caliper using the ellipse surface formula (length $\times$ width $\times \pi$)/400. All mouse procedures were performed in accordance with the institutional protocol guidelines from the Memorial Sloan Kettering Cancer Center (New York, NY).

Statistical Analysis

Mean $\pm$ SEM qRT-PCR results were considered statistically significant with p values ≤ 0.05 by Mann-Whitney U test or Student's t tests and two-tailed p values are reported. Tumor growth was assessed by two-way ANOVA between each of the mouse treatment groups with p values adjusted by the Dunnett multiple comparison test ($df = 512$).

Normalized, level 3 Agilent expression data were obtained from The Cancer Genome Atlas data portal (<https://tcga-data.nci.nih.gov/tcga/>) and analyzed by R statistical software (<http://www.r-project.org>) with existing packages and customized routines. Consensus hierarchical clustering was performed with the ConsensusClusterPlus R-package (Wilkerson and Hayes, 2010) and data analyzed by the Fisher exact p value test for association between clusters.

ACCESSION NUMBERS

The expression data reported in this paper is deposited in the cBio portal under the study name Metastatic Melanoma (MSKCC Cell, 2015).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2015.07.011>.

AUTHOR CONTRIBUTIONS

K.B.C., P.L.S., A.D., T.M., J.W., T.A.C., S.B.B., and R.S. designed experiments, performed data analyses, and wrote the manuscript. K.B.C., P.L.S., C.H., A.D., A.H., B.A., and S.B. performed experiments. N.S.R. provided *ERV-3 env* cDNA plasmid. D.S. provided ovarian cancer cell lines. H.L., A.S., V.M., D.M.P., L.M.C., M.W.B., and C.A.Z. assisted with data analyses.

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Combining Epigenetic and Immunotherapy to Combat Cancer

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Abstract

The most exciting recent advance for achieving durable management of advanced human cancers is immunotherapy, especially the concept of immune checkpoint blockade. However, with the exception of melanoma, most patients do not respond to immunotherapy alone. A growing body of work has shown that epigenetic drugs, specifically DNA methyltransferase inhibitors, can upregulate immune signal-

ing in epithelial cancer cells through demethylation of endogenous retroviruses and cancer testis antigens. These demethylating agents may induce T-cell attraction and enhance immune checkpoint inhibitor efficacy in mouse models. Current clinical trials are testing this combination therapy as a potent new cancer management strategy. *Cancer Res*; 76(7); 1683–9. ©2016 AACR.

Introduction

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

To this end, a growing body of clinical trials has shown exceptional promise. Antibodies blocking CTLA-4, an inhibitory molecule on T cells, produce durable responses for treatment of melanoma (6,7) and are currently in clinical trials for lung, prostate, and other cancers (15–17). Antibodies targeting human PD-1 (receptor on T cells) and PD-L1, the inhibitory ligand for PD-1 that is expressed at varying levels by cancer cells, have produced exceptionally durable responses in patients with highly aggressive, treatment-resistant metastatic cancers. The effects may be most apparent in patients whose tumors express PD-L1 (1–5,18). While melanoma has been the most responsive solid tumor (5), exciting results have been achieved in the most lethal of cancers, advanced non-small cell lung cancer (NSCLC;

refs. 1, 2, 5). This is of special interest as this cancer was previously considered not to be immune responsive. FDA approval for melanoma and NSCLC has resulted from the above trials (5).

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

Definition of Epigenetic Therapy

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

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

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

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

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

The Intersection of Epigenetic Therapy with Immunotherapy

Over the past several years, within the context of a Stand up to Cancer (SU2C) project to implement epigenetic therapy for cancer, our group has brought a low dose concept for use of DNMTi (5-azacytidine or 5-aza-2'-deoxycytidine) with or without HDACi to clinical trials for multiple tumor types. Signals for potential efficacy have particularly appeared for advanced, multiply pretreated non-small cell lung cancer (NSCLC; refs. 36, 37). One result in particular has driven much further emphasis in the clinic and the laboratory. A small number of patients with advanced NSCLC who progressed after receiving low-dose epigenetic therapy entered a trial for immune checkpoint therapy. Approximately 20% of the patients responded to the immune checkpoint therapy alone, passing 24 weeks without progression,

with most achieving high-grade RECIST criteria responses (1, 38). This is an astounding result for immunotherapy in NSCLC. All 5 patients who had received the prior epigenetic therapy passed the 24-week point without progression with subsequent immune checkpoint therapy and three of these developed high-grade partial RECIST criteria responses that have all been durable over 2.5 years (36, 37). These findings have prompted initiation of a larger clinical trial, which is now ongoing. Moreover, our laboratory group pursued studies to determine the mechanism(s) that might account for epigenetic sensitization to immunotherapy. Our findings to date, and those of others, support the hypothesis that there may be extraordinary potential for combined epigenetic and immunotherapy to increase the frequency of durable responses for immune checkpoint therapy in not only NSCLC but also other common tumor types.

Epigenetic Therapy Drugs Boost Immune Attraction Properties of Epithelial Cancer Cells

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

As CTAs can be recognized by the host immune system, they represent good candidates for immunotherapy, including vaccines. There is thus great potential for DNMT inhibitor treatment to upregulate CTAs on tumors, facilitating targeting by the host immune system (41). Guo and colleagues showed that the DNMTi 5-aza-2'-deoxycytidine could demethylate and upregulate the murine CTA P1A in 4T.1 mammary carcinoma cells in syngeneic mice. P1A was presented and recognized by H-2L dJ-restricted P1A-specific T cells, and combined therapy with 5-aza-2'-deoxycytidine and adoptive transfer of these T cells significantly reduced lung metastases in this mouse model (45). The novel DNMT inhibitor SGI110, which has longer *in vivo* stability than 5-azacytidine or 5-aza-2'-deoxycytidine and has shown clinical activity in patients with MDS and AML (46), also upregulates CTAs. In AML xenografts, SGI110 upregulates NY-ESO-1 and MAGE-A and induces cytotoxicity by CD8⁺ T cells specific for NY-ESO-1 (47). Similar results were observed in epithelial ovarian cancer (EOC) xenografts (48). These promising results led to a phase I clinical trial in EOC in which Odunsi and colleagues added 5-aza-2'-deoxycytidine to NY-ESO-1 vaccine combined with doxorubicin chemotherapy in patients with relapsed EOC. They observed DNA hypomethylation at the NY-ESO-1 promoter.

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

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

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

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

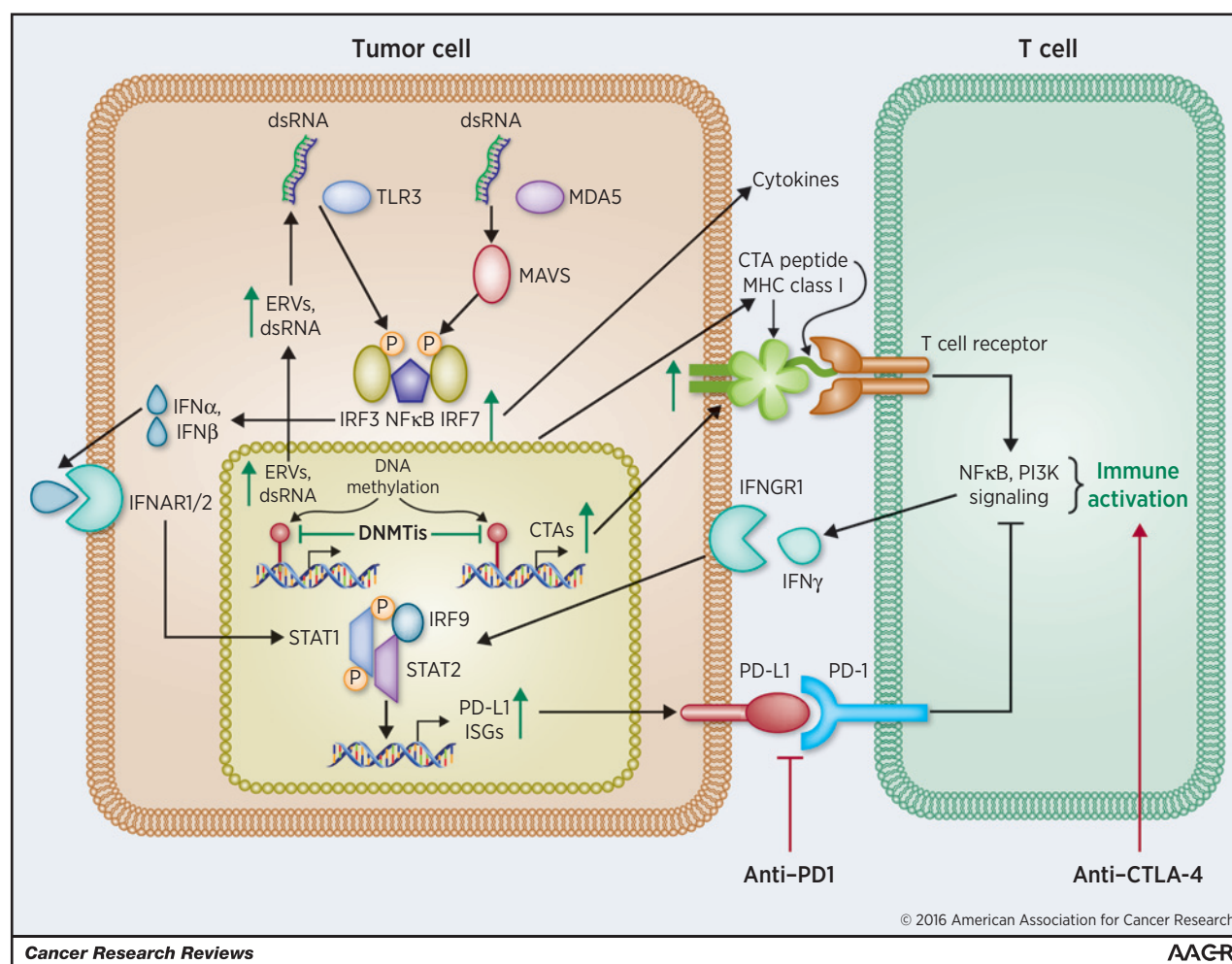
Recent work from our group and by de Carvalho and colleagues (56, 57) shows that one key way in which DNMTi upregulate immune signaling in cancer is through the viral defense pathway. In ovarian cancer cell lines, DNMTi activate a canonical IFN

signaling pathway, inducing IFN β and JAK/STAT signaling, through upregulation of dsRNA that activates the cytosolic dsRNA sensors TLR3 and MDA5. One type of RNA triggering this response is transcribed from hypermethylated endogenous retroviruses (ERVs) (56). Roulois and colleagues showed similar involvement of dsRNA and the MDA5 sensor in colon cancer cells and demonstrated that this IFN response was essential to the inhibition of colon cancer stem cells by DNMTi (57). Blocking the IFN response rescued about half of the DNMTi-induced apoptosis in ovarian cancer cells (56).

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

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

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

**Figure 1.**

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

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

Indeed, synergy of epigenetic and immune therapies was shown in ovarian cancer by Wang and colleagues (64). Treating a syngeneic mouse model of ovarian cancer with low-dose 5-aza-2'-deoxycytidine treatment caused upregulation of chemokines that recruit host natural killer (NK) and effector CD8⁺ T cells to the tumor. In addition, 5-aza-2'-deoxycytidine boosted the production of IFN γ and TNF α from effector T cells, while combining 5-aza-2'-deoxycytidine with anti-CTLA-4 therapy promoted differentiation of naïve T cells into effector T cells. As a result, this combination reduced tumor burden in the mice and extended their survival.

Another way in which 5-azacytidine may sensitize to immune checkpoint therapy is through upregulation of

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

Epigenetic Regulation of Host Immune Cells

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

HDACis boost antitumor immune responses. The HDACis panobinostat and vorinostat reduce tumor burden in immunocompetent mice, but not in immunocompromised RAG2γC^{−/−} and IFNγR^{−/−} mice (73). The authors found significant synergy of HDACi and IFNγ treatment in mouse models of colon cancer and lymphoma. IFNγ is secreted by cytotoxic T cells and NK cells and in these experiments it increased immunogenicity of tumor cells (73). Interestingly, B cells were a crucial component of the immune system in the response to HDACis (73). In addition, panobinostat significantly increased the effectiveness of adoptive cell transfer therapy (gp100-specific T cells) in the B16 mouse model of melanoma. Panobinostat enhanced gp100-specific T-cell survival and decreased Tregs in the peripheral blood and the tumor microenvironment. This HDACi also induced significantly higher levels of the IL2 receptor (CD25) and the costimulatory molecule OX-40 on T cells in the B16 mice. Taken together, these results suggest that HDACis boost the host immune response to tumors through B and T cells (74). In addition, inhibiting HDACs can also reduce myeloid-derived cells that induce immune tolerance to help prevent the immune system from clearing tumors (75).

Epigenetic agents may also affect the development of NK cells, which recognize virus-infected cells or newly formed tumor cells and release cytokines to kill the infected cells. Specifically, 5-aza-

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

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

Conclusions

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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Combining Epigenetic and Immunotherapy to Combat Cancer

Katherine B. Chiappinelli, Cynthia A. Zahnow, Nita Ahuja, et al.

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Unraveling the molecular pathways of DNA-methylation inhibitors: human endogenous retroviruses induce the innate immune response in tumors

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Epigenetic therapy activates type I interferon signaling in murine ovarian cancer to reduce immunosuppression and tumor burden

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Ovarian cancer is the most lethal of all gynecological cancers, and there is an urgent unmet need to develop new therapies. Epithelial ovarian cancer (EOC) is characterized by an immune suppressive microenvironment, and response of ovarian cancers to immune therapies has thus far been disappointing. We now find, in a mouse model of EOC, that clinically relevant doses of DNA methyltransferase and histone deacetylase inhibitors (DNMTi and HDACi, respectively) reduce the immune suppressive microenvironment through type I IFN signaling and improve response to immune checkpoint therapy. These data indicate that the type I IFN response is required for effective *in vivo* antitumor actions of the DNMTi 5-azacytidine (AZA). Through type I IFN signaling, AZA increases the numbers of CD45⁺ immune cells and the percentage of active CD8⁺ T and natural killer (NK) cells in the tumor microenvironment, while reducing tumor burden and extending survival. AZA also increases viral defense gene expression in both tumor and immune cells, and reduces the percentage of macrophages and myeloid-derived suppressor cells in the tumor microenvironment. The addition of an HDACi to AZA enhances the modulation of the immune microenvironment, specifically increasing T and NK cell activation and reducing macrophages over AZA treatment alone, while further increasing the survival of the mice. Finally, a triple combination of DNMTi/HDACi plus the immune checkpoint inhibitor α -PD-1 provides the best antitumor effect and longest overall survival, and may be an attractive candidate for future clinical trials in ovarian cancer.

5-azacytidine | histone deacetylase inhibitors | type I interferon | ovarian cancer | immunosuppression

Ovarian carcinoma is the leading cause of death from gynecological malignancies in the United States (1). Epithelial ovarian cancer is characterized by an immunosuppressive microenvironment (2), and patient responses to immunotherapy have been disappointing (3). In particular, the response of ovarian cancer to the immune checkpoint inhibitors α -PD-1 or α -PD-L1 has thus far been modest in comparison with the robust responses observed for melanoma, non-small cell lung cancer, and renal cell cancers (4). New treatment strategies are needed to reverse the immunosuppressive microenvironment of ovarian cancer and sensitize these tumors to immune checkpoint blockade.

One promising approach for reversing the tumor immune evasion phenotype and improving the efficacy of immune checkpoint therapy of cancers in general and ovarian cancers in particular is epigenetic therapy. The first suggestion for this approach came from a clinical observation in trials involving patients with advanced, pretreated non-small cell lung cancer (NSCLC) (5). These individuals initially received the DNA methyltransferase inhibitor

(DNMTi) Vidaza (5-azacytidine; AZA) and the histone deacetylase inhibitor (HDACi) entinostat (MS275). This coupling was based on preclinical data that DNMTis and HDACis in combination enhance reactivation of aberrantly silenced genes in tumor cells and cause reductions in tumor burden that are more effective with the combined agents (6–9). After disease progression, these patients entered the first trials of immune checkpoint therapy for NSCLC, and several of these patients had durable responses compared with those who received the immunotherapy alone (10). Clinical trials are being conducted to validate this preliminary observation (10), but preclinical work to optimize this combinatorial approach of HDACis and DNMTis has not been explored.

Significance

Therapies that activate the host immune system have shown tremendous promise for a variety of solid tumors. However, in most cancer types, fewer than half of patients respond to these immunotherapies. We propose epigenetic therapy as a mechanism to sensitize tumors to immune checkpoint therapy. We have shown that inhibiting DNA methylation triggers a viral defense pathway in tumors. Here we show that epigenetic therapy in a mouse model of ovarian cancer increases the numbers of activated immune cells, and that this is dependent on the interferon antiviral response. The combination of epigenetic therapy and immune checkpoint blockade leads to the greatest reduction in tumor burden and increase in survival, and may hold the greatest promise for patients.

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Compelling data are emerging to show that this combinatorial approach in preclinical studies induces immune signaling in both tumor and immune cells. Studies by our group and others have shown that DNMTis can up-regulate a wide range of genes involved in immune signaling in breast, lung, colon, and ovarian cancer cells (11–15). A particularly important component of this in ovarian and colon cancer cells is that DNMTis can induce an up-regulation of a cytosolic sensing double-stranded RNA (dsRNA) antiviral pathway which results in type I IFN production and downstream signaling for up-regulation of IFN-stimulated genes, including immune cell attracting chemokines and cytokines (11, 14). Furthermore, epigenetic changes, specifically *de novo* methylation, have been shown to play an important role in the promotion of CD8⁺ T-cell exhaustion, which is a barrier to immune cell activation during checkpoint inhibition therapy (16). While this study showed that demethylation is important for T-cell reactivation, our data in the present study show that treatment of isolated tumor cells with DNMT inhibitors is sufficient to increase immune cell numbers in the tumor microenvironment. Taken together, both the tumor and the immune microenvironment are responsive to epigenetic therapy.

Others have utilized mouse models of ovarian cancer to explore how epigenetic therapy affects the response to immune checkpoint blockade, finding that the DNMTi decitabine can increase activated immune cells in ovarian ascites and sensitize tumors to α -CTLA4 (cytotoxic T lymphocyte-associated protein 4) (14). Similarly, another study showed that epigenetic therapy induced tumor expression of CXCL10, which led to an increase in T effector cells in the tumor microenvironment and to an improved response to α -PD-L1 therapy (15). However, the precise mechanisms underlying their findings and the link to a DNMTi/HDACi-induced antiviral, cellular response remain to be clarified.

Recently, using human NSCLC cells and a mouse model of this disease, our group has approached this question by trying to elucidate the best HDACis to use based on their pharmacologic half-lives and K_i effects on key members of the HDAC family. Moreover, we have optimized the ability to combine DNMTis and HDACis to develop a chronic, tolerable, low-dose schema to keep constant pressure on reversing not only DNA methylation abnormalities but the repressive chromatin events which accompany these. A potent regimen has been developed for inducing preclinical antitumor responses and reversing tumor evasion (17). We now employ this improved regimen in a mouse model of ovarian cancer, outlining not only a potent potential paradigm for an ovarian cancer clinical trial combining epigenetic with immune checkpoint therapy but, importantly, mechanistically tying induction of viral defense to the above antitumor responses by finding, in a mouse model of ovarian cancer, that DNMTis and HDACis increase both the number and activation of immune cells in the tumor microenvironment, reduce tumor burden, and extend overall survival of the mice. This modulation of the tumor microenvironment stems in part from an AZA-induced, IFN-mediated, up-regulation of an immune gene signature including genes involved in viral defense, chemokines, cytokines, IFN signaling, and cancer testis antigens (11, 13). We also now verify that type I IFN signaling caused by AZA-induced immune signaling is required for this drug to increase the number of CD45⁺ immune cells in the tumor microenvironment and activation of CD8⁺ T cells and natural killer (NK) cells. Moreover, we demonstrate that when HDACis are combined with AZA *in vivo*, these drugs act on the immune compartment to further improve the activation of CD8⁺ T cells and NK cells and to decrease myeloid cells to create a less immunosuppressed tumor microenvironment. Together, the actions of these drugs on tumor cells and the tumor microenvironment indicate that combination epigenetic therapy may offer an approach to optimize immune checkpoint therapy for patients with ovarian cancer, and that AZA, the HDACi givinostat (ITF2357; ITF), and α -PD-1 may hold the most promise.

Results

Pretreatment of Tumor Epithelial Cells *ex Vivo*. To study how DNMTis and HDACis directly affect tumor epithelial cells to regulate response and immune cell interactions, we pretreated cultured, syngeneic mouse ovarian surface epithelial cancer cells, ID8-VEGF-Defensin (18–21), with epigenetic agents, injected the cells into untreated mice, and analyzed ascites volume as a measure of tumor burden (22). The ID8 parental cells were derived from ovarian surface epithelium instead of from the fallopian tube, which is hypothesized to be the origin of most ovarian tumors (23, 24). While genetic models of the development of ovarian cancer in the fallopian tube exist (25, 26), the ID8 model is one of very few routinely available syngeneic murine models of ovarian cancer (27) that can be transplanted into immunocompetent mice and creates an immunosuppressed tumor microenvironment (22), making it well suited to our studies. Parental ID8 cells (21) were modified to overexpress VEGF and Defensin (18, 20) to make them more aggressive and more like some human ovarian cancers (28, 29). The ID8-VEGF-Defensin cells also consistently generate hemorrhagic ascites ~4 wk after transplantation of only 250,000 cells. The ascites generated by the ID8-VEGF-Defensin cells is easily drained for analysis of tumor and immune cells, and is representative of tumor burden (18, 22, 30–34). The number of tumor cells in the ascites fluid correlates positively with the volume drained, while the number of immune cells is inversely correlated, showing that the increase in tumor cells is not a general increase in cellularity of the fluid (Fig. S1 *A* and *B*).

Ten or 17 (A10, A17), but not 3 (A3–10), days of AZA pretreatment of tumor cells (Fig. 1*A*) led to significantly less ascites (Fig. 1*B*), reflected as a reduction in weight gain (Fig. S1*C*) and increased mouse survival compared with vehicle (mock)- or HDACi-pretreated tumor cells (Fig. 1 and Fig. S1 *C* and *D*). Combination pretreatment of AZA and entinostat (A+MS17) or AZA and givinostat (A+ITF17) decreased ascites compared with mock, but did not decrease ascites (Fig. 1*C*) or improve survival (Fig. 1*D*) compared with AZA treatment alone. The slight antagonistic effect of AZA+MS17 compared with AZA alone is not observed when the whole mouse is treated, and may be due to effects on the tumor cells that are not observed in an *in vivo*, immunocompetent setting. Overall, the decrease in ascites volume and increase in survival with AZA or AZA+HDACi pretreatment appear to be driven by an AZA-mediated effect on tumor cells.

AZA pretreatment of tumor epithelial cells in these *ex vivo*, pretreatment studies led to changes in the immune microenvironment, with increased numbers of immune cells (CD45⁺) in the ascites of A10 pretreated tumor cells (Fig. 2*A* and *B*). Entinostat or givinostat treatment of cultured tumor cells did not alter the number of CD45⁺ cells per mL (Fig. 2*C*), but did lead to decreases in the percentage of T effector and T helper cells (Fig. 2*I* and *J*) in the ascites. Neither the addition of givinostat (A+ITF17) nor entinostat (A+MS17) to AZA increased the number of CD45⁺ cells above AZA treatment alone (Fig. 2*C*); however, there were some significant changes in the percentage of activated NK cells (Fig. 2*F*). These changes are particularly interesting in light of the fact that the immune cells, associated with the tumor microenvironment, were not treated in the *ex vivo*, pretreatment model.

AZA-Induced Immune Signaling in Tumor Cells. Treatment with AZA at doses that degrade its molecular target, DNA methyltransferase 1, in ID8-VEGF-Defensin cells (Fig. S1 *E–G*) caused an up-regulation of an immune-related viral defense signature in these murine cells, as was previously described for human ovarian cancer cells (11) (Fig. S24). This expression, also seen in cancer testis antigens (CTAs), was especially robust with prolonged treatment (Fig. S2 *A* and *B*). HDACis have been shown to synergize with DNMT inhibitors to reexpress silenced genes in cancer (7, 9), but entinostat (MS275) or givinostat (ITF) treatment (HDACi17)

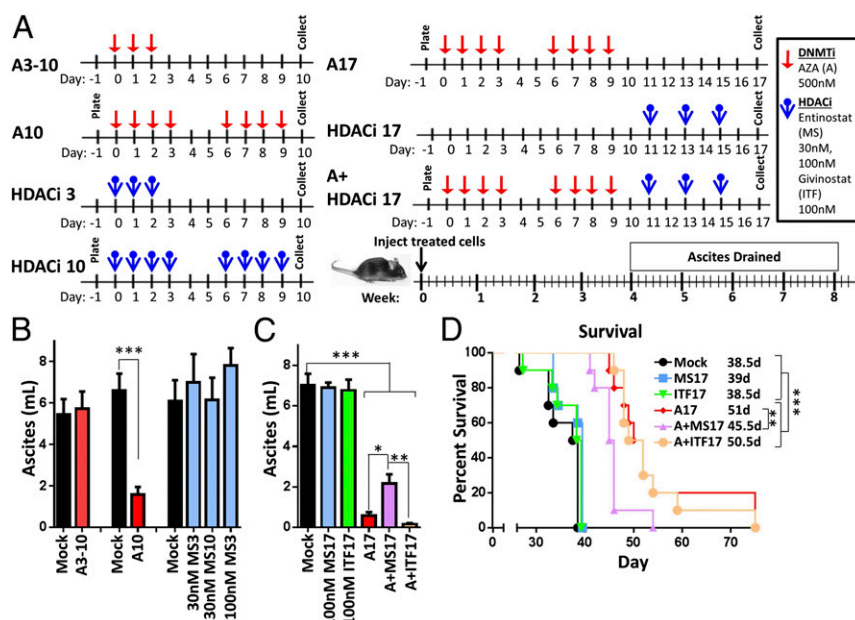


Fig. 1. Pretreatment of tumor epithelial cells with AZA and transplantation into untreated C57BL/6 mice lead to decreased tumor-associated ascites and increased overall survival. (A) Treatment schematic for in vitro treatment of cultured ID8-VEGF-Defensin cells (AZA, 500 nM; MS275, 30 or 100 nM; ITF, 100 nM). A, AZA; ITF, givinostat; MS, entinostat. (B and C) Ascites volume drained from mice 4 to 5 wk after pretreated tumor injection. Mean $\pm$ SEM is shown. A10, MS3, MS10: $n = 7$ to 30 mice, three biological replicates; A3-10: $n = 9$ mice, two biological replicates; MS17, ITF17, A17, A+MS17, and A+ITF17: $n = 9$ or 10 mice, one biological replicate. Statistical outliers were removed using Peirce's criterion, and significance was determined by Mann-Whitney t test. (D) Survival of mice in days, with median survival shown. Significance was determined using a log-rank (Mantel-Cox) test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

alone or in combination with AZA caused only small or moderate changes in the expression of the antiviral and CTA genes in these mouse ovarian cancer cells (Fig. S2 C and D).

We and others have previously demonstrated that AZA treatment of human ovarian carcinoma cell lines induced the expression of RNA from endogenous retroviruses (ERVs), which led to increased IFN signaling and viral defense gene expression (11, 14). We now demonstrate in mouse ovarian ID8-VEGF-Defensin cells that AZA significantly increased several mERVs in both cultured tumor cells (Fig. S2E) and tumor cells sorted from ascites from treated C57BL/6 mice (Figs. S2F and S4A and Table S1). While the mERVs are increased early in this treatment (day 3), they sharply decrease at later time points (days 4, 7, and 10). This is reminiscent of the increase and subsequent decrease in ERV transcripts observed in ref. 11. We hypothesize that antiviral proteins up-regulated by the IFN response may destroy the mERV RNA. Interestingly, in vivo (Fig. S2F), we observe a long-term increase in ERV transcripts. We have previously shown that these ERVs, silenced by DNA methylation, lose methylation and increase transcription upon DNMTi treatment (11).

Lastly, AZA treatment in vitro (A10) and in vivo (Fig. S4A) altered the secreted protein levels of chemokines and cytokines. In an array of 40 chemokines and cytokines, CD54, IL-1RA, CXCL10, CCL2, and CCL5 were significantly up-regulated in vitro, while CXCL12 was down-regulated. In the ascites fluid of AZA-treated mice, IL-1RA was significantly down-regulated, while CXCL10 and CXCL1 were significantly up-regulated. Only CXCL10 was significantly increased by AZA in both the media and the ascites fluid (Fig. S2 G and H).

Treatment of Mice with AZA, HDACi, and α -PD-1. Having shown that pretreatment of ovarian tumor cells led to increased immune cells in the tumor microenvironment and improved survival of the mice, we next asked whether the addition of α -PD-1 would provide added benefit. Mice bearing ovarian tumors treated intraperitoneally with AZA, an HDACi, and α -PD-1 had improved

overall survival, decreased tumor burden, and alterations of immune cell populations that would promote immune cell killing of tumor cells (Figs. 3 and 4). AZA as a single agent or in combination with either HDACi or α -PD-1 significantly reduced ascites volume (Fig. 3B, expansion), while HDACis or HDACis+ α -PD-1 were ineffective (Fig. 3B). Mirroring these ascites data, α -PD-1, HDACis alone, or HDACis plus α -PD-1 did not affect survival (Fig. S3B), while the combination of AZA with either HDACi significantly improved this key parameter over AZA treatment alone (Fig. 3C). This is in contrast to the ex vivo treatment model, where the addition of HDACi to AZA did not affect the tumor burden or overall survival (Fig. 1 C and D). The improved survival with in vivo treatment may reflect the effects of the epigenetic drugs on the host immune cells. Adding α -PD-1 to AZA+ITF further significantly increased survival over AZA+ITF or AZA+MS+ α -PD-1 (Fig. 3 D–F). In summary, the triple combination of AZA+ITF+ α -PD-1 was the most effective at decreasing ascites volume and increasing overall survival (Fig. 3F).

Immune cell subpopulations in the ascites fluid of tumor-bearing mice were changed by epigenetic therapy and α -PD-1, but immune cells in nonmalignant tissues, such as the spleen, were not affected (Fig. S4B). In the tumor microenvironment, AZA treatment moderately but significantly increased the percentage of activated CD4⁺ and CD8⁺ T cells and NK cells and, when combined with ITF or either HDACi+ α -PD-1, this activation was markedly enhanced (Fig. 4 A–C). All treatments containing AZA led to an increase in the percentage of T cells (Fig. 4F), though this effect was less consistently observed in replicate experiments than the increases in activation of CD8⁺ T and NK cells. None of the treatment groups altered the percentages of T regulatory, CD4⁺, CD8⁺, CD4⁺PD-1⁺, or CD8⁺PD-1⁺ cells or the CD4/CD8 ratio (Fig. S5). The addition of givinostat or either HDACi+ α -PD-1 to AZA therapy increased the activation of key tumor-killing subsets of immune cells.

Myeloid-derived suppressor cells (MDSCs), which aid in tumor immune evasion (35), were significantly decreased by almost

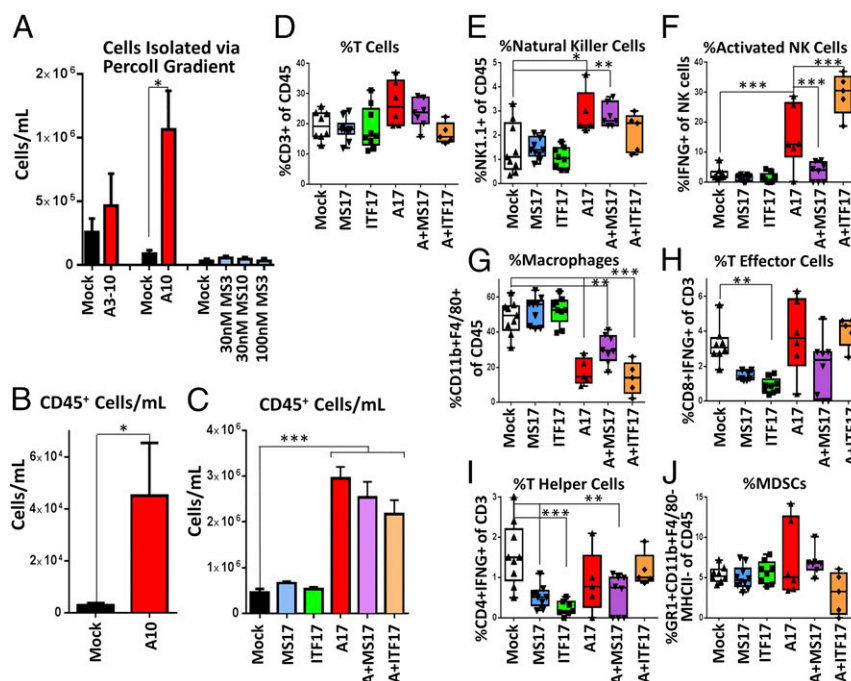


Fig. 2. Pretreatment of tumor epithelial cells with AZA and an HDACi leads to alterations in the numbers and activation of immune cell populations in tumor-associated ascites. ID8-VEGF-Defensin cells were pretreated (AZA, 500 nM; entinostat, 30 or 100 nM; givinostat, 100 nM) and injected into mice. Cells were analyzed from the drained ascites fluid (Fig. 1 A–C). (A) Immune cells per mL separated via Percoll gradient ($n = 6$ to 12 mice, two or three biological replicates). (B) CD45⁺ cells per mL identified via Percoll gradient and FACS ($n = 6$ to 11 mice, two biological replicates). Mean $\pm$ SEM is shown, and significances were determined by Mann–Whitney t test. (C–J) All cells from ascites were analyzed via FACS ($n = 5$ to 9 mice, one biological replicate). Mean $\pm$ SEM is shown, and significances were determined by one-way ANOVA. (C) CD45⁺ cells per mL of ascites. (D–J) Response of immune cell subpopulations to tumor cells pretreated ex vivo with AZA (A10). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

all therapies, with the exception of ITF and ITF+ α -PD-1; however, this exception may be due to limitations in sample number and high variability (Fig. 4D). All treatments containing AZA decreased the percentage of macrophages, which can influence tumor growth (36) (Fig. 4E). Compared with AZA alone, the addition of an HDACi significantly decreased the percentage of macrophages further. Overall, increased numbers of CD45⁺ immune cells, increased activation of CD8⁺ T cells and NK cells, and decreases in macrophages and MDSCs were the most consistent, significant changes resulting from epigenetic therapy. Differences were observed between the changes of the immune cell subpopulations in the ex vivo (Fig. 2 D–J) and in vivo (Fig. 4) models. This was not unexpected, as the immune cells were exposed to drug in the in vivo model but not in the ex vivo, pretreatment model. Further investigation is needed regarding the response of immune cell populations to epigenetic treatment.

In support of the fact that in vivo epigenetic treatment alters host immune cell populations in the tumor microenvironment, we find that AZA treatment increases expression of viral defense genes in CD8⁺ and CD4⁺ T cells and in CD11b⁺ myeloid cells in the ascites fluid of treated, tumor-bearing mice (Fig. S6). Ingenuity analysis also identified IFN-associated genes as top upstream regulators of the transcriptional program in these in vivo AZA-treated cells (Table S2). These results suggest that the AZA-induced increase in gene expression of IFN-associated genes in both tumor and host immune cells may be an integral component of the improved outcome of mice treated with epigenetic therapy and immune checkpoint inhibitors.

In summary, the combinations of epigenetic agents, as well as the addition of α -PD-1 to AZA+HDACi, increase the numbers and activation of key tumor-killing immune cells in the tumor microenvironment and decrease numbers of MDSCs and macrophages (Fig. 4). We hypothesize that these effects contribute to

the reduction in tumor burden and survival when treating with AZA, its combination with HDACis, and importantly these agents combined with α -PD-1.

Blockade of IFNAR1 Inhibits the Actions of AZA. Our expression data show that AZA treatment leads to increased IFN signaling and viral defense gene expression in ID8-VEGF-Defensin cells (Fig. S24). We therefore questioned the role and importance that IFN signaling plays in the AZA-induced decrease in tumor burden and alterations in immune cells observed in Figs. 3 and 4. To test this hypothesis, we used an antibody targeting IFN α and beta receptor subunit 1 (α -IFNAR1). Mice harboring ID8-VEGF-Defensin tumor cells were injected with α -IFNAR1 (i.p., 0.5 mg per mouse) every 3 d and simultaneously treated with AZA or vehicle control (mock) (Fig. 5A). The AZA-mediated reduction in ascites volume routinely observed in our experiments was inhibited by treatment with α -IFNAR1 (Fig. 5B), and total numbers of CD45⁺ cells in the ascites were not increased as with the AZA and IgG control treatment but remained near mock values (Fig. 5D). Likewise, activation of CD8⁺ T effector cells and NK cells in response to AZA treatment was also completely blocked by α -IFNAR1 (Fig. 5E and F), and there was no survival benefit for mice treated with AZA and α -IFNAR1 (Fig. 5C). Previous in vitro studies have demonstrated that up-regulation of a dsRNA sensing pathway by AZA triggers the activation of an intact type I IFNAR1 (11, 14), and here the use of α -IFNAR1 prevented the AZA-induced increase in expression normally observed for antiviral genes, such as ISG15, IFIT1, and ICAM1, in vitro (Fig. S7A–C). Our α -IFNAR1 data indicate that the type I IFN response is, indeed, required for effective in vivo anti-tumorigenic actions of 5-azacytidine, including reduced tumor burden, extended survival, and increased numbers and activation of immune cells.

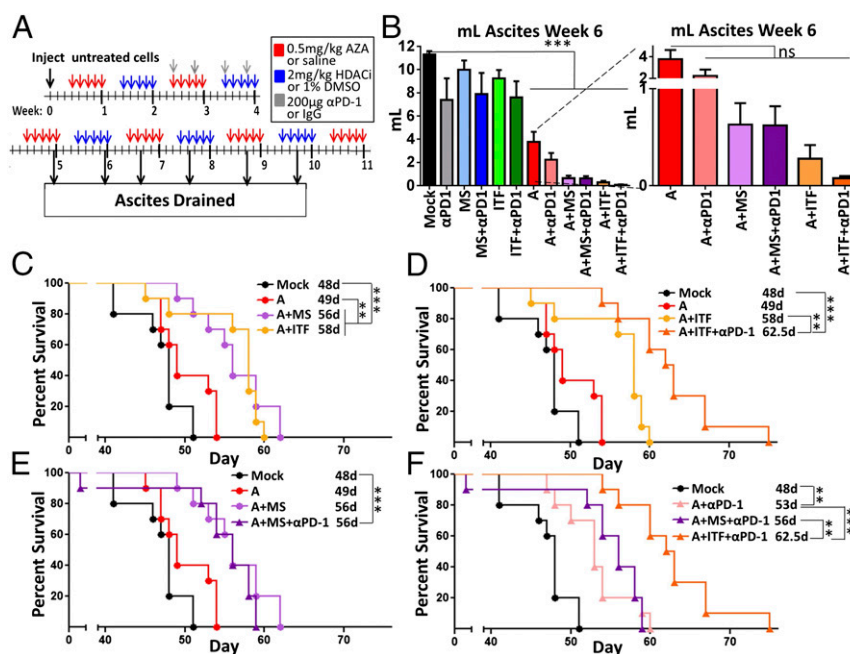


Fig. 3. Addition of immune checkpoint inhibition to epigenetic therapy in an intact mouse model decreases tumor burden and increases survival. (A) In vivo treatment schematic of AZA (0.5 mg/kg), entinostat (2 mg/kg), givinostat (2 mg/kg), and α -PD-1 (200 μ g per mouse). (B) Volume of ascites fluid drained at week 6. Mean $\pm$ SEM is shown and significances were determined by one-way ANOVA. All significances are compared with mock; ** $P < 0.01$, *** $P < 0.001$; ns, not significant; $n = 8$ to 10 mice per group. (C–F) Survival of the mice in days, with median survival shown. Significances were determined by log-rank (Mantel–Cox) test; $n = 10$ mice per group.

AZA+HDACi Efficacy Requires a Treated Immune System. To further assess the role of the immune cells in the antitumorigenic response, we compared the response to epigenetic agents in treated immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice that lack functional B, T, and NK cells (37) (Fig. 6A) with the response in the treated immunocompetent mice (Fig. 3). In the NSG immunodeficient mice, AZA treatment reduced ascites volume and increased survival as in the C57BL/6 mice, and HDACi treatment alone did not significantly affect ascites volume or survival in either mouse model. Combination treatment was more effective than AZA as a single agent in the treated immunocompetent C57BL/6 mice (Fig. 6B and C) and not in the immunodeficient mice or in the pretreatment model (Fig. 1C and D), suggesting that when combined with AZA, HDACis may act on the immune microenvironment to reduce tumor burden. Taken together, these data imply that AZA can act on both tumor and immune cells, while the added benefit of the combination with the HDACi may rely on the treatment and presence of an intact host immune microenvironment. Specifically, the activation of T and NK cells and decreases in macrophages were all significantly enhanced by the HDACi addition to AZA, and may be responsible for the reduction in tumor burden in the treated mice in an immune-intact setting (Fig. 4A–C and E). Interestingly, α -IFNAR1 treatment (Fig. 5A) did not inhibit the reduction in ascites burden in AZA-treated NSG mice (Fig. 6D), as it did in the C57BL/6 mice, shown again here for comparison, suggesting that the role IFN signaling is playing in the response to AZA is dependent on functional immune cells in the tumor microenvironment. These data also suggest that there are additional mechanisms responsible for the antitumorigenic effect of AZA that are not dependent on IFN signaling.

AZA Has Direct Antitumorigenic Effects. Even in the absence of tumor-killing immune cells in the NSG model, we noted increased numbers of dead cells in the CD45⁺ (nonimmune cell) population with AZA and AZA+ITF treatment, the two groups with the longest median survival (Fig. 6E). This could be explained by the

fact that the antitumor effects of AZA can be mediated through mechanisms that are not immune- or IFN-dependent, such as apoptosis and disruption of the cell cycle (11, 38–40). Indeed, 3 or 10 d of in vitro treatment of the tumor cells (A3-3, A10) with 500 nM AZA caused a significant decrease in cultured cell numbers (Fig. 7A and B) associated with signs of apoptosis reflected by increased cleaved-PARP protein levels and percentage of cells positive for annexin V and 7-AAD (Fig. 7C–F). These data confirm that nanomolar doses of AZA induce a low level of apoptosis in cancer cells, as we have previously described (11, 38), which appears to be too small to account for the large decrease in tumor cells observed in culture (Fig. 7B). A more important factor in the decrease in tumor cell number could be that A3 and A10 treatment in vitro decreased the percentage of tumor cells in S phase and increased those in G2-M arrest (Fig. 7G and H), as has been observed in other models (39, 40). In summary, AZA directly affects intrinsic, antitumorigenic mechanisms in the tumor cells, leading to increased apoptosis and cell-cycle arrest. However, the changes in the cell cycle and small increases in apoptosis were not inhibited in vitro by treatment with the α -IFNAR1 blocking antibody described previously (Fig. S7D and E), suggesting that while IFN signaling is important for the recruitment and activation of immune cells, AZA also has non-immune-related and IFN-independent effects.

Overall, our data demonstrate that AZA reduces tumor burden and increases the number of immune cells in the tumor microenvironment, in part through effects on the tumor cells themselves. AZA treatment up-regulates immune gene expression in tumor cells and in immune cells, and type I IFN signaling is required for some antitumorigenic effects of in vivo AZA, such as decreased ascites burden, extended survival, and activation of immune cells. When tumor-bearing mice are treated in vivo, the addition of an HDACi to AZA further reduces tumor burden and increases survival, perhaps due to an increase in activated T and NK cells and a decrease in macrophages. Finally, the combination of AZA,

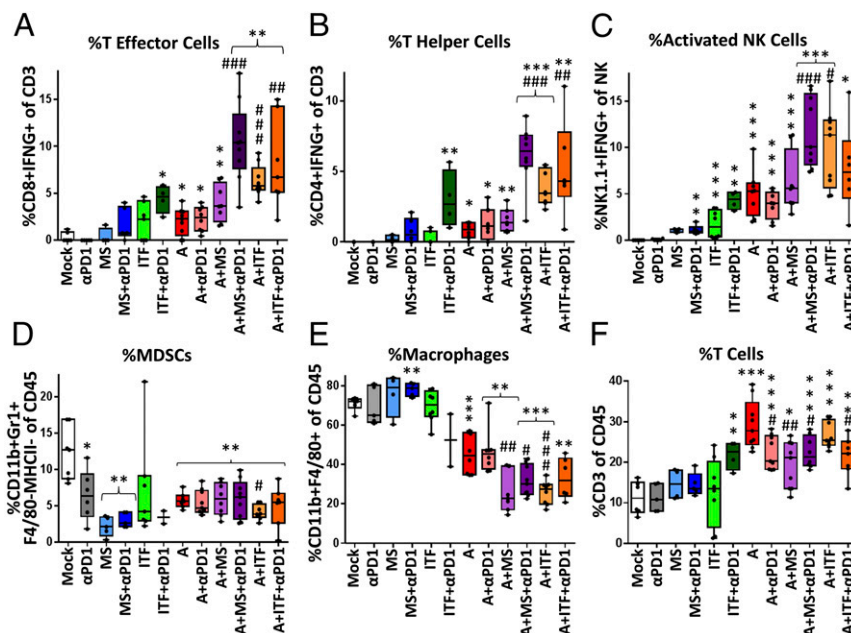


Fig. 4. Epigenetic therapy and α -PD-1 increase the number and activation of immune cells in the tumor microenvironment. Mice were treated as described in Fig. 3A. Cells from ascites fluid drained at week 6.5 were analyzed via FACS. Median, 25th and 75th percentiles, and range are plotted for each experimental arm, and significances were determined by Mann–Whitney t test. Significances compared with mock are marked with *, and significances compared with AZA are marked with #. $^{*}/\#P < 0.05$, $^{**}/\#\#P < 0.01$, $^{***}/\#\#\#P < 0.001$. (A) % T effector cells ($CD8^{+}IFN\gamma^{+}$) of T cells. (B) % T helper cells ($CD4^{+}IFN\gamma^{+}$) of T cells. (C) % activated NK cells ($NK1.1^{+}$, $IFN\gamma^{+}$) of $NK1.1^{+}$ cells. (D) % myeloid-derived suppressor cells ($GR-1^{+}$, $CD11b^{+}$, $F4/80^{-}$, $MHCII^{-}$) of $CD45^{+}$ cells. (E) % macrophages ($CD11b^{+}$, $F4/80^{+}$) of $CD45^{+}$ cells. (F) % $CD3^{+}$ T cells of $CD45^{+}$ cells. (A–C and F) $n = 4$ to 9 mice per group. (D and E) $n = 2$ to 9 mice per group.

givinostat, and α -PD-1 was the most effective in improving overall survival.

Discussion

The use of different treatment models in this study has enabled us to understand how 5-azacytidine and HDACis act individually and in combination on ovarian tumor epithelial cells and immune cells in the microenvironment to establish antitumor responses and to enhance immune checkpoint therapy. Low doses of AZA, but not HDACis, directly induce multiple antitumorigenic mechanisms in tumor cells, most notably increased immune signaling, increased apoptosis, and disruptions of the cell cycle, as well as increasing immune cell activation in the tumor microenvironment via type I IFN signaling. When an HDACi, especially givinostat, is combined with AZA in vivo, these agents can enhance the activation of specific immune subsets such as T and NK cells. Our data now show that the addition of an HDACi to a DNMTi may be optimal to achieve a maximal sensitization to checkpoint inhibitors. We hypothesize that other DNMTis such as decitabine may behave similarly to AZA used in this study, as decitabine has also been shown to trigger an antiviral response in tumor cells (11, 41). The triple combination of AZA, givinostat, and α -PD-1 extended median survival by 14.5 d compared with mock-treated mice. This change is significantly greater than the benefit derived from paclitaxel treatment, a common chemotherapy for ovarian cancer, in this fast-growing model (42). The addition of givinostat, but not entinostat, to AZA was able to sensitize the tumors to α -PD-1 therapy. The reasons why givinostat outperforms entinostat in overall survival when combined with AZA and α -PD-1 are currently under investigation. Other studies have shown that HDAC inhibition can affect tumor-associated immune cells, with one finding that B cells as well as $IFN\gamma$ receptor signaling in the tumor cells were important for the antitumorigenic effect of the HDACis vorinostat and panobinostat in syngeneic models of colon cancer and lymphoma (43).

In our study, blockade of type I IFN signaling with an antibody against $IFNAR1$ impairs the antitumorigenic effects of AZA. Although it had been observed that AZA treatment could stimulate IFN pathway induction by viral defense pathway signaling, it was not known which in vivo consequences would be linked to this pathway up-regulation. Importantly, our data now demonstrate that many of the antitumorigenic actions of AZA in ovarian cancer are mediated via $IFN\alpha$, β receptor subunit 1, including decreased tumor burden, increased $CD45^{+}$ immune cells in the tumor microenvironment, and increased activation of $CD8^{+}$ T and NK cells. We show that type I IFN signaling is responsible for the antitumor effects of AZA in immunocompetent mice (Fig. 6D). However, AZA has an effect that leads to decreased tumor burden in NSG mice that is not mediated by the IFN response (Fig. 6D). Thus, we conclude that the antiviral response caused by AZA primarily reduces tumor burden by recruitment and activation of immune cells to kill tumor cells in this model. Separately (Fig. S7), we show that AZA also has IFN-independent effects such as cell cycle disruptions in ID8-VEGF-Defensin cells (Fig. S7 F and G), which may account for the antitumorigenic effect of AZA in the NSG mice. AZA induced low levels of apoptosis that were not significantly affected by blocking $IFNAR1$ (Fig. S7 D and E), in contrast to previous work from our group and others that indicated an intrinsic apoptotic effect of AZA in human ovarian tumor cells (11, 14). Thus, in this mouse model, the primary effect of the AZA-induced IFN response is through recruitment and activation of host immune cells, while AZA also causes IFN-independent signaling to inhibit tumor cell growth.

Interestingly, P53 was identified as an upstream regulator of signaling induced by AZA in immune cells. P53 and DNMTs may cooperate to regulate repetitive elements that when transcribed trigger the IFN response (44). P53 is known to transcriptionally regulate specific IFN-stimulated genes, including $IRF9$ and $TLR3$. In addition, $STAT1$ and PKR up-regulate P53, leading to transcription of proapoptotic genes (45). Previous work has shown

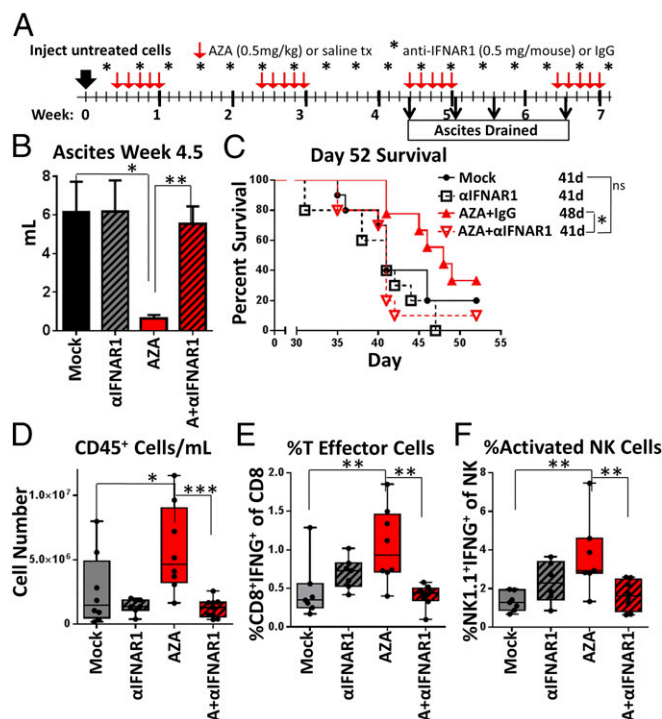


Fig. 5. Blockade of IFNAR1 inhibits the actions of AZA. (A) Treatment schematic for the mice. Mice were treated with AZA (0.5 mg/kg) or saline as described in Fig. 3. Anti-IFNAR1 was injected i.p. (0.5 mg per mouse) every 3 d, beginning 1 d before the AZA regimen. (B) Volume of ascites drained from the mice at week 4.5. Mean $\pm$ SEM is shown, and significances were determined by Mann-Whitney *t* test; *n* = 8 to 10 mice per group. (C) Survival of the mice in days, with median survival shown. Significances were determined by log-rank (Mantel-Cox) test; *n* = 10 mice per group. (D–F) Median, 25th and 75th percentiles, and range are plotted, and significances were determined by Mann-Whitney *t* test; *n* = 6 to 9 mice per group. (D) CD45⁺ cells per mL of ascites. (E) % T effector cells (CD8⁺IFN γ ⁺) of CD3⁺ T cells. (F) % activated NK cells (NK1.1⁺, IFN γ ⁺) of NK1.1⁺ cells. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

up-regulation of P53 through the DNA damage repair pathway by the DNMTi decitabine (46). Thus, P53 signaling may be activated by either the IFN response or DNA damage in the immune cells in question.

Others have tested whether the IFN response can play a role in sensitization to immune checkpoint blockade, as stabilization of IFNAR1 improves the efficacy of α -PD-1 therapy (47) and the loss of IFN- γ pathway genes is a mechanism of resistance to anti-CTLA-4 (48). IFN- γ has also been shown to suppress genes related to M2-like function in macrophages (49). On the other hand, prolonged tumor IFN signaling has been shown to induce resistance to immune checkpoint blockade over time (50), implying that perhaps the timing and duration of the response are important. Our study provides a greater understanding of how IFN signaling may sensitize tumors to immune checkpoint blockade.

The AZA-induced increases in immune signaling observed in ID8-VEGF-Defensin mouse ovarian cancer cells are consistent with our results for AZA-treated human ovarian cancer cells (11, 13). Thus, in both the human and murine tumor cells, AZA treatment leads to an increase in the expression of endogenous retroviral transcripts, viral defense genes, cancer testis antigens, and chemokines/cytokines (11, 13, 14). The chemokine CXCL10 was significantly increased in both the cell-culture media of AZA-treated murine cells and the ascites fluid of AZA-treated mice. CXCL10 has been identified in humanized models of ovarian cancer (15), validating the relevance of the ID8-VEGF-Defensin

mouse model of ovarian cancer to human disease and suggesting that these chemokines/cytokines may play a role in the AZA-induced recruitment of immune cells to the tumor-associated ascites (51–53). In fact, CXCL10 is linked to decreased tumor burden in the ID8 model (54).

Our work now provides mechanistic insight for previous studies showing that epigenetic agents may alter the tumor-associated microenvironment to potentially sensitize tumors to immunotherapy. Our study defines the cellular targets of AZA and HDACi using a more comprehensive panel of immune cells and proves a requirement for type I IFN signaling in the AZA-induced immune response. We have also shown that the addition of an HDACi to AZA can increase the activation of immune subsets, using doses of epigenetic therapy that are clinically relevant and can be immediately applied in clinical trials. The preclinical data in this manuscript helped to initiate a Celgene-sponsored, phase II randomized study of pembrolizumab with or without epigenetic modulation with CC-486 (oral AZA) in patients with platinum-resistant or -refractory epithelial ovarian, fallopian tube, or primary peritoneal cancer (55). This trial started enrollment in December 2016 and will hopefully reveal that addition of a DNMTi to checkpoint inhibitor therapy provides benefit beyond that of immunotherapy alone. The addition of an HDACi to the DNMTi in future trials may provide more benefit, and our data suggest that this combination can most effectively decrease the immunosuppression in the ovarian tumor microenvironment to sensitize to immune checkpoint blockade therapy.

Materials and Methods

Cell-Culture Treatments. MOSE ID8-Defb29/Vegf-a (ID8-VEGF-Defensin) cells, which tested negative for mycoplasma in December 2016, were grown in RPMI medium and treated with AZA (500 nM) at the same “low dose” established by Tsai et al. (38). The A3 treatment paradigm had AZA in the media on days 0, 1, and 2, while the A10 paradigm was also treated with AZA on days 3, 6, 7, 8, and 9. Cells were split on days 3 and 6 and harvested on day 10 (A3 and A10).

Entinostat (MS275) treatment followed the AZA paradigms above, but with 100 nM MS275 for 3 d or 30 nM MS275 for 3 or 10 d (HDACi3 or 10).

For the sequential combination of AZA and HDACi treatment, cells were treated with the A10 paradigm described above, followed by three doses of 100 nM MS275 or ITF. The cells were collected on day 17 (AZA17, HDACi17, and AZA+HDACi17; Fig. 1A). AZA (Sigma) was suspended in 0.9% saline. Entinostat (Syndax Pharmaceuticals) and givinostat (Selleckchem) were both suspended in DMSO and diluted 1:1,000 in media, so that the percentage of DMSO did not exceed 0.1%.

Gene Expression Analysis. RNA extraction, RNA quality analysis, hybridization to Agilent 4 $\times$ 44K Human Gene Expression v2 arrays, and analysis of the arrays were done as previously described (13). In some cases, tumor cells were isolated from ascites fluid by FACS, and RNA was isolated using the Qiagen RNeasy Micro Kit (74004). After total cellular RNA was extracted using the TRIzol method (Life Technologies), RNA concentration was determined using the NanoDrop machine and software (Thermo Fisher Scientific). One microgram total RNA was used to generate cDNA with the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative reverse-transcription PCR (q-RT-PCR) of ISG15, IFIT1, and ICAM1 mRNA was performed using TaqMan assays or Custom TaqMan Gene Expression Array Cards (Life Technologies) and the Applied Biosystems 7500 Fast Real-Time PCR System and software. TBP was used as a reference gene. The $\Delta\Delta$ CT method was used to calculate relative expression levels. Reverse transcriptase negative cDNA synthesis reactions were performed for at least one sample per plate.

Mouse Endogenous Retrovirus and SINE q-PCR. Cells with A10 treatment were collected on days 3, 4, 7, and 10. RNAs were DNaseI-treated, and cDNA synthesis was performed (High-Capacity cDNA Reverse Transcription Kit; Thermo Fisher Scientific). Expression of nine mERV genes (two IAP gag genes, an IAP-LTR, Mtv 7/8/9 sequences specific for C57BL/6 mouse strains, and placental mERVs including syncytin-A, mErv-3, Peg11, and Mart8) and the B1 SINE gene was quantified by q-PCR (ABI 7300) (see Table S1 for primer sequences and q-PCR methodology). Mouse housekeeping genes 18S rRNA, β -actin, and GAPDH were used for normalization (Table S1). Ascites tumor cells were sorted using FACS at week 4.5 following in vivo AZA treatment

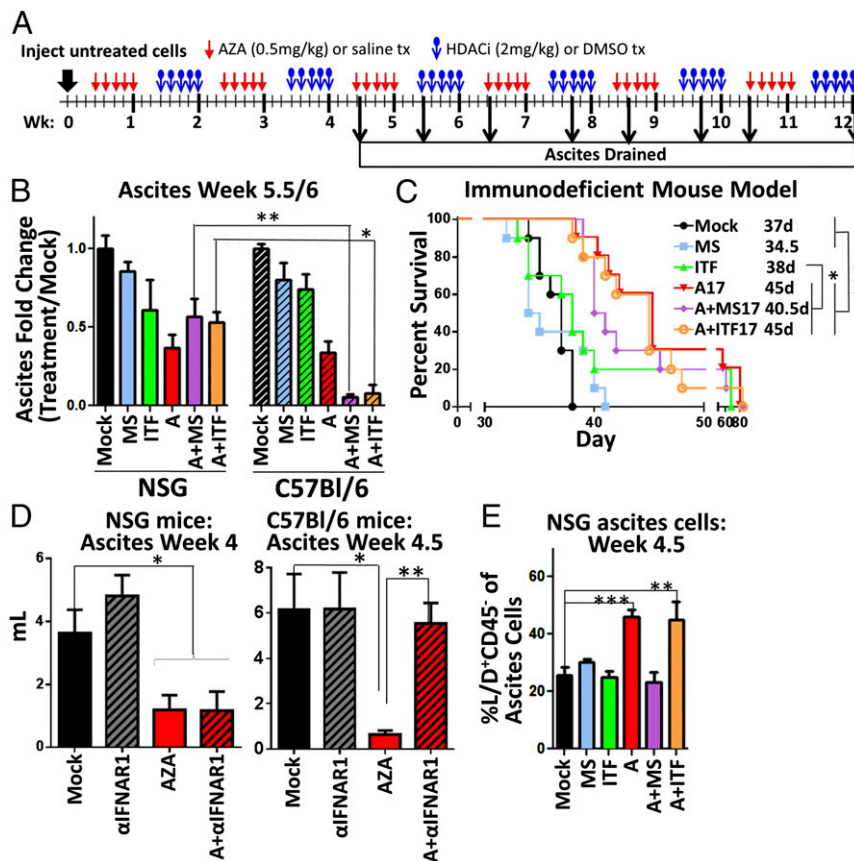


Fig. 6. AZA+HDACi combination therapy is less effective at reducing tumor burden and increasing survival in an immunodeficient mouse model. (A) Treatment schematic for in vivo treatment of NSG mice with AZA and HDACis entinostat or givinostat. (B) Fold change in ascites volume drained at week 5.5 (NSG) or 6 (C57BL/6). The C57BL/6 data from Fig. 3B are shown here for direct comparison; $n = 3$ to 10 mice per group. (C) NSG mouse survival in days, with median survival shown. Significances were determined by a log-rank (Mantel-Cox) test; $n = 10$ mice per group. (D) Ascites volume drained at week 4.5 or 4 from C57BL/6 or NSG mice, respectively, treated with AZA and anti-IFNAR1 as shown in Fig. 5 A and B; $n = 8$ to 10 mice per group. (E) % dead, CD45⁺, nonimmune ascites cells (Live/Dead stain⁺, CD45⁺) from NSG ascites fluid; $n = 5$ to 10 mice per group. (B, D, and E) Mean $\pm$ SEM is shown, and significances were determined by Mann-Whitney t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Fig. S6A). We examined gene expression for all nine mERVs and B1 SINE gene expression in sorted ascites tumor cells. The full q-PCR protocol was previously described (56, 57).

All Mouse Experiments. Tumor burden was assessed via measurement of body weight and the amount of ascites drained from the mice at the point where they had gained 20 to 30% of their body weight. Statistical outliers were removed using Peirce's criterion. Mice were cared for in accordance with the policies of The Johns Hopkins University Animal Care and Use Committee. $n = 10$ mice for all treatment groups. While each experimental group started with 10 mice, at the time ascites was drained some mice had died, and other treated mice did not have ascites yet. Furthermore, some mice only yielded enough cells to run one panel for flow cytometry, which led to decreases in mouse numbers and differences between groups in some figures.

Mouse Experiments with ex Vivo Epigenetic Treatment of Cancer Cells.

Single-agent therapy. Cells [2.5×10^5 (A10) or 5×10^5 (HDACi3, HDACi10, and A3)] were injected i.p. into 8- to 10- (A10) or 6- to 8- (HDACi3, HDACi10, and A3) wk-old female C57BL/6 mice. Immune cells were isolated from the ascites fluid via a Percoll gradient and stained for FACS.

Combination therapy. Cells (2.5×10^5) treated with A17, HDACi17, or A+HDACi17 schedules were injected i.p. into 8- to 10-wk-old female C57BL/6 mice. All cells from the ascites fluid were filtered and stained for FACS.

Mouse Experiments with in Vivo Treatment. ID8-VEGF-Defensin cells (2.5×10^5) were injected i.p. into 8- to 10-wk-old female C57BL/6NHSd (C57BL/6) mice or NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice 3 d after injection. AZA (0.5 mg/kg) or saline was given i.p. for 5 d a week. The following week, 2 mg/kg givinostat or entinostat or 1% DMSO in saline was injected i.p. for 5 d. For the rest of

the experiment, the treatment alternated AZA/HDACi every other week. α -PD-1 (200 μ g per mouse) was given on days 17, 20, 24, and 27 after injection in the C57BL/6 mouse experiment. α -PD-1 (1 mg/mL in saline) has been described previously (58, 59). Blocking of IFNAR1 was achieved with the anti-mouse IFNAR1 antibody (clone MAR1-5A3), injected every 3 d (0.5 mg per mouse) (60). Anti-IFNAR1 and the mouse IgG isotype control were purchased from Leinco Technologies and diluted in PBS.

Flow Cytometry. Ascites was drained or spleens were collected from 5 to 10 mice per group and incubated in ACK buffer (Thermo Fisher Scientific) to lyse red blood cells for 10 min and then washed. Ascites from each mouse was individually lysed and prepared for flow cytometry. The mononuclear cells collected were cultured for 4 h in RPMI with 5% FBS and in the presence of Cell Stimulation Mixture (plus protein transport inhibitors; eBioscience). Cells were then washed and stained for cell-surface markers including Live/Dead (eBioscience), CD45 (BD Biosciences), CD3 (BD Biosciences), CD4 (BD Biosciences), CD8 (BD Biosciences), PD-1 (eBioscience), NK1.1 (BD Biosciences), F4/80 (BioLegend), MHC II (isotype control 400627; BioLegend), GR1 (isotype control 400635; BioLegend), and CD11b (BioLegend). After incubation, the cells were permeabilized (FoxP3 staining buffers; eBioscience). Intracellular staining was performed for FoxP3 (isotype control 12-4321; eBioscience) and IFN- γ (isotype control 554686; BD Biosciences). Flow cytometry acquisition was performed on an LSR II cytometer (BD Biosciences), and data were analyzed using FlowJo software version 10.2.

Chemokine and Cytokine Array. Cultured cells were treated with an A10 treatment schedule, and media were collected at day 10. Ascites from mice treated with AZA as described above was collected at week 4 after injection of cells. Cells were removed from the ascites and the supernatant

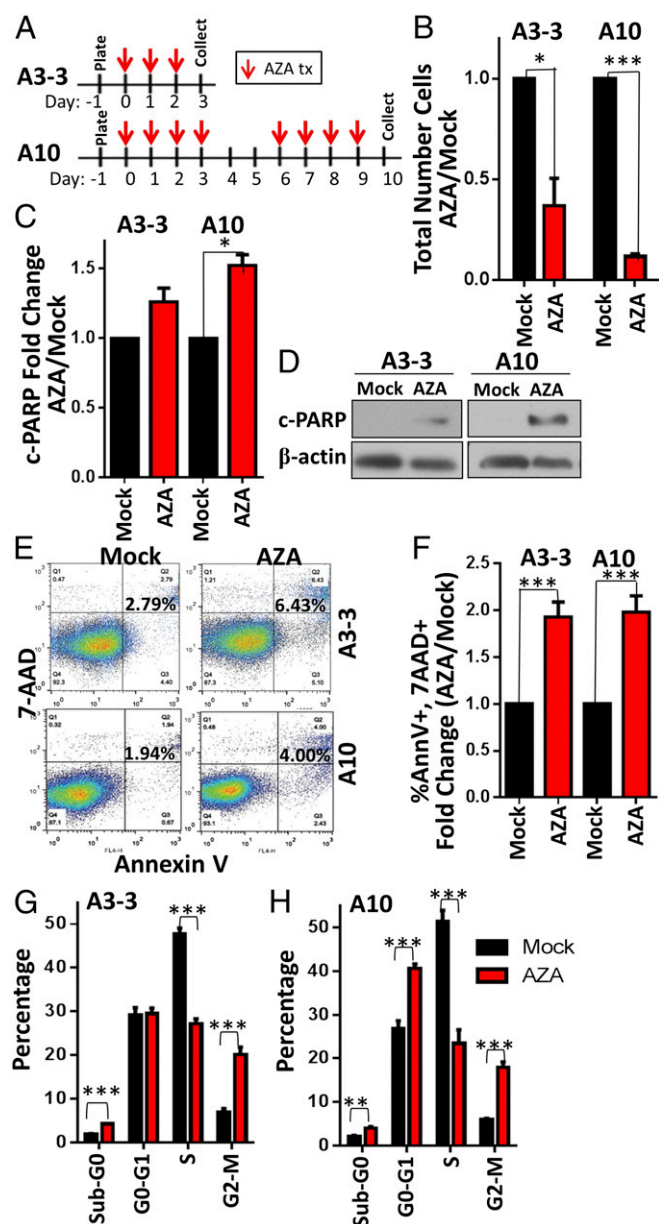


Fig. 7. Ex vivo treatment of ID8-VEGF-Defensin cells with low-dose AZA decreases viable cell number, increases apoptosis, and disrupts the cell cycle. (A) Three or 10 d in vitro treatment with 500 nM AZA. (B) Total number of cells relative to mock; $n = 3$. (C) Quantification of c-PARP levels in AZA-treated cells relative to mock; $n = 3$. (D) A representative Western blot of c-PARP levels. (E and F) Percentage of annexin V⁺ and 7-AAD⁺ apoptotic cells. Representative flow cytometry data are shown (E) along with quantification (F); $n = 3$. (G and H) Cell-cycle analysis, determined by BrdU incorporation and 7-AAD staining of DNA content; $n = 3$. Mean $\pm$ SEM is shown, and significances were determined by Mann-Whitney t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

was collected. Media and ascites samples were analyzed with the Proteome Profile Array, Mouse Cytokine Panel A (R&D Systems) according to the manufacturer's instructions.

Cell-Cycle and Apoptotic Analysis. Cells were treated with an A10 treatment schedule and collected on days 3 and 10. For cell-cycle analysis, BrdU (10 μ M; Sigma) was incubated with cells for 2 h. Cells were fixed, treated with DNase (300 μ g/mL), and stained with anti-BrdU (BioLegend) and 7-AAD (Life Technologies). For apoptosis analysis, cells were stained for FACS and measured as apoptotic based on positive annexin V (eBioscience) and 7-AAD (Life Tech-

nologies) staining. Flow cytometry was performed on a FACSCalibur cytometer (BD Biosciences), and data were analyzed with FlowJo v10 software.

Western Blots. Protein extracts were quantified and immunoblotted using the 4 to 20% Mini-PROTEAN TGX gel system (Bio-Rad) and PVDF membranes (Millipore). β -Actin or GAPDH was used as a loading control. Antibodies used were as follows: polyclonal rabbit anti-mouse cleaved PARP (1:1,000; Cell Signaling), polyclonal rabbit anti-DNMT1 (1:1,000; Sigma), mouse anti- β -actin (1:10,000; Sigma), and polyclonal rabbit anti-GAPDH (1:10,000; Trevigen). Band intensities were quantified using Adobe Photoshop Elements 6.0.

RNA Extraction and Sequencing Library Generation for Immune Cells. CD8⁺ and CD4⁺ T cells and CD11b⁺ myeloid cells were sorted using a FACSaria II from ascites derived from mock- and AZA-treated mice. Approximately 10,000 cells were collected for each sorted population, based on viability and size and lineage exclusion. Cells were pelleted at 300 $\times g$ for 10 min. The supernatant was carefully removed and 100 μ L of Arcturus PicoPure extraction buffer (Thermo Fisher Scientific) was added. Total RNA was extracted using the Arcturus PicoPure RNA Isolation Kit according to the manufacturer's protocol. Low-input RNA-sequencing libraries were generated from 200 pg of total RNA using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech). All samples were subjected to 13 PCR amplification cycles to minimize PCR biases. Amplified cDNA libraries were later fragmented through sonication to obtain 200- to 500-bp fragments. Standard Illumina sequencing libraries were prepared using the ThruPLEX DNA-Seq Kit (Rubicon Genomics) according to the manufacturer's protocol. Sample barcoded libraries were sequenced on Illumina's NextSeq 500 instrumentation using the NextSeq 300 Cycle Kit, High Output, V1 reagents (Illumina), and data analysis workflow bcl2fastq v2.17.1.14 to obtain 150-bp paired-end reads.

Paired-end RNA-sequencing reads were trimmed to remove Illumina's adapter sequences. Sequencing reads were further processed to remove poor-quality reads and/or reads mapping to mouse rRNA and tRNA sequences using the Array Studio package (www.omicsoft.com/array-studio). The following criteria were used to remove poor-quality reads: trim reads with base quality score (Sanger quality score) < 10 ; filter out reads if trim length is < 25 bp; filter out reads if maximal base quality score is < 15 ; filter out reads if average quality score is < 10 ; filter out reads if polyAGCT rate is $\geq 80\%$; and filter out the pair if either read fails the filtering criteria. Sequencing reads were aligned to the mouse reference genome (Build38) using OSA version 4 (61). To obtain transcript count data, RSEM package (62) and National Center for Biotechnology Information mouse RefSeq gene model (release July 2015) annotations were used. Transcripts with zero counts in more than two-thirds of the samples were discarded from downstream analysis to reduce noise in the expression data. Filtered count data were later normalized using quantile normalization, and differentially expressed transcripts were identified using Limma Voom (63). A P -value cutoff of 0.05 was used to classify transcripts as differentially expressed in the treatment condition. Upstream regulator and pathway analyses of differentially expressed transcripts were performed using Qiagen's Ingenuity Pathway Analysis (www.qiagen.com/ingenuity).

Statistical Analysis. Data were graphed in GraphPad Prism 5.0, and significance was determined by a Mann-Whitney t test or by multiple pairwise comparisons using the one-way ANOVA test with Bonferroni correction. Significances in survival data were determined by Mantel-Cox (log-rank) test. Differences were deemed significant with a P value of less than 0.05. Outliers were removed from ascites volume datasets and ascites immune cell datasets using Peirce's criterion (64). Significances are shown as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

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AUTHOR'S VIEW

Unraveling the molecular pathways of DNA-methylation inhibitors: human endogenous retroviruses induce the innate immune response in tumors

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ABSTRACT

Loss of DNA methylation can activate endogenous retroviral expression and dsRNA in cancer cells. This leads to induction of toll-like receptor signaling stimulating an antiviral interferon response. Recent findings provide a therapeutic rationale for combining DNA methylation inhibitors with blockage of immune checkpoint proteins to fight cancer.

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In the recent years, exciting therapeutic approaches that activate the host immune system have proven effective toward eliminating diverse solid tumors. These include humanized antibodies targeting various immune checkpoint regulators like CTLA-4, PD-1 and PD-L1.¹ Current data has also shown that epigenetic therapies, including the DNA-methylation inhibitors 5-Azacytidine (Aza) and 5-Aza-2'-deoxycytidine (5-Aza-dC) (Decitabine), boost immune signaling of tumor cells.^{2,3} Therefore, cancer treatments combining inhibition of DNA methylation with blockage of immune checkpoint proteins are a promising new therapeutic direction. Two recent publications shed light on the basic molecular and cellular efficacy regarding the above therapies,^{3,4} where one common link implicates the innate immune system.

Until the discovery of the toll-like receptors (TLR) in *Drosophila melanogaster* and subsequent functional translation to humans the innate immune system was thought to be less sophisticated than the adaptive immune system.⁵ The function of TLRs is to sense “danger” signals, which include nucleic acids or membrane components from exogenous viruses or bacteria. All 10 human TLRs described to date are subdivided by cellular localization (plasma membrane or endosomes) and activation (external membrane lipids or proteins and external nucleic acids).⁶ Examples of receptors specific for sensing foreign nucleic acids include TLR3 for dsRNA, TLR7/8 for ssRNA and TLR9 for RNA:DNA hybrids (Fig. 1). TLR-nucleic acid binding leads to interferon α/β signaling, downstream activation of interferon stimulated genes (ISGs) and anti-viral and apoptotic functions. Administration of synthetic dsRNA (polyI: C) in humans lead to activation of innate immune pathway members like TLR3, RIG-I, MDA5 and gene expression of ISGs.⁷ Interestingly, it has been shown that mouse Tlr3, Tlr7

and Tlr9 are essential for control of endogenous retroviruses (ERV).⁸

ERVs are derived from past exogenous retroviral infections and constitute approximately 10% and 8% of mouse and human genomes, respectively. In this regard, Tlr3, Tlr7 and Tlr9 deficient mice show no induction of innate immune genes and the type I interferon response and these gene deficiencies result in high expression of ERV RNA leading to viremia and tumorigenesis.⁸ Like exogenous viruses, activation of TLRs via a variety of endogenous viral nucleic acids represents the initial step for downstream induction of NF- κ B and/or IRF signaling pathways and stimulation of the interferon type I response (Fig. 1). Besides RIG-I and MDA5 the interferon promoter-stimulating factor 1 (IPS-1) (MAVS) is also essential for TLR signaling.⁹ MAVS is the sole adapter for both RIG-I and MDA5 signaling and mediates effective responses against viral RNA (Fig. 1). The Laboratory of Genetics and Physiology 2 (LGP2) gene binds dsRNA, facilitating MDA5 to induce innate immunity via interferon transcription (Fig. 1).¹⁰ Following interferon protein secretion and receptor binding ISGs become expressed and lead to immune cell recruitment, cytokine production and cell death to promote viral clearance.

Recently, we and others showed that Aza or 5-Aza-dC treatment of epithelial ovarian and colorectal cancer cell lines led to an induction of ERV dsRNA, which triggered innate type I interferon signaling and apoptosis as if in response to a viral infection.^{3,4} Critical pathway members in these responses include TLR3, MAVS, MDA5, IRF7, interferon β (IFN- β) and its receptor. Aza or 5-Aza-dC-mediated demethylation and subsequent activation of ERVs led to a cellular viral “infection” alarm, which originated within the tumor cell. Many tumors evolve the ability to mediate a strong suppression of the immune system within the tumor microenvironment. Aza treatment remarkably sensitized melanoma tumor cells

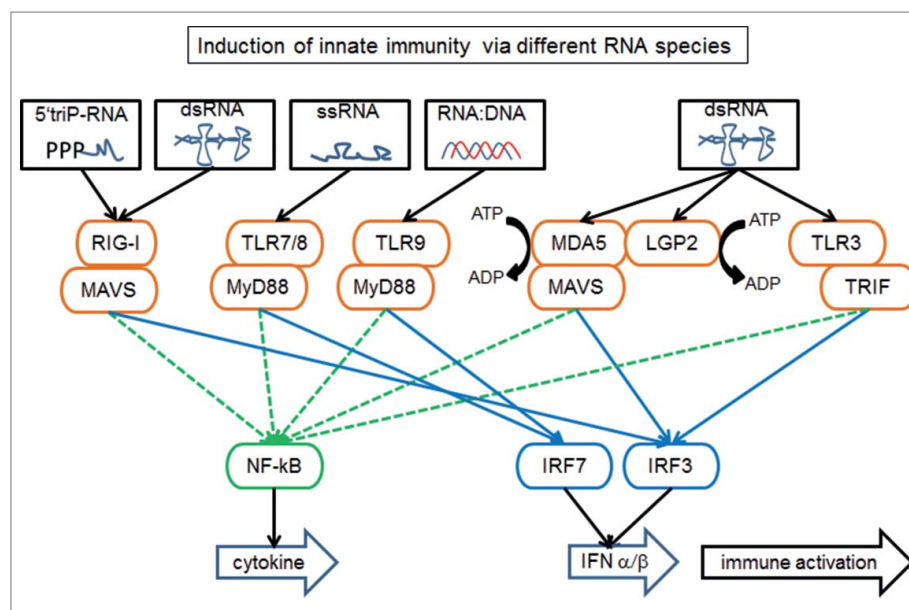


Figure 1. Different exogenous and endogenous RNA species induce the innate immune system via TLR, RIG-I and MDA5 resulting in cytokine and interferon signaling. The main ERV RNA species inducing the immune response include dsRNA and ssRNA. RIG-I, retinoic acid inducible gene-1 (or RARRES3); MAVS, mitochondrial antiviral signaling protein (or IPS1); TLR, toll-like receptor; MyD88, myeloid differentiation primary response 88; MDA5, melanoma differentiation-associated 5 (or IFIH1); LGP2, laboratory of genetics and physiology 2 (or DHX58); TRIF, TIR domain-containing adaptor-inducing interferon- β (or TICAM1); NF-kB, nuclear factor kappa-light-chain-enhancer of activated B-cells; IRF, interferon regulatory factor; IFN, interferon.

in a mouse model to anti-CTLA-4 immune checkpoint therapy demonstrating a significantly reduced tumor burden compared to each compound alone.³ Furthermore, we uncovered that a core group of ISGs, defined as a viral defense signature, divided tumor cell lines upregulated by Aza and primary ovarian, breast, melanoma and colon carcinomas into low and high ISG expressing groups. Impressively, ISG expression of ovarian carcinomas positively correlated with low and high ERV expression.³ We also showed that high expression of the viral defense genes in melanoma patients predicted a lasting clinical response to anti-CTLA-4.³ These results support a link of ERV expression with ISG response in primary tumors, which needs to be investigated further. In light of the sophisticated ways in which tumors suppress the immune system via regulation of immune checkpoint proteins, our findings have high translational connotations for considering combinatorial treatments of patients with DNA-methylation inhibitors and other checkpoint inhibitors. These combinatorial treatments could activate the immune response and facilitate tumor clearance so that patients with both low and high ISG and ERV expressing tumors would benefit.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Epigenetic Therapy Ties MYC Depletion to Reversing Immune Evasion and Treating Lung Cancer

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SUMMARY

Combining DNA-demethylating agents (DNA methyltransferase inhibitors [DNMTis]) with histone deacetylase inhibitors (HDACis) holds promise for enhancing cancer immune therapy. Herein, pharmacologic and isoform specificity of HDACis are investigated to guide their addition to a DNMTi, thus devising a new, low-dose, sequential regimen that imparts a robust anti-tumor

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SUPPLEMENTAL INFORMATION

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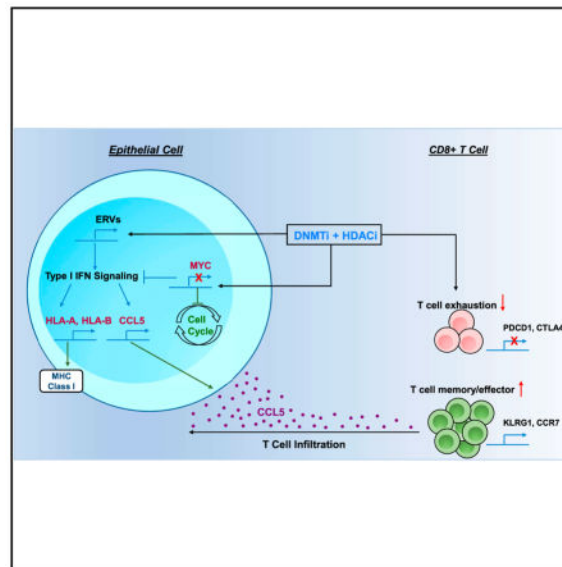
AUTHOR CONTRIBUTIONS

M.J.T., M.V., and S.B.B. designed experiments, performed data analyses, and wrote the manuscript. M.J.T., M.V., and K.B.C. performed experiments. P.L.S. and R.S. analyzed ERV data. C.D.S., A.W., and J.H. performed immunohistochemistry (IHC). V.A., N.N., and V.E.V. analyzed patient data. M.B. and P.T.T. performed and analyzed computed tomography (CT) scans. C.A.Z. assisted with xenograft studies. M.D.H. and M.S. helped in manuscript review. R.-W.C.Y. provided cell lines used in study.

effect for non-small-cell lung cancer (NSCLC). Using *in-vitro*-treated NSCLC cell lines, we elucidate an interferon α/β -based transcriptional program with accompanying upregulation of antigen presentation machinery, mediated in part through double-stranded RNA (dsRNA) induction. This is accompanied by suppression of MYC signaling and an increase in the T cell chemoattractant CCL5. Use of this combination treatment schema in mouse models of NSCLC reverses tumor immune evasion and modulates T cell exhaustion state towards memory and effector T cell phenotypes. Key correlative science metrics emerge for an upcoming clinical trial, testing enhancement of immune checkpoint therapy for NSCLC.

In Brief

Myc depletion through combined epigenetic therapy reverses immune evasion and enables effective treatment of lung cancer.



INTRODUCTION

The development of effective therapeutic approaches for patients with lung cancer, the world's leading cause of cancer-related death, is a critical medical need. The advent of immune checkpoint therapy has been a tremendous step forward (Borghaei et al., 2015; Garon et al., 2015; Reck et al., 2016), but only a minority of unselected patients benefit. Thus, identifying rational combinations to augment anti-tumor immunity and increase the response to immune checkpoint therapy represents a major challenge for the field. Durable responses were observed in patients with non-small-cell lung cancer (NSCLC) who were initially treated with a DNA methyltransferase inhibitor (DNMTi) combined with intermittent histone deacetylase inhibitor (HDACi), followed by immune checkpoint therapy (Juergens et al., 2011). Combination epigenetic therapy regimens most commonly utilize DNMTis in combination with HDACis on the premise that the latter can enhance re-expression of abnormally silenced genes mediated by abnormal gene promoter DNA methylation (Cameron et al., 1999; Jones et al., 2016). However, little attention has been

paid to examining the specific pharmacologic features of HDACi, optimal dosing strategy, and underlying mechanisms mediating maximal synergy with DNMTis. The zinc-chelating HDACis comprise 3 major classes: benzamide, hydroxamic acid, and cyclic tetrapeptides (Bradner et al., 2010; Falkenberg and Johnstone, 2014; Rasheed et al., 2007; Zahnow et al., 2016). Benzamide class HDACis target class I HDAC isoforms, while hydroxamic acid class HDACis target both class I (HDAC1, 2, and 3) and class IIb (HDAC6), although differences in isoform targeting can be noted (Bradner et al., 2010).

We have previously shown that low doses of DNMTi impart durable changes in both genome-wide DNA methylation and transcriptome while avoiding acute cytotoxicity (Tsai et al., 2012). Additionally, we and others have discovered that DNMTis can induce an epithelial immune signature for NSCLC and other cancers (Li et al., 2014; Moreno-Bost et al., 2011; Oi et al., 2009; Weiser et al., 2001; Wrangle et al., 2013). A key mediator of this signaling is a cytoplasmic, double-stranded RNA (dsRNA)-mediated interferon response induced through the activation of endogenous retrovirus (ERV) transcription (Chiappinelli et al., 2015; Roulois et al., 2015). How the distinct classes of HDACis contribute to enhancing immune therapy specifically is unknown.

Herein, testing individual HDACis based on both their isoform-specific inhibitory constant (K_i) and pharmacokinetic parameters, we demonstrate the best pairing of these agents with Aza for achieving anti-tumor effects, which include immune responses. The combination of HDACi + Aza achieves robust anti-tumor responses mediated by reducing MYC-driven cell proliferation with enhancement of immune signaling. We hypothesize dual epigenetic therapy will synergize with immune checkpoint blockade in NSCLC patients to drive a potent anti-tumor response.

RESULTS

Combination Aza + ITF-2357 Induces Profound Drug Synergy when Applied to Human NSCLC

We tested HDACis by pairing these agents with low-dose (500 nM) Aza. Clinically relevant HDACis (ITF-2357, MGCD-0103, and MS-275) were selected and paired with Aza based on their known pharmacokinetic parameters (serum maximum concentration [C_{max}] and half-life) and enzymatic K_i against key HDAC isoforms (Figure S1A) (Bradner et al., 2010; Falkenberg and Johnstone, 2014; Furlan et al., 2011; Gore et al., 2008; Rasheed et al., 2007; Ryan et al., 2005; Zahnow et al., 2016). We initially examined the effect of sequential Aza + HDACi on cell proliferation across a panel of 16 cell lines representing the diverse oncogenotypes of NSCLC (Figures 1A, S1B, and S1C). These data revealed that the antiproliferative effect of this treatment regimen correlated with the subtype and mutational status of the NSCLC cell line queried. The hydroxamic acid class HDACi (ITF-2357) in combination with Aza demonstrated the most potent antiproliferative phenotype. Among our four most responsive cell lines, three were RAS mutant subtype. Therefore, we selected these cell lines for further studies of therapeutic scheduling and HDAC isoform targeting delineation. Using a comparative analysis, wherein Aza and ITF-2357 were both sequentially and concurrently employed (Figure S1C), only the former schedule induced both synergy and robust potentiation of HDACi by Aza (Figures 1B, 1C, S1D, and S1E). We

thus used this schema of sequential treatment in our further studies. To delineate the enhanced antiproliferative effect of ITF-2357 over the others, we next tested the function of the individual HDAC isoforms in the Aza priming response to HDACi. We found, through the application of HDAC isoform-specific inhibitors, an Aza induced priming to both class I and IIb targeting HDACis (Figures 1D and 1E). These data suggest the observed Aza + ITF-2357 antiproliferative phenotype to be the result of broader targeting of HDACs, including both nuclear (HDAC1, 2, and 3) and non-nuclear isoforms (HDAC6).

As a further characterization of the antiproliferative effects of Aza in combination with HDACi, we deployed equal molar concentrations of MS-275 and ITF-2357 and assessed cell proliferation specifically. In agreement with the previous data, we found 100 nM ITF-2357, a concentration roughly one-third C_{max} (Furlan et al., 2011), demonstrated a more robust inhibition of proliferation than 100 nM MS-275 (Figure S1F). We next sought to define the contribution of individual HDAC isoforms to the antiproliferative effect noted from 100 nM ITF-2357. To accomplish this end, we first determined equivalent doses based on isoform pharmacodynamic targets, these being histone acetylation for HDAC1 and 2 and tubulin acetylation for HDAC6 (Asthana et al., 2013; Fournel et al., 2008; Khan et al., 2008). The resulting equivalent doses were 200 nM MGCD0103 and 1,000 nM Tubastatin A to mimic 100 nM ITF-2357 (Figure S1G). Selection of 2,000 nM RGFP996 for HDAC3 inhibitor was based on extrapolation of K_i , due to a lack of known pharmacodynamic markers for these isoforms (Malvaez et al., 2013). These normalized doses of HDAC isoform-specific inhibitors identified HDAC1, 2, and 3 as key arbiters of the Aza + ITF-2357 antiproliferative actions (Figure S1H). Additionally, to elucidate the specificity of Aza priming to HDACi, we deployed small hairpin RNA (shRNA)-mediated DNMT1 depletion, the major pharmacodynamic target of Aza (Cai et al., 2014). We observed potent augmentation of the HDACi antiproliferative effect when utilized in combination with DNMT1 depletion (Figures S1I and S1J).

Finally, we tested the efficacy of sequential Aza + HDACi *in vivo* using immune-incompetent mice. We observed a significant reduction of tumor burden across 3 xenograft models, two for established NSCLC cell lines and one using a *KRAS*, *KDR*, *TP53* mutant primary patient-derived xenograft (PDX) model when utilizing Aza + ITF-2357 (Figures 1F–1H). The combination of Aza + MS-275 did not have significant efficacy in the H460 xenograft model, the most sensitive cell line tested, at the doses used in our study (Figure 1F). Aza + MGCD0103 achieved significant efficacy in H460 xenografts (Figure S1K) and modest efficacy in the patient-derived xenograft (Figure S1L).

Effects of the Drug Combination Paradigm on the Transcriptome in NSCLC Lines

Assessment of drug combination-induced effects on gene expression yields important insight into signaling pathway alterations that may relate to eventual clinical efficacy. The combination epigenetic treatment induced an increase in differential gene expression relative to HDACi mono-treatment conditions, with a clear advantage noted from the application of Aza + ITF-2357 (Figures 2A, S2A, and S2B). Clustering of the top 500 differentially expressed genes showed a divergence between HDACi mono-treatment and Aza-containing regimens (Figure 2B). Database for Annotation, Visualization and Integrated Discovery

(DAVID) pathway analysis revealed the most differentially expressed pathways to be immune, MYC, and metabolism related (Figure 2C). Gene set enrichment analysis (GSEA) identified three significantly induced pathways by combination epigenetic treatment, two of which are related to immune signaling (Figures 2D and 2E). The interferon alpha/beta (IFN α/β)-signaling pathway was the most prominent of these altered pathways (Figure S2C). Comparative analysis of combination epigenetic treatment-downregulated pathways by GSEA revealed a conserved cell cycle repression signature across the drug combinations tested, with a noted advantage for Aza + ITF-2357 (Figures 2F, 2G, and S2D). The above transcriptional downregulation of cell cycle-related pathways elicited by combinatorial epigenetic treatment matched with the observed proliferative arrest induced by combination epigenetic treatment (Figures S1F).

The Potential of Combinatorial Epigenetic Treatment to Stimulate Specific Immune-Related Genes

A primary goal for combined epigenetic therapy is to increase the efficacy of immune checkpoint and other immunotherapies. We found the combination of Aza + ITF-2357 to be the most effective relative to other conditions tested in terms of induction of IFN α/β pathway-related genes, including those associated with antigen presentation (Figures 3A, 3B, and S3A). To demonstrate whether this efficacy of immune gene induction by Aza + ITF-2357 was potency or isoform targeting based, we used isoform-specific HDACi at concentrations mimicking 100 nM ITF-2357. We observed the inhibition of HDAC1, 2 (MGCD0103), and HDAC6 (Tubastatin A) in combination with Aza, induced IFN α/β pathway-related genes, including antigen presentation, while HDAC3- (RGFP996) specific inhibitors demonstrated no significant induction of these genes (Figures 3C–3F, S3B, and S3C).

We also compared the efficacy of ITF-2357 against another hydroxamic acid derivative HDACi (SAHA). Using doses at equal ratio of C_{max} for the respective inhibitors, we observed a superior augmentation of interferon genes by ITF-2357 when deployed in combination with Aza (Figures S3D and S3E). The above data demonstrates an induction of interferon-stimulated genes by Aza, which are further augmented by HDACi's targeting isoforms 1, 2, and 6.

Cancer/testis antigens are epigenetic-treatment responsive and have an established role in facilitating the recognition of tumors through immune surveillance (Moreno-Bost et al., 2011; Oi et al., 2009; Weiser et al., 2001). We found, in agreement with previous studies, the expression of cancer/testis antigens are Aza inducible, but a significant additional benefit is noted with the combination of Aza + ITF-2357 (Figures S4A–S4D).

The augmentation of the IFN α/β pathway by Aza partially relies on increasing dsRNA species, including ERV transcripts (Chiappinelli et al., 2015; Roulois et al., 2015; Stengel et al., 2010; Strissel et al., 2012). Indeed, combination HDACi with Aza transcriptionally induced multiple ERVs across all cell lines tested (Figures 3G, 3H, S4E, and S4F). We found ERV9-1 to be the most differentially expressed ERV induced by Aza + ITF-2357. Using isoform-specific inhibitors in combination with Aza, we determined its transcription is potentiated most robustly by inhibition of HDAC6 (a cytoplasmic HDAC) (Figures 3I and

3J). Further, we demonstrated using DNMT1-targeting shRNA that ERV9-1 induction specifically resulted from DNMT1 depletion in our combination treatment (Figure S4G). The inducibility of interferon signaling from ERV transcription was linked in our NSCLC cells, as single ERV overexpression induced transcription of OASL, an interferon-responsive gene (Figure S4H).

In summary, HDACis enhance Aza-induced interferon-responsive gene transcription, particularly with the inhibition of HDAC1, 2, and 6. Class I antigen presentation is augmented through the inhibition of HDAC1, 2, 3, and 6 in combination with Aza. The induction of cancer/testis antigen transcription requires potent HDACis, such as ITF-2357, to mediate significant upregulation over Aza alone.

Perturbation of MYC by Aza Drives Sensitization to HDACi

As described earlier, DAVID analysis of the top 500 differentially expressed genes revealed that three of the top eight pathways are MYC related (Figure 2C), suggesting the perturbation of MYC as a key target of our epigenetic therapy. Critically, in the tested NSCLC cell lines, Aza produced a significant transcriptional downregulation of MYC, with an at least 1.4-fold reduction observed across all cell lines assayed (Figure 4A). These transcription data correlated with downregulation of MYC protein in A549 and H460 cells (Figure 4A). Additional assessment of MYC protein downregulation by Aza and priming to sequential HDACi across a panel of NSCLC cell lines of varying oncogenotypes revealed a significant correlation between the degree of downregulation and HDACi sensitization by Aza (Figures 4A and S5A). Combinatorial Aza + HDACi acted to further deplete MYC over Aza treatment alone (Figure S5B). The application of Aza and Aza + HDACi treatment induced potent repression of the top 200 MYC targets (Figure 4B). Importantly, knockdown of MYC protein phenocopied the Aza sensitization of NSCLC to HDACi-induced cytostasis (Figures 4C and S5C). The stable over-expression of MYC partially rescued the Aza-induced sensitization to HDACi-induced cell depletion (Figures 4D and S5D). These data indicated an Aza-induced MYC depletion as a potent sensitizer to HDACi-induced proliferative arrest, and, accordingly, they implicate depletion of MYC signaling as a driver of these events.

High MYC tumors have been reported to display resistance to IFN γ signaling and the action of cytotoxic T lymphocytes (Casey et al., 2016; Schlee et al., 2007a, 2007b). In this regard, exogenous MYC overexpression resulted in antagonism of interferon-stimulated gene induction and antigen presentation induced by epigenetic treatment (Figures 4E and 4F). Thus, the MYC depletion signature imparted by Aza sensitizes to the actions of HDACi-induced cytostasis and augments NSCLC interferon signaling.

Combination Epigenetic Treatment Exerts a Robust Anti-tumor Effect and Decreases myc and myc Pathway Signaling in Mouse Models of NSCLC

The translational potential for utilizing our chronic schema of alternating, low-dose 1-week Aza followed by 1-week ITF-2357 was apparent from its robust efficacy when tested in two mouse models of NSCLC. The first, the LSL-Kras^{G12D} mouse model of NSCLC, represented an interception model, as we began treatment when lung adenomas emerged 16

weeks following the lung-specific activation of the mutation (Jackson et al., 2001). Mice were treated with the combination drug treatment for 3 months using the schema as shown in Figure S6A. The therapy was well tolerated, and, while mock-treated mice developed large adenocarcinoma lesions in the lungs, combination epigenetic treatment prevented the occurrence of these macroscopic lesions and caused over a 60% reduction of tumor area in the treated mice (Figures 5A, 5B, and S6B). Consistent with all the studies in the human NSCLC cells, proliferation as assessed by Ki67 positivity was markedly reduced in any remaining histologic, adenomatous lesions (Figure 5C). To evaluate the implications of this treatment regimen in combination with immune checkpoint blockade, we added treatment with anti-PD1 antibody to the described regimen (Figure S6C). However, the combination epigenetic therapy alone proved as above to be so efficacious that it fully dominated the therapeutic effect (Figure S6D).

The translational potential for our combination epigenetic drug efficacy for established, aggressive NSCLC was apparent utilizing a second mouse model of NSCLC, Lewis Lung Carcinoma (LLC). This murine model has demonstrated high correlation between response in the animal and response in the clinic for 2 therapeutic regimens (Kellar et al., 2015). Treatment with our alternating schedule of Aza + ITF-2357 and of Aza + MGCD0103 for 1 month not only reduced growth of established and rapidly growing primary tumor explants of the LLC cells but also dramatically reduced metastatic occurrence and burden (Figures 5D, 5E, S6E, and S6F).

Importantly, as outlined below, the above efficacy of our combination therapy was accompanied by profound immune effects. First, in the LSL-Kras^{G12D} mice, the combination epigenetic treatment profoundly altered the tumor transcriptome with 5,167 significantly upregulated and 4,540 downregulated genes (Figure 5F). This corresponded to 18 gene sets upregulated and 52 gene sets downregulated by GSEA, with the former predominantly associated with immune parameters and the latter with cell cycle pathways (Figure 5G). Similar to the results for human NSCLC lines, 12 of the 18 gene sets upregulated involved strong induction of cytokine- and interferon-related gene sets (Figure 5H) as well as for 24 genes involved with inflammatory responses (Figure 5I). Concomitantly, as for the human NSCLC lines, most of the downregulated cell cycle pathways involved myc pathway signaling (Figure 5J), with decreases in 30 myc pathway-related genes (Figure 5K). Likewise, we confirmed the downregulation of MYC protein in the LLC cells (Figure S6G). These data in total demonstrate a potent reduction in tumor proliferation, growth, and burden is induced by the combination epigenetic treatment, which is mediated by repression of myc and myc-related signaling.

Combination Epigenetic Treatment Alters the Tumor Immune Microenvironment

In the above mouse models, deployment of epigenetic treatment reversed tumor immune evasion status, and a significant perturbation of tumor-associated immune cell subsets appeared to underlie the efficacy of the drug combinations. First, there was a robust alteration in the distribution of F4/80⁺ macrophages, which have been shown to have an established role in the progression of the LSL-Kras^{G12D} mouse model (Okayama et al., 2013). These cells have been shown to basally infiltrate tumors induced in this model, and

our combination treatment resulted in a significant decrease in their numbers (Figure 6A). Transcriptional profiling of tumor-associated macrophages (TAMs) isolated by fluorescence-activated cell sorting (FACS) of F4/80^{hi}, CD11b⁺ macrophages from the lungs, following the 3 months of combination epigenetic treatment, revealed differential expression of 1,104 genes (Figure 6B), including significant downregulation of angiogenesis and hypoxia-responsive genes and gene sets (Figure 6C). These observed perturbations appeared to be a direct effect of the treatment and not a function of tumor stage, as treatment of normal bone marrow-derived macrophages (BMDMs) *in vitro* induced angiogenic signature downregulation as part of the 2,295 differentially expressed genes (Figures 6D and 6E).

The above altered pathways in TAMs may have implications for mediating CD8⁺ T cell intra-tumor infiltration. The inhibition of angiogenesis has proved efficacious in the MMTV mouse model through facilitating an increase in CD8⁺ tumor-infiltrating lymphocytes (Huang et al., 2012). This anti-angiogenic-based strategy for the induced infiltration of T lymphocytes depends on the concept of vascular normalization (Huang et al., 2013). Therefore, the repression of angiogenic programs in macrophages may act locally in the tumor to create a more immune infiltrate-permissive environment in the LSL-Kras^{G12D}.

Critically, there were increased CD8⁺ tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment of treated mice (Figure 6F). This enhanced TIL accumulation was accompanied by an IFN γ -responsive signature in the transcriptional profile of LSL-Kras^{G12D} whole-tumor lysates (Figure 6G). As a validation of this signature, we profiled TILs by FACS in the LLC model, and, although we did not observe changes in total CD8 numbers in this short-term treatment model, there was a 2.25-fold increase in CD8⁺ IFN γ positivity, as the result of exposure to epigenetic treatment (Figures 6H, S6H, and S6I).

Transcriptional profiling of TILs from control and treated tumors in the LSL-Kras^{G12D} mice suggested a key role of epigenetic therapy on these cells. TILs are known to acquire an exhaustion state in response to continuous stimuli and interaction with inhibitory ligands (Wherry and Kurachi, 2015; Zehn and Wherry, 2015). Therefore, the reversion or prevention of this exhausted state remains a major hurdle for durable T cell-mediated anti-tumor responses. A recent study by Ghoneim et al. (2017) established the utility of demethylating agents to reverse CD8⁺ T cell exhaustion, thereby allowing this population to be actionable by PD-1 blockade. Our epigenetic treatment regimen induced alteration of the TIL transcriptome. Comparative analysis of our 3,698 differentially expressed TIL genes revealed downregulation of exhaustion-associated genes and induction of activation- and memory-associated genes, as defined by Wherry et al. (2007). Our gene signature reflected an admixture of increased memory and/or effector T cells (Figures 6I–6K). The expansion of effector/memory T cell population is associated with durable responses to immune checkpoint blockade (Ribas et al., 2016). Thus, through the deployment of epigenetic treatment in two animal models, we confirmed epigenetic treatment can both increase activated CD8s in an inflamed tumor and induce the attraction of CD8s to an immune desert tumor.

Combination Epigenetic Treatment Inhibits Tumor Growth through CD8⁺ T Cells and MYC-Dependent Mechanisms

The above results demonstrated that epigenetic therapy can potentially reduce a key barrier to immune therapy, namely, the failure of tumors to attract functional CD8⁺ T cells. Depleting CD8⁺ T cells in the LLC mouse model attenuated the efficacy of epigenetic treatment on the reduction of primary tumor burden (Figures 7A and S6J). Relative to the Aza + ITF-2357 efficacy established in Figure 5, we also observed an increase in both metastatic frequency and burden as the result of CD8 depletion. In the CD8-depleted environment, we noted a metastatic frequency of 33% in mock- and 20% in Aza + ITF-2357-treated mice (Figure 7B).

Additionally, in response to our combined epigenetic treatment, there was transcriptional augmentation of CCL5, a secreted chemokine involved in lymphocyte attraction (Figures 7C and S6K). Importantly, protein levels of CCL5 also accumulated in bronchoalveolar lavage of mice treated with combination epigenetic therapy (Figure 7D). This protein and its ligand have been identified as the primary factors influencing T cell infiltration of melanoma post-chemotherapy (Hong et al., 2011), thus suggesting that the enhanced levels of CCL5 induced by the epigenetic treatment may contribute to the increased number of CD8⁺ T cells observed in tumors of treated mice.

Several key findings suggest the importance of the above CCL5 results and link this parameter with the combination treatment-mediated downregulation of MYC signaling. First, expression of exogenous MYC in human A549 NSCLC cells acts as a potent repressor of CCL5 transcriptional induction in response to epigenetic treatment (Figure 7E). This links perturbation of MYC by epigenetic treatment to the alteration of immune correlates. Second, the potentially repressive role of MYC on CCL5 is also apparent in The Cancer Genome Atlas (TCGA) project primary NSCLC, lung adenocarcinoma (LUAD) samples, wherein there is an inverse relationship between CCL5 and MYC RNA transcription (Figure 7F).

Finally, our new findings in a small, previously reported cohort of patients with NSCLC treated with immune checkpoint therapy (Anagnostou et al., 2017) provide a suggestion for the importance of the present pre-clinical findings for the role of MYC signaling in response to immune therapy. In these four patients, all but one achieved durable clinical benefit, with partial responses for greater than 6 months as defined by RECIST 1.1. Genome-wide copy-number variation analysis on the tumors of these patients revealed a significant amplification of the *MYC* locus only in the one patient who did not achieve durable benefit (Figures 7G and 7H). Of note, the tumors from all four patients had characteristics predicting favorable response to checkpoint blockade therapy (Anagnostou et al., 2017), including high PD-L1 protein expression and high mutation density (Rizvi et al., 2015; Tumei et al., 2014). These data, which must be validated in larger patient cohorts, suggest a predictive value when considering both the genetic and expression status of MYC for monitoring outcomes and practicing precision medicine for patients with NSCLC and other cancers receiving immune checkpoint therapy.

DISCUSSION

We have derived pre-clinical data supporting a role for epigenetic therapy in decreasing MYC signaling and driving a substantial anti-tumor response associated with a reversion of an immune evasion phenotype. We describe a novel regimen using careful selection of specific HDACis in combination with Aza. We find the inhibition of HDAC1, 2, and 3 isoforms is key for inducing a profound proliferative arrest (Figure S1H). The inhibition of HDAC6 appears to play a more important role in interferon signaling (Figures 3C–3F), possibly involving its known function for regulating Stat-1-dependent nuclear factor κ B (NF- κ B) signaling (Krämer et al., 2006).

We find these HDACis can complement the therapeutic effects of Aza across a diverse panel of NSCLC cell lines, thus suggesting its applicability for the treatment of NSCLC (Figures 1A and S1B). Our observation of a strong correlation between both MYC basal levels and degree of downregulation by Aza suggests the importance of MYC assessment for the delineation of patients most likely to respond to epigenetic treatment (Figures 4A and S5A).

The best indication of the translational potential for our combination epigenetic therapy are the results achieved by treating two mouse models of NSCLC (Figure 5). These models allow for our observations that the epigenetic treatment alters the intra-tumor localization and functional status of key immune cell populations (Figure 6). This occurs concurrently with a decrease in MYC pathway signaling (Figures 5K and S6G). The importance of MYC in driving tumor formation in the LSL-Kras^{G12D} mouse model has been established, wherein the inhibition of MYC and its targets has been shown to eradicate lung tumor lesions (Soucek et al., 2013).

Although the above anti-tumor responses are partly driven by the direct growth inhibitory effects of the therapy as observed in immune-incompetent mice (Figures 1F–1H, S1K, and S1L), we also observed a significant alteration of immune populations mediated by our therapeutic paradigm when treating immune-competent mouse models (Figures 6A–6K, 7A, 7B, S6I, and S6J). The crucial role of the CD8⁺ T cells in driving the anti-tumor response is apparent from the significant attenuation of the anti-tumor response in the immune-competent mice following CD8⁺ T cell depletion (Figures 7A, 7B, and S6J). Our observation of decreased angiogenic potential of TAMs post-treatment has implications in tumor vascularization, which is essential for the growth of large adenocarcinoma lesions (Figures 6B and 6C). Key among these findings is the effect of epigenetic treatment duration on the modulation of T cell fate. A critical observation is that application of epigenetic treatment for limited duration induces the accumulation of activated T effector cells (Figure 6H and S6I), while chronic, long-term treatment induces modulation away from an exhausted T cell phenotype, inducing a profile more indicative of the formation of effector-memory T cells (Figures 6J and 6K). This acquisition of a memory phenotype coupled with intra-tumor accumulation of activated T cells imparts an immunological landscape primed for durable response to immune checkpoint therapy (Ribas et al., 2016).

A final point for high translational implications is our linking epigenetic therapy-induced attraction of CD8⁺ T cells to the tumor microenvironment and the relationship of this event

with decreased MYC signaling. An important parameter altered, which may have implications for the induced infiltration of CD8⁺ T cells into the tumor microenvironment, is the simultaneous upregulation of CCL5 protein secretion into the bronchoalveolar compartment (Figure 7D). The forced overexpression of this secreted protein has been found to induce recruitment of T cells (Lavergne et al., 2004). Notably, others have found higher levels of CCL5 to be an independent prognostic indicator for longer overall survival, with an induction of an active tumor-associated lymphocyte compartment in NSCLC patients (Moran et al., 2002).

In patient tumors, CCL5 augmentation by epigenetic therapy may prove crucial both functionally and as a biomarker in pre- and post-epigenetic treatment. As a biomarker, these data indicate that such findings for basal levels of CCL5 may be especially valuable when combined with the other markers studied, such as the genetic and expression status of MYC. Considering these relationships may enable the prediction of those patients most likely to respond to a combination of epigenetic and immune therapy. Specifically, based on our corollary hypothesis, patients with MYC-high, CCL5-low tumors could be those who most benefit from this combination.

In summary, we believe that combination epigenetic therapy-induced depletion of MYC can remove a barrier to interferon responsiveness, potentiate T cell attraction, and cause direct antiproliferative actions. This coupled with the reversion of T cell exhaustion and acquisition of a T cell memory fate suggests the potential for use of epigenetic therapy in combination with immune checkpoint blockade. Our early findings for *MYC* amplification in the one of four patients who had a much less robust initial response to immune checkpoint response than the others who achieved durable clinical benefit are in keeping with the need to further pursue these findings in the clinic (Figures 7G and 7H). In this regard, the work in our present study and the hypotheses derived are being pursued in the aforementioned clinical trial for patients with NSCLC, testing our combined epigenetic therapy with the DNA-demethylating agent Guadecitabine plus the HDACi Mocetinostat plus anti-PD1 (NCT03220477).

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-β-Actin	Sigma-Aldrich	Cat#A5441; RRID: AB_476744
Rat monoclonal anti-CD8a	BioXCell	Cat#BE0061; RRID: AB_1125541
Rat monoclonal anti-CD16/CD32	BD Biosciences	Cat#553141; RRID: AB_394656
BV510 Rat monoclonal anti-CD45	BD Biosciences	Cat#563891
Rat monoclonal anti-CD3	BD Biosciences	Cat#560527; RRID: AB_1727463
FITC Rat monoclonal anti-CD8a	Biolegend	Cat#100706; RRID: AB_312745
Brilliant Violet Rat monoclonal anti-F4/80	Biolegend	Cat#123137; RRID: AB_2563102

REAGENT or RESOURCE	SOURCE	IDENTIFIER
APC Rat monoclonal anti-CD11b	Biolegend	Cat#101212; RRID: AB_312795
APC Rat IgG2b, kappa Isotype Ctrl antibody	Biolegend	Cat#400611; RRID: AB_326555
Rat monoclonal anti-IFN γ	BD Biosciences	Cat#562018; RRID: AB_10896992
Rat anti-PD-1 IgG2a (RMP1-14)	BioXCell	Cat#BE0146; RRID: AB_10949053
Rat anti-IgG2a Isotype Control Clone 2A3	BioXCell	Cat#BE0089; RRID: AB_1107769
Rat monoclonal anti-CD8a	eBioscience	Cat#14-0808; RRID: AB_2572860
Rat monoclonal anti-F4/80	Serotec	Cat#MCA497; RRID: AB_2335598
Rabbit monoclonal anti-Ki67	Cell signaling	Cat#12202; RRID: AB_2620142
Rabbit monoclonal anti-c-MYC	Cell signaling	Cat#13987; RRID: AB_2631168
Rabbit monoclonal anti-Acetyl α Tubulin	Cell signaling	Cat# 5335; RRID: AB_10544694
Rabbit monoclonal anti-Acetylated Histone 3	Cell signaling	Cat#4499; RRID: AB_10544537
Rabbit polyclonal anti-Histone H3	Cell signaling	Cat#9715; RRID: AB_331563
Rabbit polyclonal anti-DNMT1	Sigma Aldrich	Cat#D4692; RRID: AB_262096
Anti-rabbit secondary (western blot)	GE Healthcare	Cat#NA934V
Anti-mouse secondary (western blot)	GE Healthcare	Cat#NA931V
Mouse monoclonal IgG1 Isotype Control	Cell Signaling	Cat#5415; RRID: AB_10829607
Rabbit anti-rat Biotinylated IgG	Vector	Cat#BA-4001; RRID: AB_10015300
Bacterial and Virus Strains		
Ad-CMV-Cre	Viral Vector Core, University of Iowa	Cat#VVC-U-IOWA-5
Biological Samples		
Patient-derived xenografts (PDX)	The Jackson Laboratory	Cat#TM00302
Chemicals, Peptides, and Recombinant Proteins		
Azacidine	Sigma-Aldrich	Cat#A2385
ITF-2357	Apexbio	Cat#A4093
MS-275	Syndax	MTA
MCGD0103	Apexbio	Cat#A4089
RGFP996	Apexbio	Cat#A8803
Tubastatin	Apexbio	Cat#A4101
Vorinostat	Cell signaling	Cat#12520
Puromycin	Sigma-Aldrich	Cat#P7255
DMSO	Sigma	Cat#D2660
RPMI1640	Corning	Cat#10-040
Fetal Bovine Serum	Hyclone	Cat#SH30910.03
0.25% Trypsin	Corning	Cat#25-053
DMEM	Corning	Cat#10-013
Pen/Strep	GIBCO	Cat#15140-122

REAGENT or RESOURCE	SOURCE	IDENTIFIER
m-CSF murine	Peprtech	Cat#315-02-100UG
ECM gel	Sigma Aldrich	Cat#E2170-10ML
Lipofectamine 2000	Invitrogen	Cat#11668-022
Fixable Viability Dye	eBioscience	Cat# 65-0865-14
eBiosciences Stim cocktail	eBioscience	Cat# 00-4970-03
Propidium Iodide	eBioscience	Cat#00-6990-50
Critical Commercial Assays		
RNeasy Mini kit	QIAGEN	Cat#74104
RNeasy Microkit	QIAGEN	Cat#74004
RNase free DNase Set	QIAGEN	Cat#79254
qScript cDNA SuperMix	Quanta	Cat#95048-025
iTaq universal sybr green supermix	BioRad	Cat#1725124
Homogenizer mini column	Omega	Cat#HCR003
Purelink midiprep	Invitrogen	Cat#K210014
Cell titer AQueous one	Promega	Cat#G3582
BrdU Cell Proliferation ELISA	Calbiochem	Cat#QIA58
Mouse Cytokine Array Q1	Raybiotech	Cat#QAM-CYT-1-1
Sigma Whole Transcriptome Amplification kit	Sigma Aldrich	Cat#WTA2-10RXN
Deposited Data		
Raw and analyzed data for gene expression	This paper	GEO: GSE104244
Experimental Models: Cell Lines		
NCI-H1755 (	ATCC	RRID: CVCL_1492
NCI-H520	ATCC	RRID: CVCL_1566
NCI-H1650	ATCC	RRID: CVCL_1483
NCI-H1975	ATCC	RRID: CVCL_1511
NCI-H441	ATCC	RRID: CVCL_1561
NCI-H661	ATCC	RRID: CVCL_1577
NCI-H596	ATCC	RRID: CVCL_1571
HCC4006	ATCC	RRID: CVCL_1269
NCI-H1703	ATCC	RRID: CVCL_1490
NCI-H838	ATCC	RRID: CVCL_1594
NCI-H2170	ATCC	RRID: CVCL_1535
NCI-H1792	ATCC	RRID: CVCL_1495
NCI-H460	ATCC	RRID: CVCL_0459
NCI-H23	ATCC	RRID: CVCL_1547
A-549	ATCC	RRID: CVCL_0023
NCI-H1299	ATCC	RRID: CVCL_0060
HCC827	ATCC	RRID: CVCL_DH92

REAGENT or RESOURCE	SOURCE	IDENTIFIER
LLC1	ATCC	RRID: CVCL_4358
Experimental Models: Organisms/Strains		
Mouse: B6.129S4- <i>Kras</i> ^{tm4Tyj/J}	The Jackson Laboratory	RRID: IMSR_JAX:008179
Oligonucleotides		
shMYC (TRCN0000010391)	RNAi Consortium	Cat# TRCN0000010391
Primers for qRT-PCR, see Table S1	This paper	N/A
shDNMT1		
MYC vector	(Cheng et al., 2013)	Addgene plasmid # 46970
pLV-AcGFP-N1	Clontech	Cat#632154
Recombinant DNA		
<i>Syngtin-1</i> (5' LTR-Syn-1-pGL3)	(Chiappinelli et al., 2015)	N/A
<i>ERV-3</i> (p3Xflag-CMV-14)	(Chiappinelli et al., 2015)	N/A
pE-GFP-N1	Clontech Laboratories	Cat#632469
pMD2.G		Addgene # 12259
psPAX2	Addgene	Addgene plasmid #12260
Software and Algorithms		
GraphPad Prism	GraphPad Software	https://www.graphpad.com . RRID: SCR_002798
Gene set enrichment Analysis	(Subramanian et al., 2005)	http://www.broadinstitute.org/gsea . RRID: SCR_003199

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Stephen B. Baylin (sbaylin@jhmi.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and cell culture—Non small cell lung cancer cell lines used in the study were obtained from ATCC. The cells were cultured in RPMI1640 media (Corning) containing FBS (Hyclone) at 10% v/v. Cells were maintained at 37°C in the presence of 5% CO₂. Cells were passaged every 3 days. Human Non Small Cell Lung Cancer cell lines: NCI-H1755 (female), NCI-H520 (male), NCI-H1650 (male), NCI-H1975 (female), NCI-H441 (male), NCI-H661 (male), NCI-H596 (male), HCC4006 (male), NCI-H1703 (male), NCI-H838 (male), NCI-H2170 (male), NCI-H1792 (male), NCI-H460 (male), NCI-H23 (male), A-549 (male), NCI-H1299 (male), HCC827 (female)

In Vivo Mouse Studies

Xenograft studies in NOD/SCID mice: We used male NOD-SCID mice (5–6 weeks old) for these studies. Mice were bred and housed at the Johns Hopkins Animal care facility. All animal experiments were approved by the Johns Hopkins Animal Care and Use Committee. All animal care and protocols followed were in accordance with guidelines of the institutional Animal Care and Use Committee (IACUC). Mice were injected subcutaneously in the flank with 2.0×10^5 viable cells for H1299 and 5.0×10^4 viable cells H460 cells in

0.2 mL of RPMI with Matrigel (1:1). Drug treatments were started 10 days post injection for H460 and 14 days post injection for H1299, when palpable tumors could be discerned and continued as indicated in the figure. Mice were treated with the dosing schedules shown in (Figure S6A). Treatment was continued for the entire duration of the study and mice were sacrificed before tumors volume exceeded 2000 mm³. Tumor volume determined by measurements obtained from digital caliper and calculated as $V = 0.5(L \times W \times H)$.

PDX mouse model—A PDX mouse model (TM00302) was obtained from Jackson Laboratories. This model was derived from metastatic lung adenocarcinoma in a female patient with the following mutations KRAS^{G12D}, KDR^{Q472H}, TP53^{R158L}. The tumor was harvested from a JAX provided donor mouse and sectioned into 2 mm³ fragments. These fragments were then implanted subcutaneously in the flank of male NSG mice (5–6 weeks old). Once implanted tumors were palpable, mice were randomized into three treatment groups and dosed as depicted in Figure S6A. Mice were treated for the duration of the study and at culmination of treatment mice were sacrificed. Tumors were excised from the animals and weighed as a measure of disease burden.

LSL-Kras^{G12D} mouse model—LSL-Kras^{G12D} mice (Jackson et al., 2001) were obtained from Jackson laboratories and bred with wild-type C57BL/6 mice to generate heterozygous LSL-Kras^{G12D} mice. LSL-Kras^{G12D} mice (8–10 weeks, both genders) were infected intratracheally with Adenoviral vectors encoding Cre (obtained from University of Iowa) to activate Kras. LSL-Kras^{G12D} mice harbor a latent point mutant allele of Kras (Kras^{G12D}) and infection with an adenoviral vector encoding Cre leads to Cre-mediated deletion of a transcriptional termination sequence (Lox-Stop-Lox, LSL) and the oncogenic Kras production, thereby resulting in very high frequency of lung tumors (Jackson et al., 2001). All experimental animal protocols were performed in accordance with guidelines approved by the animal care and use committee at the Johns Hopkins University (Baltimore, MD). Mice were examined for presence of disease at 16 weeks post infection with AdenoCre. Mice were sacrificed and lungs were examined histologically. At this stage most of the mice showed presence of significant regions of hyperplasia as well as adenomas. Drug treatments were started as per the schedule shown in Figure S6A. Mice were divided in two groups. One group was injected with vehicle control while the other was injected with combination epigenetic therapeutics. Mice were uniformly distributed into the two treatment groups so that each group had comparable numbers of age and weight matched male and female mice. Drug treatments were continued for 12 weeks, following which mice were sacrificed and the lungs processed for histological studies, RNA extraction and analysis of cytokine levels in lung bronchoalveolar lavage (BAL) fluid as described as described in main methods.

Lewis lung Carcinoma (LLC) model—LLC cells (LLC1, ATCC) 2.0×10^4 were injected subcutaneously in flanks of male C57BL6 mice (6–8 weeks old) obtained from Harlan. LLC1 cells were suspended in 100uL RPMI + Matrigel (1:1). Drug treatments were started 7 days post injection when palpable tumors could be discerned. Mice were injected with drugs as per the dosing schema in Figure S6A. Treatment was continued for a month and at the culmination of study mice were sacrificed, tumors were harvested and weighed.

Lungs of these mice were inflated and fixed in formaldehyde for 24 hours prior to sectioning and staining with H&E stains.

LLC model CD8 depletion model—CD8a antibody (BioXcell, 2.43 clone) mediated CD8 depletion of male C57BL6 mice was initiated 3 days prior to onset of treatment (Day 4 post implantation). Implantation and therapeutic treatment of CD8 depleted LLC model is described in Lewis Lung Carcinoma model section of methods. For CD8 depletion mice were injected IP with 150ug of CD8a antibody (BioXcell, 2.43 clone), three times per week throughout the duration of study. CD8 depletion was verified by assessment of CD8% of CD45 population in the spleen of depleted sample versus control samples. Treatment was continued for one month and at the culmination of study mice were sacrificed, tumors were harvested and weighed. Lungs of these mice were inflated and fixed in formaldehyde for 24 hours prior to sectioning and staining with H&E stains.

METHODS DETAILS

Drug Reagents—Azacitidine (Sigma) was dissolved in PBS at 500uM (*in vitro*) and 7.1 mg/mL (*in vivo*), aliquoted, and stored at -80°C for single use. ITF-2357 (Apexbio) was dissolved in DMSO to concentrations of: 1mM, 500uM, 250uM, 100uM, 50uM and 25uM (*in vitro*) and 50mg/mL (*in vivo*), aliquoted and stored at -20°C . MS-275 (Syndax) was dissolved in DMSO to concentrations of: 2.5mM, 1mM, 500uM, 250uM, 125uM (*in vitro*) and 50mg/mL (*in vivo*), aliquoted and stored at -20°C . MGCD0103 (Apexbio) was dissolved in DMSO to concentrations of: 1mM, 500uM, 250uM, 100uM, 50uM and 25uM (*in vitro*) and 10mg/mL (*in vivo*), aliquoted and stored at -20°C . RGFP996 (Apexbio) was dissolved in DMSO to concentrations of: 10mM, 5mM, 2.5uM, 1uM, 500uM and 100uM (*in vitro*), aliquoted and stored at -20°C . Tubastatin A (Apexbio) was dissolved in DMSO to concentrations of: 10mM, 5mM, 2.5mM, 1mM, 500uM and 250uM (*in vitro*), aliquoted and stored at -20°C . Vorinostat (Cell signaling) was dissolved in DMSO to concentrations of 300uM (*in vitro*), aliquoted, and stored at -20°C . Puromycin (Sigma), dissolved in PBS at 1mg/mL, aliquoted and stored -20°C .

Azacitidine drug treatments—Cell lines were plated at the following densities for Azacitidine treatments per T75 flask: NCI-H1755 (2.0×10^5), NCI-H520 (2.0×10^5), NCI-H1650 (2.0×10^5), NCI-H1975 (1.0×10^5), NCI-H441 (2.0×10^5), NCI-H661 (2.0×10^5), NCI-H596 (2.0×10^5), HCC4006 (2.0×10^5), NCI-H1703 (2.0×10^5), NCI-H838 (1.0×10^5), NCI-H2170 (2.0×10^5), NCI-H1792 (1.0×10^5), NCI-H460 (1.0×10^5), NCI-H23 (2.0×10^5), A-549 (1.0×10^5), NCI-H1299 (1.0×10^5), HCC827 (2.0×10^5). Cells were allowed to adhere for 24 hours before the onset of treatment. Cells were treated with 500uM Azacitidine diluted 1:1000 or PBS vehicle in complete RPMI1640 media every 24 hours for the indicated treatment duration. Drug treatments with HDACi are assay specific and are detailed in the relevant sections.

Cell Viability Assays (MTS colorimetric)—Cells treated as described in Azacitidine treatment section, were trypsinized, enumerated, and assessed for viability by trypan blue exclusion assay. Equal numbers of viable cells were plated in 96 well plates at the following densities per well, in technical triplicate: NCI-H1755 (2.0×10^3), NCI-H520 (2.0×10^3), NCI-

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H1650 (2.0×10^3), NCI-H1975 (1.0×10^3), NCI-H441 (2.0×10^3), NCI-H661 (2.0×10^3), NCI-H596 (2.0×10^3), HCC4006 (2.0×10^3), NCI-H1703 (2.0×10^3), NCI-H838 (1.0×10^3), NCI-H2170 (2.0×10^3), NCI-H1792 (1.0×10^3), NCI-H460 (1.0×10^3), NCI-H23 (2.0×10^3), A-549 (1.0×10^3), NCI-H1299 (1.0×10^3), HCC827 (2.0×10^3). Cells were allowed to adhere for 24 hours in the presence of complete media prior to the onset of treatment. Adhered cells were incubated with 100uL drug supplemented media changed every 3 days, treated with DMSO(vehicle) at 0.1% or the following drugs/concentrations standardized to 0.1% DMSO final concentration. ITF-2357: 25nM, 50nM, 100nM, 250nM, 500nM, 1uM; MGCD0103: 25nM, 50nM, 100nM, 250nM, 500nM, 1uM; RGFP996: 250nM, 500nM, 1uM, 2.5uM, 5.0uM and 10uM; Tubastatin A: 250nM, 500nM, 1uM, 2.5uM, 5.0uM and 10uM; MS-275: 125nM, 250nM, 500nM, 1uM, 2.5uM. Treatments were applied for a period of 5 days, afterwhich, MTS colorimetric assay (promega) was conducted. MTS reagent was diluted 20uL per mL in complete media and vortexed to ensure the resultant solution was homogeneous. Drug treated media was gently removed from treated cells by multichannel pipette and replaced with MTS supplemented media. Cells were incubated in 37C incubator to allow the colorimetric reaction to occur. After incubation 490nm absorbance was read using the BioRad iMark microplate reader for media+MTS, vehicle treated+MTS and drug treated+MTS cells. Absorbance values were imported to Graphpad prism. Data were normalized and drug doses were log transformed. These normalized, log transformed data were analyzed by 4 parameter nonlinear regression to generate log dose response curves for each cell line and drug condition and determination of drug effect at dose. The resultant dose response curves are a representation of these data, with the indicated biological replicates being a representation of the mean for each condition. These dose response data were utilized for drug synergy analysis (Compusyn). The fractional effect data for each drug condition were imported for the inhibitors deployed both alone and in combination with Aza. These data were analyzed and used to generate combination index output as an indicator of drug synergy.

BrdU Cell Proliferation Assay, colorimetric ELISA—Cells were plated in technical triplicate in 96 well format at the densities described in the Cell viability section. Vehicle or Aza treated cells were allowed to adhere for 24 hr prior to the onset of treatment. Cell were subsequently treated with the following inhibitors for 3 days. MGCD0103: 200nM, ITF-2357: 100nM, Tubastatin A: 1uM, RGFP996: 2uM. On day 3 of treatment, Stock BrdU (Calbiochem BrdU Cell Proliferation Assay) was diluted 1:1000 in complete media and added to 100uL of drug treated media. Cells were incubated with BrdU at 37C for 5 hours to allow for DNA synthesis based incorporation. The remainder of protocol was carried out as described in Calbiochem protocol, with the lot specific 1:2400 dilution of secondary antibody. 450nm absorbance values derived from ELISA were measured using the Biorad iMark plate reader. The triplicate measurements per condition were imported to graphpad prism and the data background subtracted, normalized to vehicle treated controls to generate the normalized proliferation. Error bars depicted as SEM between the means of experimental replicates. Statistical significance is the result of two way t test conducted between the experimental conditions depicted.

Gene expression analysis by microarray—RNA expression analyses were conducted by microarray for the conditions described in the relevant figures. The R/Bioconductor package limma was used to process expression data. Within- and between-array normalizations were performed using the loess and aquantile methods, respectively. The normexp option was used for background correction. Raw files read in using the read.maimages function. Log2 fold change in transcription for drug treated conditions over mock treated was obtained for each sample at each time point studied. Ranked lists of log2 fold change were analyzed using Gene Set Enrichment Analysis (GSEA) by the Broad Institute and data packages (Subramanian et al., 2005). Pathways enriched with a false discovery rate less than 0.25 were selected with cutoff of 2.0 normalized enrichment score in human and 1.5 normalized enrichment score cutoff in mouse studies. Median absolute deviation analysis of Log2 fold change transcription data were obtained using Morpheus program (Broad). Top 500 genes were selected and depicted as heatmap representative of minimum to maximum value ranking for each gene across cell lines and conditions.

DAVID analysis of median absolute deviation derived genes (KEGG Pathways)

—Top 500 genes derived by median absolute deviation analysis were obtained as described in Gene expression by microarray methods section and were analyzed for KEGG Gene Ontology (GO) enrichment using the DAVID Bioinformatics resources database (Huang et al., 2009a, 2009b). Only categories that were below the DAVID p value of 0.05, and containing at least 5 genes per pathway are reported.

MYC targets heatmap—MYC targets heatmaps are based on hierarchical clustering of Log2 fold change over mock using Euclidean distance and complete linkage of MYC hallmarks gene set v2.

ERV absolute quantitative real time PCR (qPCR)—Eleven codogenic and partially codogenic ERV envelope (env) gene families (see Figure 3 and Figure S4 for ERV env genes used in this investigation), one ERV gag (ERV-W5) and two ERV pols (ERVFXA34, erv9-1) were quantified using qPCR and have been previously described (Chiappinelli et al., 2015). Additionally, some of these ERV primers could potentially hybridize with other ERV transcripts among the ERV family members, e.g., the ERV-K env primers could hybridize with 8 transcripts derived from ERV-K102 (1q22), -107 (5q33.3), -108 (7p22.1), -109 (6q14.1), -113 (19p13.11), -115 (8p23.1), -17833 (19q12), -74261 (12q14.1) according to sequence alignments (Chiappinelli et al., 2015).

The qPCR methodology detecting the ERV genes has been previously described (Chiappinelli et al., 2015). Briefly, ERV genes were amplified by qPCR from 40 ng of cell line cDNA with SYBR-green technology and then analyzed with an ABI7300 (ABI, Darmstadt, Germany). Expression values were calculated as molecules per ng total RNA using a standard curve of each cloned gene determined by real time PCR and calculated as mean $\pm$ SEM. TBP and beta-actin were used as housekeeping genes where a mean from both genes was used for normalization of NSCLC cell lines.

IFN α / β qPCR—qPCR methodology for detecting IFN α / β related genes (ISG15, OASL, HLA-A, HLA-B) differential expression was conducted on 20ng of cell line cDNA with

SYBR green technology. Expression values were calculated as fold change over mock by delta-delta ct. Beta-actin was used as housekeeping gene for normalization of NSCLC cell line expression.

Taqman microfluidic genecard—1 μ g of total RNA was used for cDNA reaction and diluted to 50 μ L total volume in molecular grade water. Diluted cDNA was combined with 50 μ L of Taqman universal PCR mastermix and loaded onto a Taqman microfluidic genecard with the following primer/probe sets preloaded: IRF7, IFI27, STAT1, IFNB1, MX1, OASL, IFI6, beta actin, TBP. Expression values were calculated as fold change over mock by delta-delta ct. Beta actin and TBP were used as housekeeping genes for normalization of NSCLC cell line expression.

ERV Overexpression and OASL qPCR—*Syncytin-1*, *ERV-3*, and empty vectors were transfected using standard lipofectamine 2000 methods. Vectors for ERV overexpression assay are described in Chiappinelli et al., 2015. Four days post transfection of either empty or indicated ERV vector assessment of OASL and ERV3 or Syn1 RNA transcription by qPCR was conducted.

Immunoblotting—Protein was extracted by 4% SDS mediated lysis, followed by processing of lysates through homogenizer column (Omega). Protein concentrations were estimated by BCA (Pierce Biotechnology). Lysates were processed using PAGE with 4%–12% Bis-Tris BOLT gel (Life Technologies) and transferred to PVDF membrane (Millipore). Membranes were blocked in 10% milk/TBST and immunoblotted with the following antibodies.: rabbit monoclonal c-MYC (Cell Signaling, 1:1000), Rabbit monoclonal anti-Acetyl α Tubulin (Cell Signaling, 1:1000), Rabbit monoclonal anti-Acetylated Histone 3 (Cell Signaling, 1:1000), Rabbit polyclonal anti-Histone H3 (Cell Signaling, 1:1000), Rabbit polyclonal anti-DNMT1 (Sigma Aldrich, 1:2000), Mouse monoclonal anti- β -Actin (Sigma Aldrich, 1:10000). The loading control antibodies (anti- β -Actin, anti-Histone H3) in all cases were applied after membrane stripping.

DNMT1 Knockdown Cell Viability Assay—DNMT1 knockdown was initiated see reference for sequence (Cai et al., 2017) and control vector shGFP 5'-GCAAGCTGACCCTGAAGTTCAT3-3'. Clonal selection was initiated with puromycin selection. Selected clones were plated at cell densities, treated and analyzed as described in Cell viability Assay section of methods. Error bars are representative of mean $\pm$ SEM between experimental replicates.

MYC Knockdown Cell Viability Assay—MYC knockdown was initiated using Broad TRCN0000010391 clone and control vector shGFP 5'-GCAAGCTGACCCTGAAGTTCAT3-3'. Clonal selection was initiated with puromycin selection. Selected clones were plated at the cell densities and treated as described in Cell viability assay section of methods. MTS was used to quantify numbers of proliferating cells. Bar graphs depicted are representative of the percent of MYC knockdown cells present as a percentage of the empty vector treated controls for same dose of drug. Error bars are representative of mean $\pm$ SEM between experimental replicates. Statistical significance was determined by two tailed t test between empty vector and MYC shRNA treated cells at each dose.

MYC Overexpression Cell Viability Assay—MYC vector (Addgene plasmid # 46970) (Cheng et al., 2013) and empty vector (Clontech cat# 632154). Clonal selection was initiated with puromycin, 2 days post infection. Selected clones were plated at cell densities and treated as described in Cell viability assay section of methods. MTS was used to quantify numbers of proliferating cells. Bar graphs depicted are representative of the percent of MYC overexpression cells present as a percentage of the empty vector treated controls for same dose of drug. Error bars are representative of mean $\pm$ SEM between experimental replicates. Statistical significance was determined by two tailed t test between empty vector and MYC overexpression vector treated cells at each dose.

In-vivo Drug Studies—Mice were treated as follows: Azacitidine 0.5 mg/kg (PBS vehicle) IP injected daily for the first 5 days of every 14 day treatment cycle. *In vivo* HDACi was applied on day 7 of 14 day treatment cycle and injected daily for 5 days of 14 day cycle. ITF-2357 2.0 mg/kg (1% DMSO in PBS vehicle) IP injected daily for 5 days sequentially, every 14 days. MS-275 2.0 mg/kg (1% DMSO in PBS vehicle) IP injected daily for 5 days sequentially, every 14 days. MGCD0103 4.0 mg/kg (5% DMSO in PBS vehicle) IP injected daily for 5 days sequentially, every 14 days. InVivoMAb mouse Anti-PD1 (BioXcell RMPI 14 clone) injected 10ug/kg on the 12th day of a 14 day cycle. InVivoMAb Rat IgG2a Isotype control (BioXcell 2A3 clone) injected 10ug/kg on the 12th day of a 14 day cycle. CD8a depletion antibody (BioXcell, 2.43 clone), 3 times per week at 150ug per animal, see LLC CD8 depletion model for details about the treatment schema. For xenograft studies, treatment was initiated when palpable tumors could be discerned and continued for the duration of study. For Kras G12D mouse model studies, drug application initiated 16 weeks post Ad-Cre instillation, and applied for 12 weeks as described in the Kras G12D animal model section. For LLC model treatment was initiated 7 days after subcutaneous implant of flank tumor and mice were treated as described in (Figure S6A). Mice were injected IP with 150ug of CD8a antibody (BioXcell), three times per week throughout the duration of study. CD8 depletion was verified by assessment of CD8% of CD45 population in the spleen of depleted sample versus control samples.

Assessment of lung tumor formation—Mice were sacrificed and the left lung was fixed in formalin for histologic examination. The right lung in some mice were lavaged by instilling with 1.0ml of sterile PBS to collect Bronchoalveolar lavage (BAL) fluid. In some mice right lung was used to macro dissect tumors for RNA extraction.

Bronchoalveolar lavage (BAL) fluid cytokine analysis—1.0 mL of BAL fluid was instilled into the right lung of tumor bearing KrasG12D mice. 50uL of this BAL was utilized for cytokine analysis as described by the manufacturer (Raybiotech, QAM-CYT-1). Cytokine array was scanned by the manufacturer. Cytokine quantification was conducted using a standard curve of each cytokine present at known concentrations supplied by the manufacturer. p values for cytokine data were derived using Graphpad Prism software and were defined as < 0.05 by unpaired two tailed t test when comparing treated condition versus control, biological replicates were used for the derivation of significance. Data are mean $\pm$ SD between biological replicates.

Histological analysis of mouse tissues—Lungs tissues were fixed in 10% formalin overnight, and subsequently transferred into 70% ethanol, embedded in paraffin and sectioned (5 μ M) at 3 levels at regular intervals. Sections were stained with Hematoxylin and eosin (H&E) and scanned using the Aperio whole slide scanning system (Scanscope CS). The entire left lung lobe was scanned for presence of adenomas/adenocarcinomas using the Aperio ImageScope analysis software. For each sample, sections from 3 levels were analyzed. Unstained sections of the left lung were also used for Immunohistochemistry (IHC) staining. All Images were scanned at 20x magnification using the Aperio Scanscope system.

Immunohistochemistry (IHC)—Immunostaining of lung sections was performed with the PowerVision kit according to the manufacturer's protocol (Leica Bio-systems). Briefly, slides were heated at 60°C for 10 min, deparaffinized and hydrated through xylene, graded ethyl alcohols, dH₂O, dH₂O with 20% Tween 20 (P-7949, Sigma-Aldrich). After antigen retrieval (45 minutes of steaming in Target Retrieval Solution (Dako S170084-2) using Black and Decker Handy Steamer Plus), sections were treated 5 minutes with Dual Endogenous Enzyme Block (S2003, Dako). Sections with primary antibodies Ki67 (Cell signaling, 9101, 1:500) were incubated at room temperature for 45 minutes. Additional blocking steps were used for CD8 and F4/80 slides using DakoCytomation Biotin Blocking System (X0590). Antibody incubations for CD8 (eBiosciences, 14-0808, 1:800) and F4/80 (Serotec, MCAP497, 1:1000) were carried out at room temperature for 45 minutes, soaked an additional 45 min in PBS-Tween, and followed by mouse adsorbed biotinylated anti Rat IgG (Vector, BA-4001, 1:500) for 15 minutes. For all, the secondary used was anti-rabbit IgG-reagent provided in the Powervision kit (PV6119, Leica Biosystems) for 30 minutes. Immunostaining was visualized with DAB chromogen (D4293, Sigma-Aldrich) and sections were counterstained with Mayer's hematoxylin. Control slides: No primary for each, CD8 (spleen), F480 (tumor)

F4/80 Intratumor Macrophage Analysis—F4/80 IHC stained sections were scanned used the Aperio system as described earlier, these images were analyzed using image-scope software. To generate the false color images depicted used to demarcate pixel saturation by macrophage infiltration, the images were analyzed by the positive pixel v4 algorithm.

Isolation of murine bone marrow derive macrophages (BMDMs)—Mice (6–8) week old were euthanized and hind legs were harvested under aseptic conditions. Femurs and tibia were obtained and flushed with RPMI medium. Cells were then centrifuged and treated with ACK buffer to lyse RBC's. Cell pellets were then washed with and resuspended in RPMI medium and plated in six well plates in the presence of M-CSF. Medium was changed every 2 days. Treatments with Aza and HDACi were started after 4 days and the *in vitro* drug treatment schema was 5 days of 500nM Aza followed by either vehicle or 100 nM ITF-2357 for 5 days. Cells were then collected and RNA extracted.

TCGA Analysis—Broad Morpheus tool was used to analyze TCGA LUAD data for the relationship between CCL5 and MYC. First the 2 genes were selected from available RNA seq data of 576 patients available. These data were used to generate a new heatmap. This

new heatmap was sorted based on CCL5 and MYC expression and the samples were separated into low, intermediate and high expression groups. These subsets were then compared using the profile interaction tool to generate the depicted plots. 576 LUAD samples were ranked by expression of CCL5 and MYC into: low, intermediate and high expression groups. These 3 expression categories for MYC and CCL5 were then compared as follows on the y axis, where gene(y) is sorted based on low, intermediate or high expression of other gene: MYC high:CCL5(y), MYCintermediate:CCL5(y), MYClow:CCL5(y), MYC(y):CCL5high, MYC(y):CCL5intermediate, MYC(y):CCL5low.

Patient and sample characteristics used for MYC amplification studies—The studies were conducted in accordance with the Declaration of Helsinki and were approved by the Institutional Review Board (IRB), and patients provided written informed consent for sample acquisition for research purposes. We analyzed pre-treatment exome data derived from a recently published set of NSCLC patients treated with immune checkpoint blockade (Anagnostou et al., 2017). Patient and sample characteristics as well as treatment and response assessments are described in detail elsewhere (Anagnostou et al., 2017). In brief, our study group consisted of two NSCLC patients treated with PD-1 blockade (CGLU117T1 and CGLU127T1) and two NSCLC patients treated with combined PD-1 and CTLA4 blockade (CGLU116T1 and CGLU161T1). All patients achieved a partial response of more than six months in duration (durable clinical benefit-DCB) apart from CGLU117T1, who achieved stable disease (22% tumor regression by RECIST 1.1) of 4 months duration before he developed disease progression (non-durable clinical benefit-NCB). An additional NSCLC tumor sample from a patient with sustained response to PD-1 blockade was obtained for comparative analysis of CD8+ T-cell density.

Somatic Copy Number Analysis—Whole exome sequence data from a previously published cohort of four patients with NSCLC (Anagnostou et al., 2017) was evaluated to determine the somatic copy number of MYC locus in pre-treatment tumor samples. The sequencing depth of coverage of the tumor sample in bins spanning coding and non-coding regions of the genome were compared to the matched normal sample from each patient. The log₂ read ratio values were corrected for biases resulting from the exome capture process and sequence composition using CNVKit (0.7.6) (Talevich et al., 2016), and were adjusted to account for variable normal cell contamination as previously described (Anagnostou et al., 2017).

FACS sorting and profiling of macrophages and T cells LSL-Kras^{G12D}—For FACS sorting of macrophages and CD8 T cells from LSL-KRas^{G12D} mice, lungs were harvested from mock or treated mice following three months of treatment. Tissue was then digested for 30 minutes at 37°C in digestion buffer (RPMI, FBS (10%), Collagenase Type 1 (0.2%), Collagenase Type 2 (0.2%) and DNase I (50u/mL). Tissue was then minced and strained through a 40um cell strainer to obtain a single cell suspension. RBC's were then lysed with ACK buffer and the cell pellet was washed twice in RPMI prior to suspension in RPMI medium. Cells were then counted and blocked with Rat monoclonal anti-CD16/CD32 (Fc block Antibody) in PBS for 30 min at 4°C. Cells were then stained with antibodies against CD45, Cd11b and F4/80 for macrophages or CD45, CD3, CD8a for CD8⁺ T cells.

Propidium Iodide was used as a viability marker (5uL/100uL total volume). Cells were sorted and pellets were lysed and RNA extracted using RNeasy kit. RNA was then quantitated and amplified using the Sigma Whole Transcriptome Amplification kit. Amplified RNA was then processed and analyzed on Mouse Agilent 4x44 single color microarray. Post sort purity checks were utilized and the sorted populations were determined to be more than 95% pure population.

FACS profiling T cells LLC—For FACS profiling of CD8⁺ T cells from LLC mice, primary tumors were harvested from mock or treated mice following one month of treatment. Tissue was then digested using a mixture of collagenase, hyaluronidase and DNase. The resulting single cell suspension was counted and plated in complete media (RPMI+10% FBS) with or without eBiosciences stimulation cocktail (1:1000) for 4 hours. Cells were then collected and blocked with Rat monoclonal anti-CD16/CD32 (Fc block Antibody) in PBS for 30 min at 4°C. Cells were then stained with antibodies against Live/dead, CD45, CD3, CD8a, and IFN γ for CD8⁺ T cells. Cells were then profiled by FACS.

QUANTIFICATION AND STATISTICAL ANALYSIS

Dose response data—Four parameter, non-linear regression of normalized and log transformed colorimetric absorbance response to increasing concentrations of therapeutics as described in each figure. Data analyzed using Graphpad prism, error bars are mean $\pm$ SEM between biological replicates unless indicated otherwise.

BrdU proliferation data—Colorimetric absorbance was used as an indicator of proliferating cell numbers. Bar graphs depicted are representative of the percent of treated cells normalized to untreated controls. Error bars are representative of mean $\pm$ SEM between experimental replicates. Statistical significance was determined using Graphpad Prism software and are two tailed t test between control and Aza treated cells or Aza + MS275 and Aza + ITF-2357 at dose indicated in the figure.

Microarray data—Differential gene expression was defined as 0.5 log₂ fold change in treated condition versus control. Significantly enriched gene sets were defined using an FDR cutoff < 0.25 . p values for microarray data were derived using R programming language, limma package, and were defined as < 0.05 when comparing treated condition versus control.

qRT-PCR data—Differential gene expression was defined as 0.5 log₂ fold change in treated condition versus control. p values for qRT-PCR data were derived using Graphpad Prism software and were defined as < 0.05 by unpaired two tailed t test when comparing treated condition versus control, biological replicates were used for the derivation of significance.

MYC knockdown/overexpression data—Colorimetric absorbance was used as an indicator of proliferating cell numbers. Bar graphs depicted are representative of the percent of MYC knockdown cells present as a percentage of the empty vector treated controls for same dose of drug. Error bars are representative of mean $\pm$ SEM between experimental

replicates. Statistical significance was determined using Graph-pad Prism software and are two tailed t test between empty vector and MYC knockdown or overexpression treated cells at each dose.

Tumor area quantification data—Tumor area was quantitated as total area of left lung presenting with lesions in LSL-Kras^{G12D} mice using Aperio Imagescope software. p values for tumor area quantification data were derived using Graphpad Prism software and defined as < 0.05 by unpaired two tailed t test when comparing treated condition versus control, independent animals were used for the derivation of significance. Error bars are representative of mean $\pm$ SEM between experimental replicates.

Immune cell infiltration data—IHC quantification- tumor infiltrating CD8 T cells were enumerated in field of view for hotspots of infiltration for the conditions indicated in the figure. The n values depicted indicate individual animals scored for infiltration. p values for IHC quantification data were derived using Graphpad Prism software and were defined as < 0.05 by unpaired two tailed t test when comparing treated condition versus control, independent animals were used for the derivation of significance. FACS infiltration data- tumor infiltrating CD8 T cells were profiled from whole digested tumors. The n values depicted indicate individual animals profiled by FACS. p values for FACS data were derived using Graphpad Prism software and were defined as < 0.05 by unpaired two tailed t test when comparing treated condition versus control, independent animals were used for the derivation of significance.

Hypergeometric test of cumulative distribution function—The number of overlapping differentially expressed genes present in the Wherry et al., 2007 and our RNA expression dataset (> 0.5 log2 fold change over mock treated, p value < 0.05) were used to calculate over representation of these genes. p values were calculated using hypergeometric test of cumulative distribution function using p value/representation calculator (<http://systems.crupp.ucla.edu/hypergeometric/>).

Cytokine data—Data are array based fluorescence intensity. Cytokine quantification was conducted using a standard curve of each cytokine present at known concentrations supplied by the manufacturer p values for cytokine data were derived using Graphpad Prism software and were defined as < 0.05 by unpaired two tailed t test when comparing treated condition versus control, n value represents biological replicates and data are mean $\pm$ SD between biological replicates.

TCGA data—Broad Morpheus tool was used to define low, medium and high groups for MYC and CCL5 expression. These groups were then compared as defined in the figure using the built-in profile interaction tool (Morpheus).

MYC amplification data—Whole exome sequencing depth of coverage of the tumor sample in bins spanning coding and non-coding regions of the genome were compared to the matched normal sample from each patient. The log₂ read ratio values were corrected for biases resulting from the exome capture process and sequence composition using CNVKit

(0.7.6) (Talevich et al., 2016), and were adjusted to account for variable normal cell contamination as previously described (Anagnostou et al., 2017).

DATA AND SOFTWARE AVAILABILITY

The accession number for microarray data reported in this paper is GEO: GSE104244.

ADDITIONAL RESOURCES

Clinical trial identifier: NCT03220477: <https://clinicaltrials.gov/ct2/show/NCT03220477>

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Azacitidine depletes MYC and sensitizes NSCLC to HDACi
- Combination epigenetic treatment induces a potent anti-tumor response *in vivo*
- Epigenetic treatment potentiates anti-tumor responses by modulating T cell phenotypes
- MYC status determines tumor immunophenotype

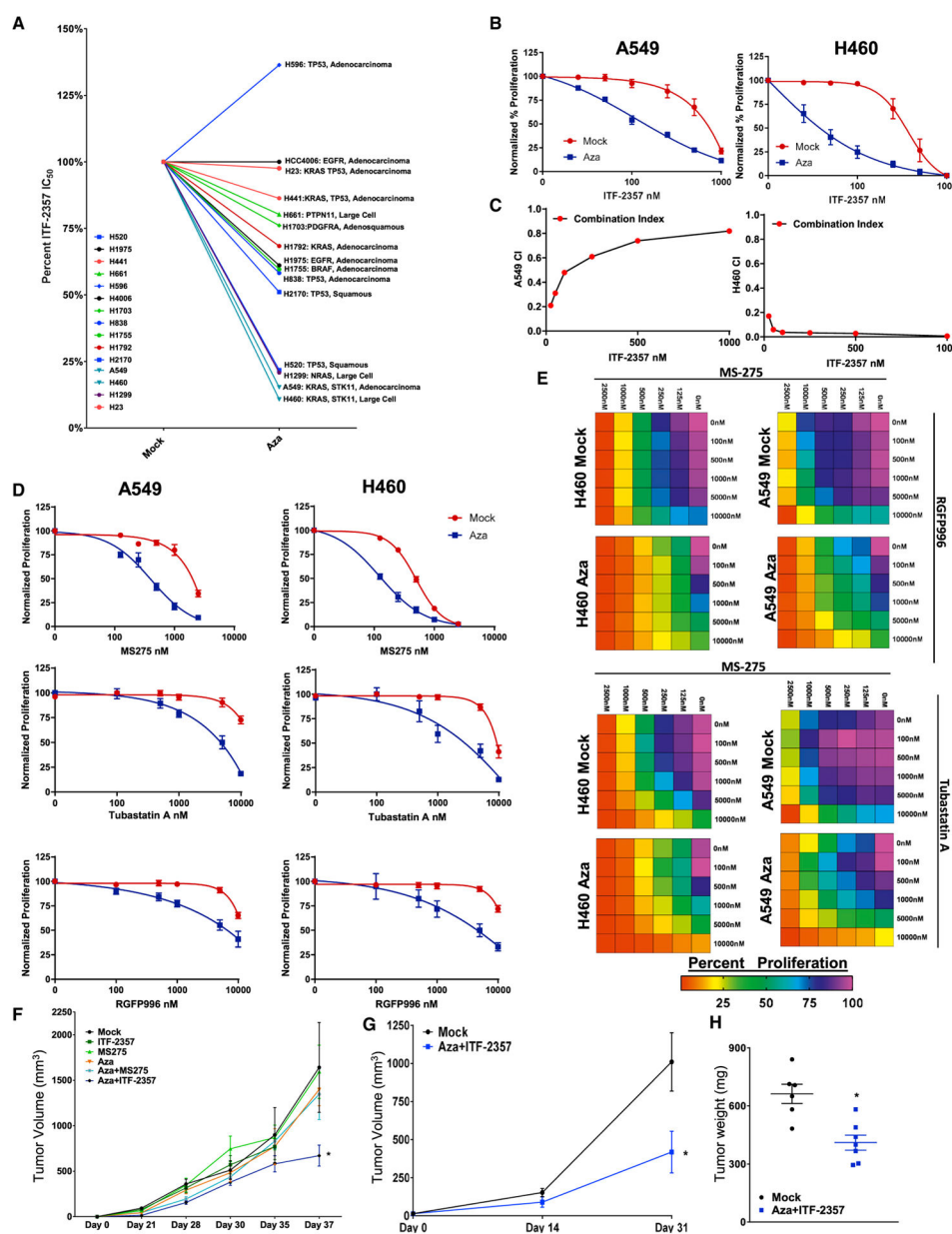


Figure 1. Azacitidine Synergizes with Sequential HDACi for Reducing Cell Proliferation
 (A) Composite representation of ITF-2357 IC_{50} as determined by four parameter dose-response analysis in the presence of mock or 500 nM Aza pre-treatment. Individual dose-response curves are in Figures 1B, S1B, and S1E. H1650 not depicted due to lack of sensitivity to epigenetic agents deployed in study.
 (B) Sequential treatment with (mock or 500 nM Aza) + ITF-2357 log dose-response curves for growth inhibition of A549 and H460 cells (day 11, $n = 5$, data represented as mean $\pm$ SEM).
 (C) Combination index (CI) plots for sequential application of Aza + ITF-2357 in A549 and H460 cells ($n = 5$).

(D) Sequential treatment with (mock or 500 nM Aza) + MS-275, RGFP996 (HDAC3i), or Tubastatin A (HDAC6i) log dose-response curves for growth inhibition of A549 and H460 cells (day 11, n = 3, data represented as mean $\pm$ SEM).

(E) Sequential treatment (mock or 500 nM Aza) with MS-275 plus either RGFP996 or Tubastatin A drug dose-response matrix for growth inhibition of A549 and H460 cells (day 11, n = 3, color gradation indicates percentage viability at the indicated dose combination).

(F and G) Mean volumes of tumor xenografts obtained from NOD-SCID mice subcutaneously injected with H460 cells (F) or H1299 cells (G) and treated with the agents as indicated in the figure. Data are presented as mean $\pm$ SEM (n = 5). *p < 0.05 calculated using two tailed t test.

(H) Tumor weights for patient-derived xenografts treated with the agents as outlined in the figure (28 days of treatment duration; data are presented as mean $\pm$ SEM; n = 6 mock and n = 7 Aza + ITF-2357). *p value < 0.05 calculated using two-tailed t test.

See also Figure S1.

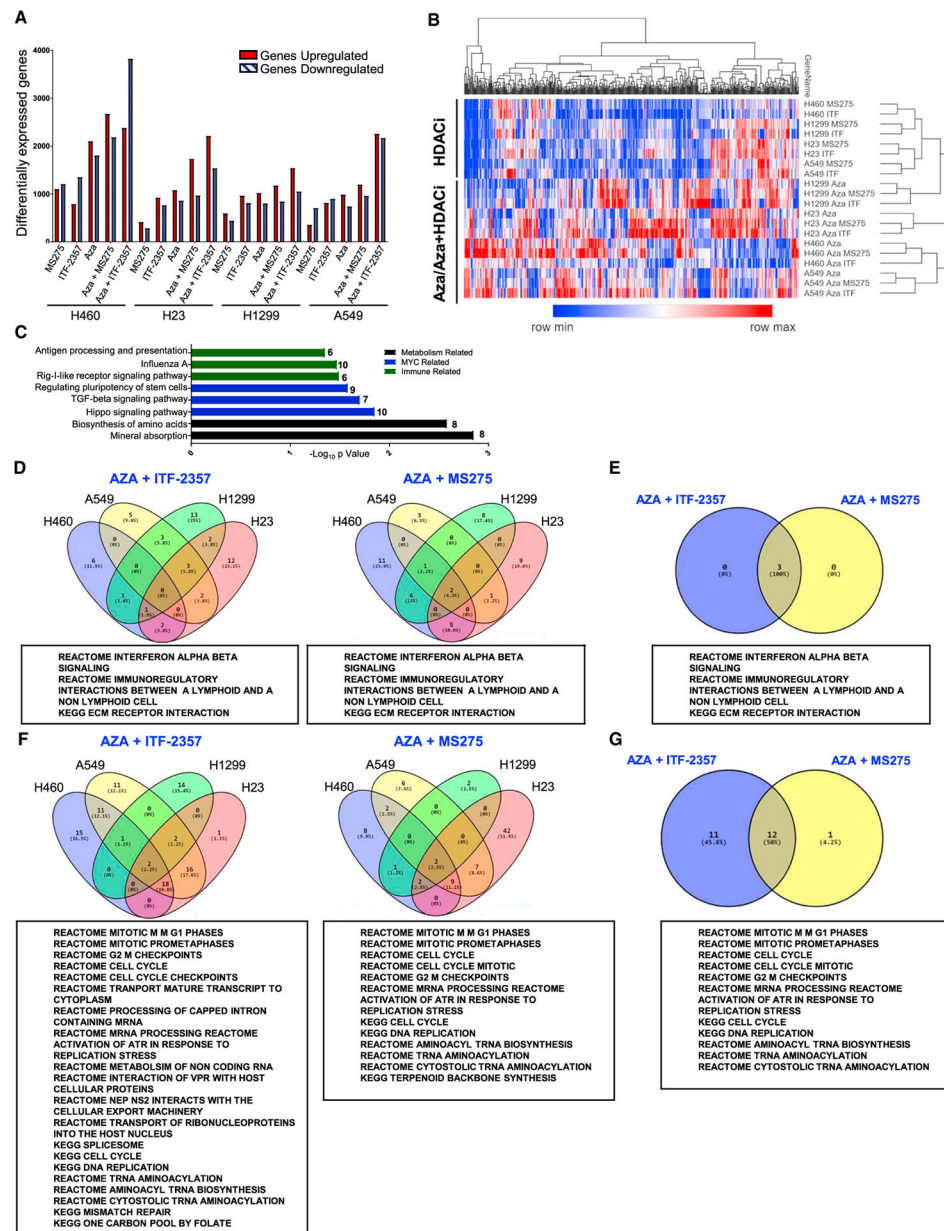


Figure 2. Epigenetic Treatment of NSCLC Cell Lines Induces Robust Alteration of Cell Transcriptome

(A) Quantitation of differentially expressed genes (cutoff Log2 fold change over mock >0.5) for each treatment condition.

(B) Unsupervised hierarchical clustering of relative RNA expression by median absolute deviation (MAD). RNA expression Log2 fold change over mock; blue to red color gradation is based on the ranking of each condition from minimum (blue) to maximum (red). The top 500 genes are depicted.

(C) DAVID analysis of the top 500 MAD genes using Kyoto Encyclopedia of Genes and Genomes (KEGG) gene ontology.

(D) Venn diagrams depicting GSEA-derived overlapping and unique pathways induced by combination treatment in at least 3 cell lines with the respective HDACi (normalized enrichment score [NES] > 2.0, false discovery rate [FDR] < 0.25).

(E) Venn diagram of pathways commonly upregulated by combination treatment with Aza and the respective HDACi.

(F) Venn diagrams depicting GSEA-derived overlapping and unique pathways downregulated by combination treatment in at least 3 cell lines with the respective HDACi (NES < 2.0, FDR < 0.25).

(G) Venn diagram of pathways commonly downregulated by combination treatment with Aza and the respective HDACi.

The above data are derived from microarray analysis of RNA from cells treated with 500 nM Aza, 100 nM ITF-2357, and 100 nM MS-275.

See also Figure S2.

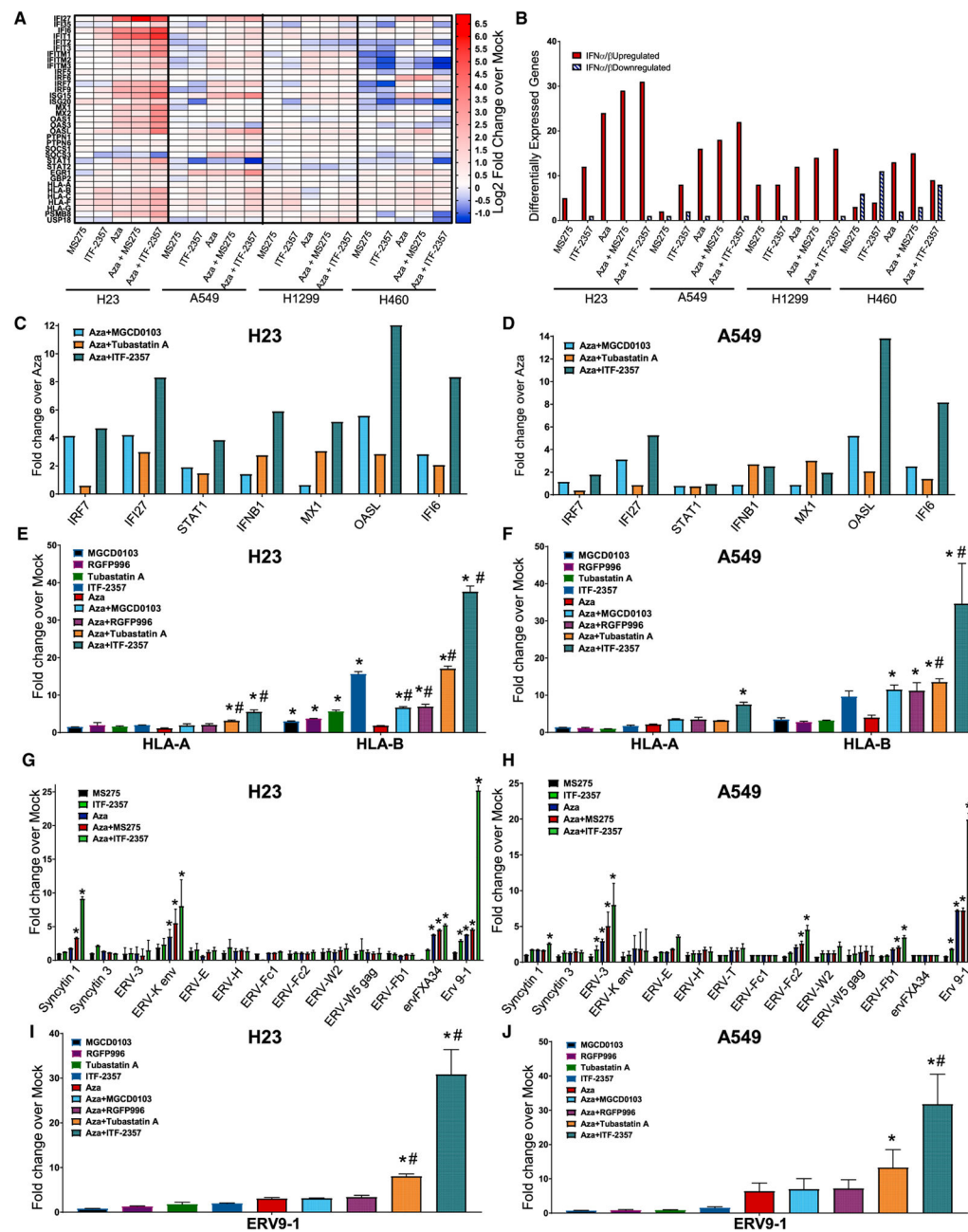


Figure 3. Combination Epigenetic Treatment Augments IFN α / β Pathway-Associated Immune Genes and ERV Transcription

(A) Heatmap of relative RNA expression for IFN α / β -signaling pathway core-enriched genes for the indicated cell lines (microarray, day 8; 500 nM Aza, 100 nM MS-275, and 100 nM ITF-2357).

(B) Quantification of IFN α / β pathway core-enriched genes differentially expressed by the indicated conditions (microarray, day 8; 500 nM Aza, 100 nM MS-275, and 100 nM ITF-2357; differential gene expression cutoff Log2 fold change over mock >0.5).

(C and D) Expression of viral defense gene subset of IFN α / β pathway (PCR genecard, day 8; 500 nM Aza, 100 nM ITF-2357, 200 nM MGCD0103, and 1,000 nM Tubastatin A) in H23 (C) and A549 (D) cells.

(E and F) Quantitation of selected major histocompatibility complex (MHC) class I genes of the IFN α / β pathway in response to Aza and/or HDACi in H23 (E) and A549 (F) cells (qRT-PCR, day 8; 500 nM Aza, 100 nM ITF-2357, 200 nM MGCD0103, 2,000 nM RGFP996, and 1,000 nM Tubastatin A; n = 3).

(G and H) Quantitation of ERV transcripts in response to Aza and/or HDACi in H23 (G) and A549 (H) cells (qRT-PCR, day 8; 500 nM Aza, 100 nM ITF-2357, and 100 nM MS-275; n = 4).

(I and J) Quantitation of ERV9-1 in response to Aza and/or HDACi in H23 (I) and A549 (J) cells (500 nM Aza, 100 nM ITF-2357, 200 nM MGCD0103, 2,000 nM RGFP996, and 1,000 nM Tubastatin A; n = 3).

Data are presented as mean $\pm$ SEM (*p < 0.05 relative to mock and #p value < 0.05 relative to Aza; p value determined by two-tailed t test).

See also Figures S3 and S4.

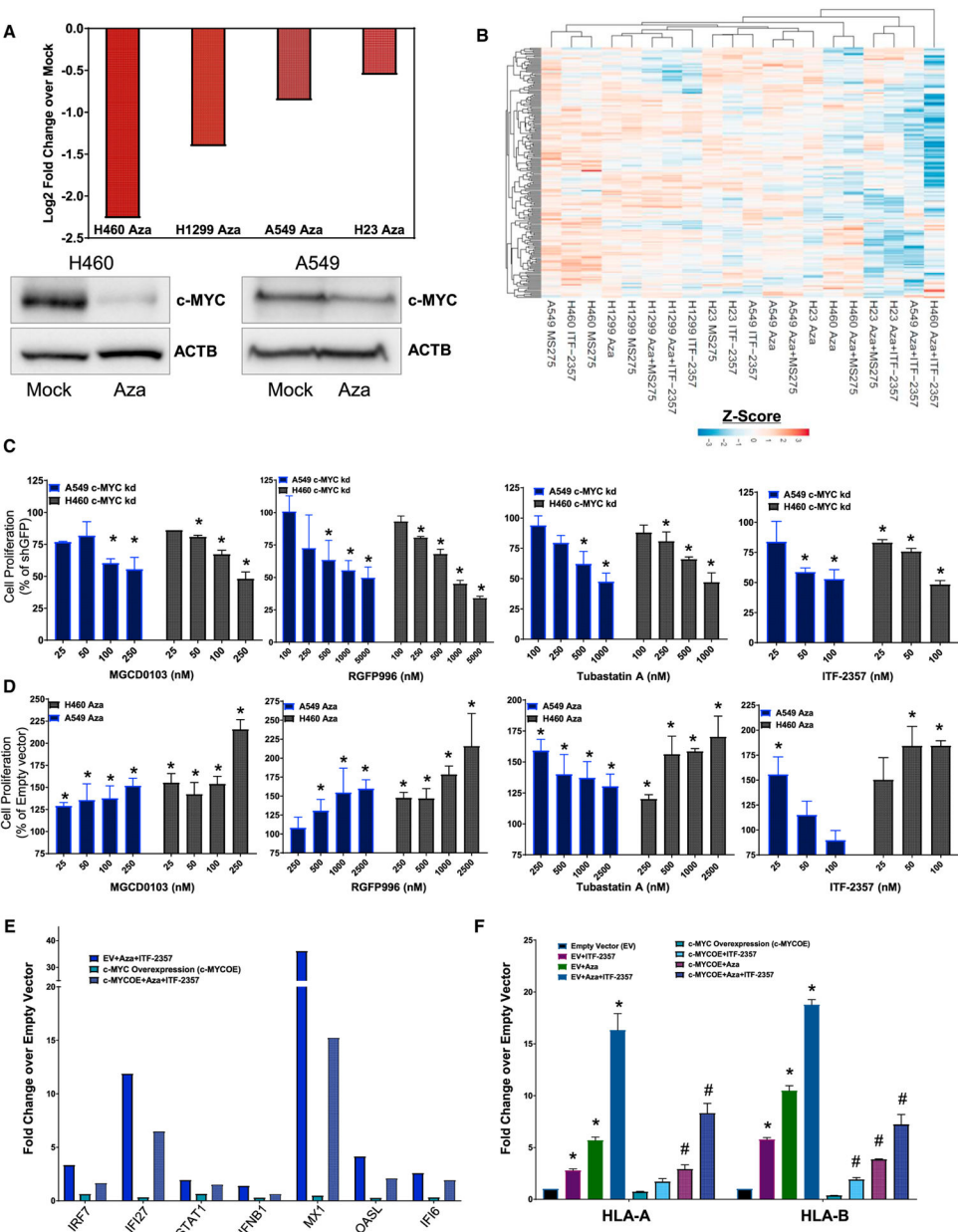


Figure 4. MYC Perturbation Drives Aza Sensitization to HDACi and IFN α / β Pathway Gene Augmentation

(A) Top panel: quantitation of relative MYC RNA expression in NSCLC cell lines following 500 nM Aza treatment (microarray, day 8). Bottom panel: immunoblot shows expression of MYC protein on day 9 of treatment. β -actin was used as a loading control (500 nM Aza, n = 3).

(B) Unsupervised hierarchical clustering of relative RNA expression for GSEA HALLMARK MYC TARGETS. Color gradation is based on Z score ranking of log2 fold change over mock (microarray, day 8; 500 nM Aza, 100 nM MS-275, and 100 nM ITF-2357).

(C and D) A549 and H460 cell line quantitation of colorimetric absorbance as an indicator of proliferating cell number, normalized to untreated control. (C) Percentage proliferation for GFP and shMYC vector-infected cells treated with the indicated HDACi for 5 days (n = 3). (D) Percentage proliferation for empty vector (EV) or MYC overexpression vector containing cells treated with the indicated HDACi for 5 days (n = 3 overexpression clones). (E and F) Relative RNA expression of IFN α / β pathway-responsive genes in A549 cells infected with EV or MYC overexpression construct and treated with mock or 500 nM Aza + 100 nM ITF-2357. (E) Quantitation of relative RNA expression for IFN α / β pathway-viral defense gene subset (genecard, day 8). (F) Quantitation of relative RNA expression for IFN α / β pathway-MHC class I genes (qRT-PCR, day 8, n = 3).

Data are presented as mean $\pm$ SEM (*p < 0.05 relative to mock and #p < 0.05 relative to EV + Epigenetic treatment; p value determined by two-tailed t test).

See also Figure S5.

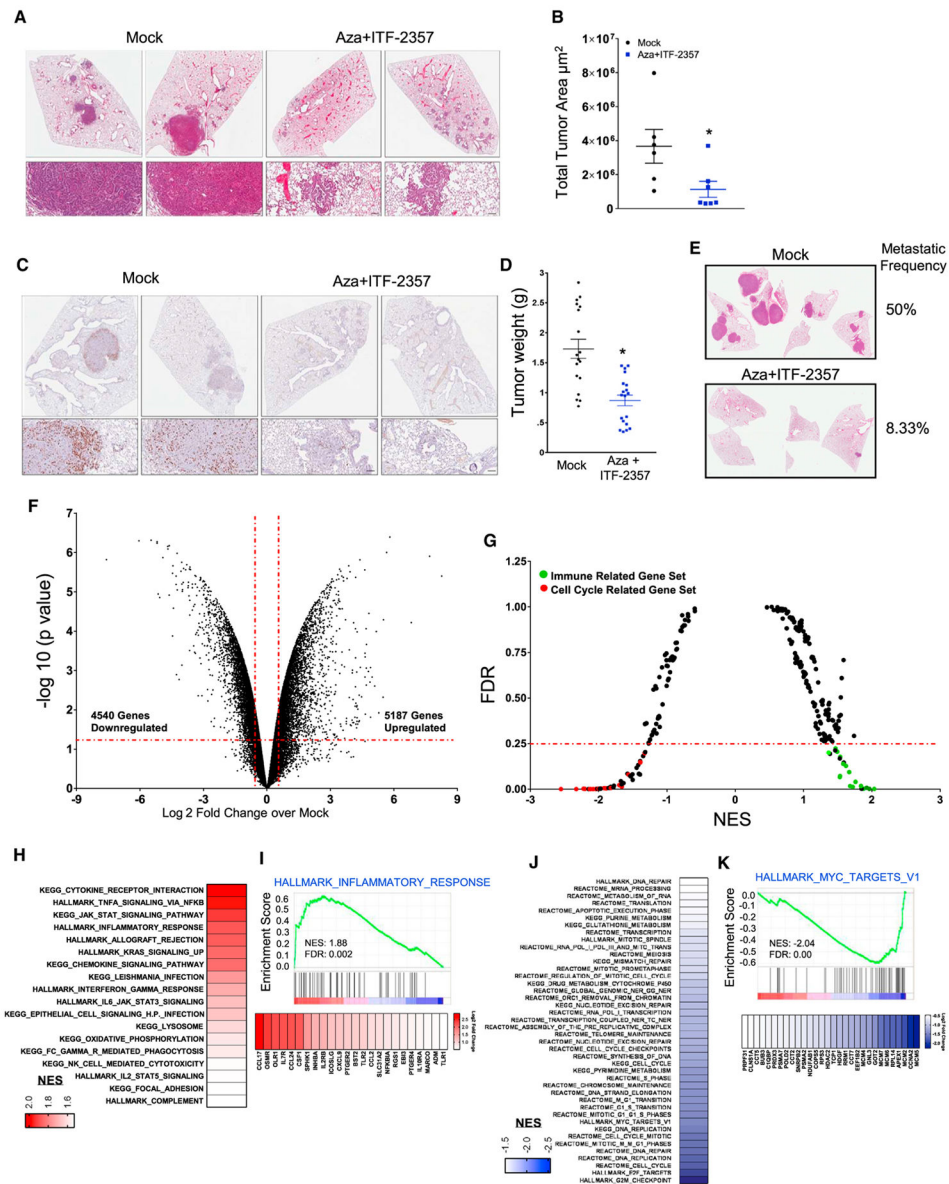


Figure 5. Combination Epigenetic Treatment Reduces Lung Tumor Burden and Progression in Mouse Models of NSCLC

(A) Representative H&E-stained images of lung sections from mice treated with mock or Aza + ITF-2357. Scale bar, 100 μ m.

(B) Quantitation of total tumor area occupied by lesions in lungs of LSL-Kras^{G12D} mice treated with mock or Aza + ITF-2357. Data are presented as mean $\pm$ SEM (p value determined by two-tailed t test; n = 6 mock and n = 7 Aza + ITF-2357 mice per group/2 sections analyzed per mouse).

(C) Representative Ki67-stained IHC images of lung sections from mice treated with mock or Aza + ITF-2357 (n = 5 per group). Scale bar, 100 μ m.

(D) Lewis Lung Carcinoma (LLC) tumor weights of subcutaneous explants from 1-month mock- and Aza + ITF-2357-treated mice (n = 19 mice per group; error bars, SEM).

(E) Representative H&E-stained images of lung metastasis from LLC mice obtained from 1-month mock- and Aza + ITF-2357-treated mice (n = 12 mice per group), with the indicated percentage frequency of metastasis.

(F) Volcano plot of relative RNA expression from LSL-Kras^{G12D} mouse lung tumors treated for 3 months with Aza + ITF-2357 as compared to mock mice. Genes in upper left and right quadrants are significantly differentially expressed (microarray, n = 2 per group).

(G) GSEA (KEGG, REACTOME, and HALLMARK) pathway distribution for Aza + ITF-2357 versus mock tumors from LSL-Kras^{G12D} mice. Horizontal line denotes FDR significance cutoff of 0.25. Immune- and cell cycle-related gene sets are demarcated by green and red dots, respectively (microarray, n = 2 per group).

(H) Gene sets upregulated in LSL-Kras^{G12D} mice (FDR < 0.25 and NES > 1.5) by Aza + ITF-2357. Color gradation is based on GSEA NES.

(I) Representative upregulated GSEA plots with corresponding core-enriched genes. Color gradation is representative of Log2 fold change over mock.

(J) Gene sets downregulated in LSL-Kras^{G12D} mice (FDR < 0.25 and NES < 1.5) by Aza + ITF-2357. Color gradation is based on GSEA NES.

(K) Representative downregulated GSEA plot with core-enriched genes. Color gradation is representative of Log2 fold change over mock-treated RNA expression.

*p < 0.05 calculated using two tailed t test. See also Figure S6.

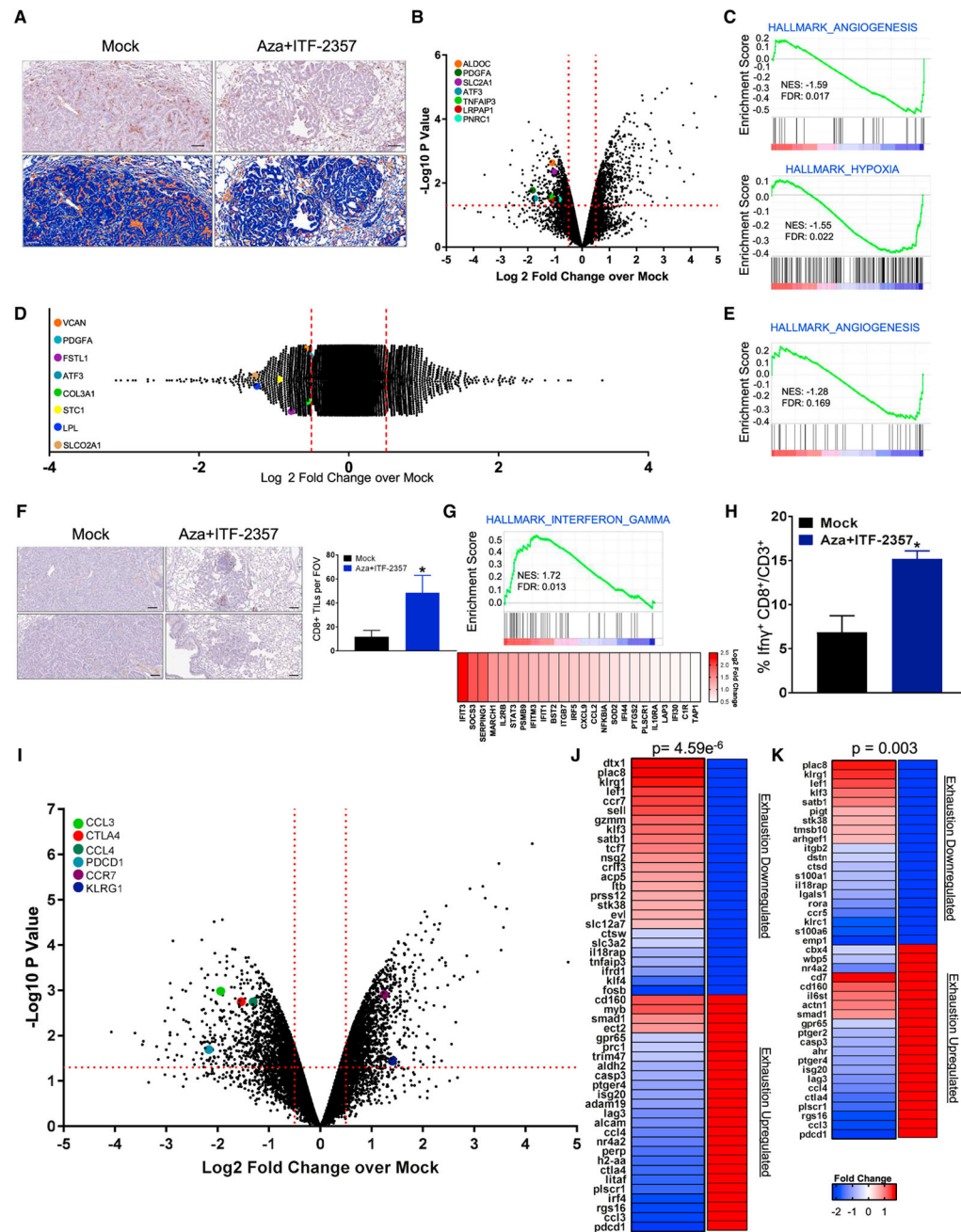


Figure 6. Effect of Combination Epigenetic Treatment on Tumor-Associated Immune Populations and Their Functional Status

(A) Representative IHC staining of F4/80⁺ macrophages in LSL-Kras^{G12D} lung tumor sections treated with mock or Aza + ITF-2357 for 3 months. Upper panel: representative F4/80⁺ IHC. Lower panel: positive pixel transformation of IHC images in upper panel using Aperio Imagescope software. Scale bar, 100 μ m.

(B) Volcano plot of relative RNA expression of CD45⁺CD11b⁺F4/80^{hi} macrophages sorted via FACS and isolated from tumor-bearing lungs from 3-month mock- or Aza + ITF-2357-treated LSL-Kras^{G12D} mice. Genes in the upper left and right quadrants are significantly

differentially expressed (microarray, n = 2 per group). Hypoxia- and angiogenic pathway-associated genes are highlighted.

(C) Key affected pathways obtained from GSEA of CD45⁺CD11b⁺F4/80^{hi} macrophage RNA isolated from tumor-bearing lungs from 3-month Aza + ITF-2357-treated LSL-Kras^{G12D} mice as compared to mock-treated mice.

(D) Log2 fold relative RNA probe distribution showing differential gene expression from bone marrow-derived macrophages (BMDMs) treated *in vitro* with mock or Aza + ITF-2357. Angiogenic pathway-associated genes are highlighted (microarray, BMDM data representative of n = 3 mice).

(E) GSEA enrichment plot for angiogenesis pathway from BMDM RNA expression.

(F) CD8⁺ IHC in lung tumors of mock- and Aza + ITF-2357-treated LSL-Kras^{G12D} mice following 3 months of treatment (scale bar, 100 μ m). The graph on the right indicates the average number of CD8⁺ T cells counted per field of view (FOV) intra-tumor for mock and treated mice (n = 6 mock and n = 7 Aza + ITF-2357 mice).

(G) Relative RNA expression-based enrichment plot for hallmark interferon gamma response gene set in tumors from LSL-Kras^{G12D} mice treated with mock and Aza + ITF-2357. Color gradation is representative of Log2 fold change over mock RNA expression (microarray, n = 2 per group).

(H) Percentage IFN γ ⁺ CD8⁺/CD3⁺ TILs by FACS in LLC subcutaneous tumors from 1-month mock- and Aza + ITF-2357-treated mice.

(I) Volcano plot of relative RNA expression for CD45⁺CD3⁺CD8⁺ FACS-obtained lymphocytes isolated from tumor-bearing lungs of 3-month Aza + ITF-2357-treated LSL-Kras^{G12D} mice as compared to mock mice. Genes in the upper left and right quadrants are significantly differentially expressed (microarray, n = 2 per group). Highlighted genes are involved in T cell fate determination.

(J and K) Fold change in expression of selected differentially expressed genes in FACS-obtained T cells from 3-month Aza + ITF-2357-treated mice. Genes shown are those that overlapped with exhaustion versus memory signatures (J) or exhaustion versus effector signatures (K) queried from Wherry et al. (2007) as defined in the Results. Genes on the left of each panel were differentially expressed by Aza + ITF-2357 and directionality in the gene set queried is on the right (red, upregulated; and blue, downregulated). The associated p value for the probability of overlap as derived by hypergeometric probability calculation is depicted above each panel. See also Figure S6.

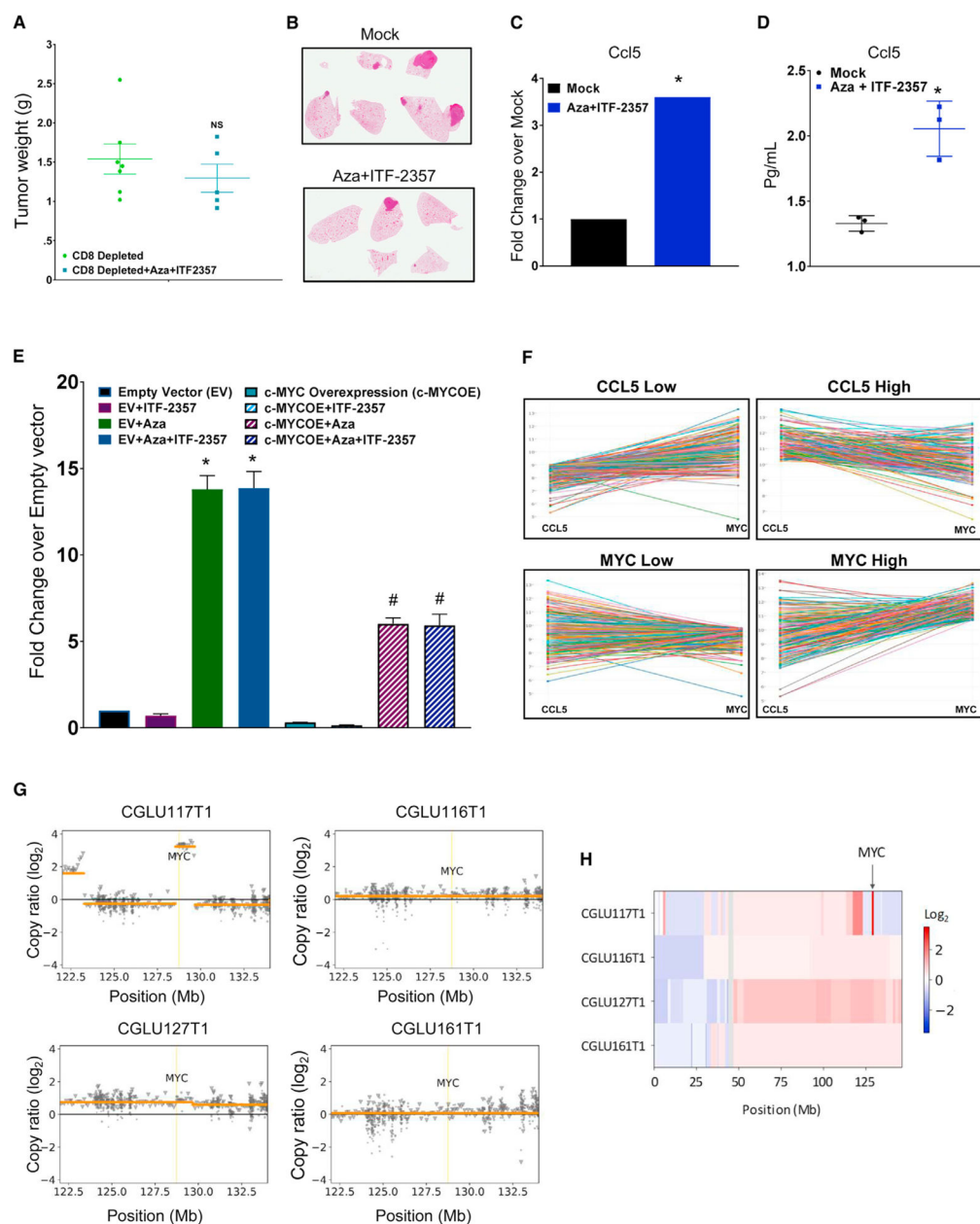


Figure 7. The Functional Role of Immune Parameters for Anti-tumor Effects of Combination Epigenetic Treatment

(A) Weights of subcutaneous LLC tumors from 1-month mock and Aza + ITF-2357 treated mice in the presence of CD8a-depleting antibody (n = 7 mock and n = 5 treated mice). NS, non-significant p value calculated by two tail t test.

(B) Representative H&E-stained images of lung metastases from the above 1-month mock and Aza + ITF-2357 treated mice in the presence of CD8a-depleting antibody (n = 7 mock and n = 5 treated mice).

(C) Expression of ccl5 RNA in lung tumors from LSL-Kras^{G12D} mice in response to 3 months of treatment with mock or Aza + ITF-2357 (microarray, n = 2, p value < 0.05 calculated by two-tailed t test).

(D) Quantitation of ccl5 protein levels in bronchoalveolar lavage from LSL-Kras^{G12D} mice treated with mock or Aza + ITF-2357 (n = 3; error bars, SD; *p value < 0.05 calculated by two-tailed t test).

(E) Relative fold change for CCL5 RNA expression in empty vector (EV) or MYC-overexpressing A549 human NSCLC cells treated with 500 nM Aza and/or 100 nM ITF-2357 (qRT-PCR, day 8, n = 3). Data are presented as mean ± SEM (*p value < 0.05 relative to EV and #p value < 0.05 relative to EV + Aza + ITF-2357, p values calculated by two-tailed t test).

(F) Profile interaction plots of TCGA RNA sequencing data for CCL5 and MYC expression across primary LUAD samples.

(G) Somatic copy number status of the *MYC* locus in NSCLC tumors. A 1.13-Mb segment overlapping *MYC* harbored a 12-fold amplification in CGLU117T1, the tumor that did not derive durable clinical benefit from immune checkpoint blockade. Orange lines represent segments of constant copy number. Circle and triangle markers indicate genomic bins in coding (target) and non-coding (off-target) regions, respectively.

(H) Copy number status of chromosome 8. The heatmap depicts segmental copy ratios after tumor purity correction, highlighting the amplification of the *MYC* locus for CGLU117T1 (arrow), but not for the other tumors analyzed.

See also Figure S6.



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Dual inhibition of DNA and histone methyltransferases increases viral mimicry in ovarian cancer cells

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Abstract

Ovarian cancer ranks as the most deadly gynecologic cancer and there is an urgent need to develop more effective therapies. Previous studies have shown that G9A, a histone methyltransferase that catalyzes mono- and di-methylation of histone H3 lysine9, is highly expressed in ovarian cancer tumors, and its overexpression is associated with poor prognosis. Here we report that pharmacological inhibition of G9A in ovarian cancer cell lines with high levels of G9A expression induces synergistic anti-tumor effects when combined with the DNA methylation inhibitor (DNMTi) 5-aza-2'-deoxycytidine (5-aza-CdR). These anti-tumor effects included upregulation of endogenous retroviruses (ERV), activation of the viral defense response, and induction of cell death, which have been termed “viral mimicry” effects induced by DNMTi. G9Ai treatment further reduced H3K9me2 levels within the long terminal repeat (LTR) regions of ERV, resulting in further increases of ERV expression and enhancing “viral mimicry” effects. In contrast, G9Ai and 5-aza-CdR were not synergistic in cell lines with low basal G9A levels. Taken together, our results suggest that the synergistic effects of combination treatment with DNMTi and G9Ai may serve as a novel therapeutic strategy for ovarian cancer patients with high levels of G9A expression.

Keywords

Epigenetic therapy; DNA methyltransferase inhibitor (DNMTi); G9A histone methyltransferase inhibitor (G9Ai); endogenous retrovirus (ERVs); ovarian cancer

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Disclosure of potential conflicts of interests

The authors declare no potential conflicts of interest.

INTRODUCTION

Recent advances in cancer genomics and epigenomics have revealed that the vast majority of human cancers harbor both genetic and epigenetic alterations (1–3). Epigenetic mechanisms, including DNA and histone modifications, as well as chromatin accessibility, determine how cells express genes and play a crucial role in regulating normal cellular functions (4). Aberrant DNA methylation occurs in almost every cancer type and is among the earliest and most common event during tumorigenesis (5). Unlike genetic abnormalities, which are difficult to reverse, epigenetic alterations are readily reversible, making them attractive therapeutic targets for cancer treatment (6).

A group of inhibitors that target epigenetic modifiers has emerged as an exciting group of compounds for use in the clinic (6). For example, the Food and Drug Administration (FDA) has approved DNA methyltransferase inhibitors (DNMTi), such as 5-aza-2'-deoxycytidine (5-aza-CdR), for the treatment of myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). Currently, there are ongoing clinical trials for solid tumors including colon, ovarian, liver, lung, and breast cancer (6,7). Proposed mechanisms underlying the clinical efficacies of DNMTis include demethylation of the promoters of tumor suppressor genes and bodies of oncogenes, thereby restoring more normal expression levels of these genes (8). Moreover, recent work has suggested a novel mechanism of action for DNMTis, termed “viral mimicry” (9–11). Viral mimicry is characterized by the up-regulation of endogenous retrovirus (ERV) transcripts, formation of cytoplasmic double-stranded RNA, and the induction of viral defense pathways (9–11). Importantly, the sensing of these ERVs by viral defense proteins leads to the death of colon cancer stem cells (11). The response to DNMTis in a subset of patients was impressive, with long-term durable anti-tumor effects (12–15).

However, primary and secondary resistance to the epigenetic therapies is common, likely because epigenetic processes are established and reinforced by both positive and negative feedback loops (16,17). For example, while DNMTis induce immediate and genome-wide demethylation of DNA, their effectiveness can be limited by a fairly rapid remethylation after the drug is removed (8), and/or by the involvement of alternative silencing mechanisms such as histone modifications (18). The future of epigenetic therapies, especially for solid tumors, relies on the rational design of combination treatments taking alternative silencing mechanisms into consideration.

DNA methylation plays an important role in silencing ERVs in somatic cells and in the male differentiating germline (19–22), yet it is dispensable for the repression of a subset of ERVs in the early germline, early embryo, and embryonic stem (ES) cells (23–25). Silencing of ERVs in early embryo and germline development depends primarily on histone methylation, notably at lysine 9 of histone H3 (H3K9). Many studies have shown that H3K9 lysine methyltransferases, including G9A, GLP, and SETDB1, are essential for repressing ERV expression in these stages (24–27). Importantly, G9A, which is responsible for catalyzing mono- or di-methylation of H3K9, is up-regulated in many types of cancers (28–30). G9A and H3K9me2 are present at DNA hypermethylated promoters of tumor suppressor genes in cancers, and are lost from the promoters when these genes are reactivated by DNMTis or in

DKO cells (31,32). The level of G9A overexpression has been associated with poor prognosis of ovarian, colon, breast, and lung cancers (30,33–36). We have previously shown that knocking down G9A sensitizes a colon cancer cell line to 5-aza-CdR treatment (37). In addition, G9Ai in combination with DNMTis has been proposed as a potential therapy for sickle cell disease, since this combination further activates fetal hemoglobin genes (38). This combination has also been shown to effectively increase the expression of immune genes in a colon cell line (39). These findings urged us to evaluate this novel combination as a potential epigenetic therapy approach for ovarian cancer. Here, we show that pharmacological inhibition of G9A synergistically enhances the efficacy of 5-aza-CdR by restoring strong promoter activity of ERVs and enhancing the viral mimicry effects in ovarian cancer cells with high levels of G9A and GLP expression.

MATERIALS AND METHODS

Cell lines and drugs

A2780, CAO3, PEO14, and OAW42 ovarian cancer cell lines were authenticated using the American Type Culture Collection (ATCC) human cell line authentication service. All cell lines were cultured according to standard mammalian tissue culture protocols using sterile technique. A2780 were maintained in McCoy's 5A medium, PEO14 cells in Roswell Park Memorial Institute (RPMI) 1640 medium and CAO3 and OAW42 in Dulbecco's Modified Eagle Medium (DMEM). All media (from GIBCO) were supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (GIBCO). Venor™ GeM Mycoplasma Detection Kit (Sigma-Aldrich) was used every three months to confirm all cell lines were mycoplasma-free. 5-Aza-CdR and G9Ai (UNC0638) were purchased from Sigma-Aldrich and APEX-BIO, respectively.

Cell viability assay

To test for dose-dependent response, 400 A2780 cells or 1000 CAO3, PEO14, and OAW42 cells were plated in each well of the 96-well plates 24 h prior to the treatment. A2780 and OAW42 cells were exposed to a dose of 5-aza-CdR (from 25 to 4800 nM) for 48 h, while CAO3 and PEO14 cells were treated with two consecutive daily doses of 5-aza-CdR (from 25 to 4800 nM) for 48 h. Subsequently G9Ai UNC0638 (from 100 to 20,000 nM) was added to the culture media until 7 d after the treatment. Cells were then incubated with CellTiter-Glo assay reagent (Promega) for 10 min and luminescence was measured using a Synergy HT multi-mode microplate reader (BioTek).

Evaluation of combination effect

Cell viability data were normalized to their corresponding untreated controls for each treatment condition and were expressed as percentage fractional affect (Fa). CompuSyn software (ComboSyn, Inc.) was used to calculate combination index (CI) values of Fa under different conditions using the Chou-Talalay equation (40) $CI = (D)_{VC} / ((D_m)_{VC} (Fa / (1 - Fa))^{1/m_1} + (D)_{Aza} / ((D_m)_{Aza} (Fa / (1 - Fa))^{1/m_2})$, where D is the concentration of G9Ai and 5-aza-CdR either alone or in combination to achieve a given Fa. The median effect dose (D_m), m_1 (G9Ai), and m_2 (5-aza-CdR) values were determined using the median-effect equation (41) $(Fa) / (1 - Fa) = ((D) / (D_m))^m$ for G9Ai and 5-Aza-CdR treatment alone. The CI values

define synergistic effect (42) when $CI < 1$, additive effect when $CI = 1$, and antagonism when $CI > 1$.

Cell-cycle analysis and quantification of dead cell percentages

Cells were harvested and then stained with the amine reactive viability dye Ghost Dye Violet 450 (Tonbo Biosciences) at 4°C for 30 minutes according to the manufacturer's protocol. Cells were then fixed with 66% ethanol and stored at 4°C until ready to stain with propidium iodide (PI). Fixed cells were pelleted and resuspended in 500 μ l of PI staining solution (PBS + 100 μ g/ml RNase A + 50 μ g/ml PI) and incubated overnight at 4°C. For flow cytometry analysis, cells were filtered through cell strainer snap caps (Fisher Scientific) and then analyzed on a CytoFLEX S (Beckman Coulter). Cell cycle was analyzed using ModFit LT software (Verify Software House, www.vsh.com) and the percentage of cells alive and dead was measured using FlowJo v10.0.7 (FlowJo, LLC).

Chromatin fractionation and western blot analysis

Cell lysis and washing steps were performed in cold buffer containing 10 mM PIPES, pH 7.0, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 1× EDTA-free protease inhibitor (Roche), 1× phosphatase inhibitor cocktail (Sigma), and 0.1% Triton X-100. Whole cell and chromatin fractions were treated with benzonase (Sigma-Aldrich) prior to western blot analysis. Whole cell, chromatin associated or soluble fractions were mixed with SDS/ β -mercaptoethanol loading buffer and resolved on a Biorad 4–15% gradient SDS/PAGE gel. Antibodies against G9A (Perseus Proteomics Inc, PP-A8620A-00), Tubulin (Cell Signaling, 86298S), TBP (Santa Cruz Biotech sc-74596), H3K9me1 (Epicyphe 13–0014), H3K9me2 (Abcam #1220), H3K9me3 (Active Motif #39161) and total H3 (Abcam #12079) were used. Proteins were visualized using the Clarity Western ECL substrate (Bio-Rad) and ChemiDoc™ XRS+ imaging system (BioRad).

RNA sequencing

Total RNA was extracted with Trizol reagent (Invitrogen), followed by clean-up and Turbo DNase I (Invitrogen) treatment with Zymo Direct-Zol RNA mini prep kit (Zymo Research) according to the manufacturer's protocol. RNA quality was assessed using Agilent 2100 bioanalyzer with RNA Nano chips (Agilent Technologies, Inc.). For directional RNA-seq with ribosomal RNA (rRNA) reduction, libraries were prepared using KAPA Stranded RNA-Seq Kit with RiboErase (HMR) (KapaBiosystems) and sequenced as single-end 75 bases on a NextSeq 500 instrument (Illumina) at the Van Andel Research Institute Genomics Core. The RNA-seq reads were mapped to the human transcriptome using TopHat version 2.1.0 with NCBI RefSeq as the reference annotation of transcripts. The transcripts were assembled and quantified using Cufflinks version 2.2.1. Differential expression is measured using edgeR package from the Bioconductor project.

Identification of bidirectionally transcribed ERVs

To quantify the transcription of repetitive element at a specific locus, we used Repeatmasker (<http://www.repeatmasker.org>) annotation as our input and considered only uniquely mapped reads (with mapping quality threshold 10) as previously described (10). For each transcript,

we separated reads mapped to the two strands. We considered a transcript as bidirectionally transcribed if the smaller read count divided by the greater read count is over 0.5.

MethyLight assay and bisulfite sequencing

On day 5 after treatment, A2780, CAOV3, PEO14, and OAW42 cells were harvested and genomic DNA was purified by phenol–chloroform extraction and ethanol precipitation. Bisulfite conversion was performed using the EZ DNA Methylation Kit (Zymo). MethyLight assays were then performed as previously described (43) using the primers listed in Supplementary Table 1. For A2780 cells, bisulfite PCR was performed using bisulfite-converted DNA with primers listed in Supplementary Table 1, and the products were cloned using the pGEM-T Vector System I (Promega) as previously described (10).

Chromatin immunoprecipitation

Single nucleosome preparation was performed according to the Dilworth lab native ChIP protocol (44). Briefly, A2780 cells (10^7 cells) before and after 5-aza-CdR, G9Ai, or combination treatment were harvested and washed twice and resuspended in ice-cold Buffer N (15 mM Tris pH7.5, 15 mM NaCl, 60 mM KCl, 8.5%(w/v) Surcose, 5 mM MgCl₂, 1 mM CaCl₂, 1 mM DTT, 200 μ M PMSF, 1X cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail (Roche)). To prepare nuclei, cells were lysed in 1 mL Lysis Buffer (Buffer N supplemented with 0.3% NP-40 substitute (Sigma)) for 10 min at 4°C, and nuclei were collected by centrifugation ($500 \times g$ for 5 min at 4°C), resuspended in 1 mL of Buffer N, then sedimented through 7.5 mL sucrose cushion (10 mM HEPES pH7.9, 30%(w/v) sucrose, 1.5 mM MgCl₂ and centrifuged $13000 \times g$ using Sorvall swinging bucket for 12 min at 4°C). To isolate single nucleosomes, the nuclei were digested with MNase (1U Worthington MNase per 70 μ g of chromatin at 37°C for 10 min), the nucleosomes were then purified by hydroxyapatite chromatography, and adjusted to a concentration of 20 μ g/mL with ChIP Buffer 1(25 mM Tris pH 7.5, 5 mM MgCl₂, 100 mM KCl, 10% (v/v) glycerol, 0.1% (v/v) NP-40 substitute) and analyzed using 2% agarose gel.

H3K9me2, H3K4me3 and H3K27ac ChIP were performed as previously described (45), using 5 μ g of nucleosome pulled down with 10 μ g of anti-H3K9me2 (Abcam ab1220), anti-H3K4me3 (Abcam ab1012), and anti-H3K27ac (Active Motif AM39133) antibody on Dynabeads Protein G (Invitrogen) for 2 h at 4°C. Initial chromatin (10%) for each IP was set aside to serve as ChIP input. The beads were washed 3 times with ChIP Buffer 2 (10 mM Tris pH 7.5, 5 mM MgCl₂, 300 mM KCl, 10% (v/v) glycerol, 0.1% (v/v) NP-40 substitute), twice with ChIP Buffer 3 (10 mM Tris pH 7.5, 250 mM LiCl, 1mM EDTA, 0.5% Na•Deoxycholate, 0.5%(v/v) NP 40 substitute), twice with 1 $\times$ TE buffer followed with two elution steps in Elution buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1% w/v SDS). After proteinase K (Roche) digestion (65°C 1h), sample DNA was purified using Agencourt AMPure XP beads (Beckman Coulter) prior to qPCR analysis. Primers used in this study are listed in Supplementary Table 1.

Accession codes

All data have been deposited at the Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) with accession code GSE108223.

RESULTS

Combination treatment with G9Ai and 5-aza-CdR induces synergistic effects to promote cell death in ovarian cancer lines A2780 and CAOV3, but not in PEO14 and OAW42.

RNA sequencing data from The Cancer Genome Atlas (TCGA) project reveals that G9A expression is up-regulated in most types of cancer, and that the highest levels are found in primary ovarian tumors amongst 33 cancer types (Figure S1A). In addition, the related histone methyltransferase GLP is also significantly overexpressed in ovarian tumors compared to normal tissues (Figure S1B). We therefore used four ovarian cancer cell lines A2780, CAOV3, PEO14 and OAW42 as our *in vitro* models for treatment. We first tested various concentrations of a G9A inhibitor (UNC0638) in A2780 cells and found that the global levels of H3K9me1 and H3K9me2 were efficiently reduced by 400 nM G9Ai at 48 h after the treatment (Figure 1A). In addition, UNC0638 at 400 nM exhibited low cellular toxicity for all four ovarian cancer cell lines (Figure S2). This is consistent with a previous report that UNC0638 has high G9A/GLP inhibition potency and low cellular toxicity with concentrations in the nanomolar range (46). We therefore used 400 nM UNC0638 to determine the optimum dosing schedules for combination treatments with low dose 5-aza-CdR (100 nM) in A2780 cells. We explored different time points to add G9Ai relative to 5-aza-CdR and varied the duration of exposure to these compounds (Figure 1B). The expression levels of HERV-Fc1, a marker for the effects of DNMTis (9,10), were used as read-out and were measured by quantitative RT-PCR. The dosing schedule in which A2780 cells were exposed to 5-aza-CdR for 48 h and subsequently G9Ai until harvest achieved the greatest effect on upregulating HERV-Fc1 (Figure 1B). Therefore this schedule was applied to A2780 and OAW42 cells in subsequent experiments (Figure 2A). Since the doubling times for CAOV3 and PEO14 cells were greater than 24 h, we treated these cells with two consecutive daily doses of 5-aza-CdR for 48 h to compensate for the fact that incorporation of the drug requires cell doubling (Figure 2B).

Following these dosing schedules, we tested whether the combinations of G9Ai and 5-aza-CdR could induce synergistic effects in ovarian cancer cells. We measured dose-dependent inhibition of cell proliferation (as fraction affected (f_a)) with various concentrations of 5-aza-CdR and G9Ai alone or in combination using the CellTiter-Glo luminescent cell viability assay (Figure S3). Combination treatments resulted in increased inhibition of cell proliferation (increased f_a) compared to single compound-treatment in A2780 and CAOV3 cells (Figure S3). The Chou-Talalay analyses (47) determined that the two compounds indeed acted synergistically (combination index (CI) values <1) under the majority of conditions in A2780 and CAOV3 cells (Figure 2C). In contrast, the synergistic effects were limited in PEO14 and OAW42 cells (Figure 2C and S3).

As further characterization of the effects by combination treatments, we next monitored changes of total cell counts upon treatments using a Coulter counter. In this experiment, A2780, CAOV3, PEO14, and OAW42 cells were cultured in 100 mm dishes and treated with 400 nM G9Ai alone or in combination with 100 nM 5-aza-CdR following the dosing schedules outlined above. The results showed that A2780 and CAOV3 cells exhibited significantly reduced total cell counts by combination treatment compared to untreated or

single compound-treated cells (Figure S4). Since this effect could be due to reduced cell proliferation rate or increased cell death, we next examined the cellular phenotypes by flow cytometry using an amine reactive viability dye and performed cell cycle analysis to test these possibilities. The reduced total cell counts in A2780 and CAOV3 cells were associated with increased cell death by combination treatment (Figure 2D), while cell cycle parameters remained unchanged compared to 5-aza-CdR treatment (Figure 2E). These data indicated that low dose of G9Ai and 5-aza-CdR could act synergistically by promoting cell death in A2780 and CAOV3 cells. To the contrary, no significant differences in total cell counts (Figure S4), percentages of dead cells (Figure 2D) and cell cycle parameters (Figure 2E) were found between combination treatment and 5-aza-CdR treated PEO14 and OAW42 cells. These data further confirmed that the two compounds have no synergistic effects on cell growth or inducing cell death in PEO14 and OAW42 cells.

Combination treatment with G9Ai and 5-aza-CdR synergistically up-regulate viral defense genes in A2780 and CAOV3, but not in PEO14 and OAW42 cells.

To investigate gene expression changes underlying the synergistic anti-tumor effects of combination treatments, we sequenced total RNA in A2780, CAOV3, PEO14, and OAW42 cells after treatments. A p-value of 0.05 and two-fold expression change were used as cut-offs to identify differential expressed genes between treated and untreated cells from two independent experiments. In general, G9Ai treatment alone did not induce global gene expression changes in the four ovarian cancer cell lines (Figure S5), with only a few genes significantly up-regulated in A2780 and CAOV3 cells (Figure 3A). Although genes up-regulated by 5-aza-CdR and combination treatment largely overlapped, more genes were up-regulated by combination treatment compared to 5-aza-CdR treatment alone in all four cell lines (Figure 3A, S5 and S6A-C). We therefore performed gene ontology (GO) analysis to identify the functions of genes that were uniquely up-regulated by combination treatment. Interestingly, the immune response pathways, especially the interferon pathways as cellular defense response to virus were overrepresented in the set of genes uniquely up-regulated by combination treatments in A2780 and CAOV3 cells (Figure 3B). We and others have previously shown that these viral defense genes can be up-regulated by 5-aza-CdR treatment and are responsible for inducing apoptosis or inhibiting proliferation of cancer cells (9–11). Therefore, the expression status of a panel of 24 viral defense genes were examined after G9Ai, 5-aza-CdR, and combination treatment compared to untreated cells (Figure 3C). Consistent with previous reports (9–11), 5-aza-CdR treatment up-regulated these viral defense genes in a cell type specific pattern (Figure 3C and S7A-B); whereas G9Ai treatment alone slightly increased the expression of these genes less than 2 fold relative to untreated cells (Figure 3C and S7A-B). These viral defense genes were further up-regulated by the combination treatment (Figure 3C and S7A-B), suggesting that the synergistic anti-tumor effects by the combination treatment were contributed in part by the increased viral defense response in A2780 and CAOV3 cells. In contrast, the same analysis showed that the viral defense pathway were not further up-regulated by combination treatments in PEO14 and OAW42 cells (Figure S6B-C and S7C-D), in which no synergy was found (Figure 2C-E). This observation strengthens our conclusion that the synergistic effects between G9Ai and 5-aza-CdR are associated with further induction of the viral defense pathway.

ERVs were up-regulated by G9Ai, 5-aza-CdR, and combination treatment in a cell line specific pattern.

Since induction of ERV expression, especially as dsRNA, is key for triggering the viral defense response by 5-aza-CdR (9–11), we next tested whether ERVs were further up-regulated by combination treatment. We mapped transcripts unique to ERV loci in two replicates of the RNA-seq data for all four cell types after the various treatment regimens. ERVs located within coding genes (including introns) were removed from analysis to focus on expression induced by their LTRs rather than as part of host genes. G9Ai treatment alone increased the total reads of intergenic ERVs in CAO3 but not in A2780 cells (Figure 4A). While 5-aza-CdR increased the total intergenic ERV counts compared to untreated A2780 and CAO3 cells, a further increase after combination treatment was found (Figure 4A).

Our previous data also showed that some ERVs could be bidirectionally transcribed and that these overlapping transcripts can pair to form dsRNA (10). In addition, dsRNA are preferred substrates for the RIG-I-like receptors (RLRs), such as RIG-I or MDA5, and are important inducers of antiviral immunity (9,11,48). We therefore analyzed the strand-specific RNA sequencing data and detected increases in bi-directional transcribed ERVs mainly by 5-aza-CdR and combination treatment, similar to total intergenic ERVs (Figure 4B). G9Ai treatment alone, however, significantly increased the total counts of the bi-directionally transcribed ERVs in both A2780 and CAO3 cells (Figure 4B). These data support a correlation between total bi-directionally transcribed ERVs and the up-regulation of viral-defense genes (Figure 3C and 4B). Therefore, combination treatment of G9Ai and 5-aza-CdR enhanced the previously reported “viral mimicry” response by 5-aza-CdR alone, with increased total ERV counts, especially the dsERV counts, that further up-regulated an anti-viral immune response in these cells.

Interestingly, we observed that G9A and DNA methylation appear to repress a distinct set of ERVs, since the majority of ERVs up-regulated by G9Ai treatment did not overlap with those up-regulated by 5-aza-CdR (Figure 4C). This is consistent with our recent finding that there is a switch in silencing mechanisms depending on the evolutionary age of ERVs (49). Evolutionary “young” ERVs tend to be CpG-rich and are mainly repressed by DNA methylation, while “intermediate aged” ERVs with lower CpG density are predominantly repressed by histone modifications, particularly H3K9 methylation (49). The combination treatment of G9Ai with 5-aza-CdR resulted in the activation of more ERVs than single compound-treated A2780 and CAO3 cells (Figure 4C and S8), the majority of which were uniquely up-regulated by the combination treatment (Figure 4C). These data suggest that these ERVs might be silenced by both G9A and DNA methylation. Alternatively, an “epigenetic switch” might happen at these ERVs which become silenced by histone methyltransferases such as G9A upon loss of DNA methylation. Similar mechanisms have been described previously (18,49–51). We also observed that different ERVs were up-regulated in A2780 and CAO3 cells, showing a cell line specific pattern of ERV up-regulation (Figure 4D). Using quantitative RT-PCR, we confirmed that HERV-Fc1 and MLT1N2 were robustly up-regulated by 5-aza-CdR and further by combination treatments in both cells (Figure 4D and S9A-B). However, LTR12C, a family of ERVs previously found to

be induced by 5-aza-CdR treatment (10,52), were not further up-regulated by combination treatment (Figure S9A-B).

In PEO14 and OAW42 cells, however, no further increases in intergenic ERVs or bi-directionally transcribed ERVs were found with combination treatments compared to 5-aza-CdR treated cells (Figure S10A-B), which might explain why no further up-regulation of the viral defense genes is elicited by combination treatments. In summary, the cell lines exhibited variable responses to the combination treatments with respect to ERV up-regulation.

G9Ai treatment does not alter DNA methylation levels at LINE-1 elements and 5'LTR of HERV-Fc1.

To address the cell-line dependent effects on ERV up-regulation by the combination treatments, we next examined the effectiveness of 5-aza-CdR in inhibiting DNA methylation. We previously observed that 5-aza-CdR treatment in HCT116 cells with stable knock-down of G9A causes a decrease in DNA methylation levels at the promoters of the G9A-targeted genes (37). We therefore used a MethyLight assay (43) to determine DNA methylation level at LINE-1 elements, an approximate measure of global DNA methylation levels. DNA methylation was not inhibited by G9Ai alone, but was more strongly inhibited by 5-aza-CdR in A2780 and CAOV3 than in PEO14 and OAW42 cells (Figure 5A and S11). No synergy in inhibition of DNA methylation was seen in any of the combination treatments (Figure 5A). We then performed bisulfite sequencing to examine the DNA methylation levels at the 5' LTR of HERV-Fc1, which serves as its promoter (illustrated in Figure 5B), since the expression levels of HERV-Fc1 were further up-regulated by combination treatment compared to 5-aza-CdR treatment alone in A2780 cells (Figure 1B). Consistent with the MethyLight assay results, bisulfite sequencing data showed no robust changes in DNA methylation induced by G9Ai treatment alone or in combination with 5-aza-CdR in A2780 cells (Figure 5C). Thus the enzymatic activity of G9A was dispensable for the maintenance or restoration of DNA methylation status at LINE-1 elements or the LTR of HERV-Fc1 after 5-aza-CdR treatment. To summarize, although DNA demethylation was required for up-regulation of a group of ERVs such as HERV-Fc1, the synergistic effects of the combination treatment to further up-regulate these ERVs did not involve further DNA demethylation by G9Ai.

Combination treatment further reduces the level of chromatin bound G9A protein and global levels of H3K9me1/2/3 in A2780 cells.

Reduction of global and gene locus-specific G9A protein and H3K9 di-methylation levels by 5-aza-CdR treatment alone have been reported previously (30–32). Since the G9Ai (UNC0638) acts as a competitive inhibitor, the combination treatment with G9Ai and 5-aza-CdR could further reduce the chromatin bound G9A protein and H3K9 methylation levels to increase promoter activity synergistically. To test this possibility, we performed chromatin association assays to quantify chromatin-bound G9A protein levels at d 5 after the treatments in A2780 cells. G9Ai treatment alone did not significantly change the chromatin-bound G9A protein levels (Figure 6A-B), but increases free G9A proteins in both whole cell and soluble fractions (Figure S12A-C). 5-Aza-CdR treatment resulted in a significant

reduction of chromatin-bound G9A protein levels, which were further reduced by combination treatment in A2780 cells (Figure 6A-B), despite the fact that more soluble G9A proteins were found after combination treatment than 5-aza-CdR treatment alone (Figure S12A and C).

We next quantified the levels of H3K9 methylation by Western blot in A2780 cells, and found that both G9Ai and 5-aza-CdR significantly reduced global H3K9me1/2/3 levels, especially for H3K9me2, and the combination treatment further reduced the global levels of these repressive marks (Figure 6C-D). Interestingly, global H3K9me3 levels were also reduced by the treatments in A2780 cells (Figure 6C-D). Although the G9A/GLP complex does not catalyze tri-methylation of H3K9, as the levels of H3K9me1/2 decreased, other lysine methyltransferase activities might have been reduced by depleting their substrates. Taken together, we observed a global reduction of chromatin-bound G9A protein and H3K9me1/2/3 levels by combination treatment compared to treatment with either compound alone, which might serve as the underlying mechanism for the synergistic effects of combination treatments in A2780 cells.

We also examined the H3K9me1/2 levels in OAW42 cells, in which no synergy was found between G9Ai and 5-aza-CdR. Interestingly, the results showed that 5-aza-CdR treatment alone efficiently reduced H3K9me1/2 levels to less than 20% of those in untreated cells (Figure S13A-B), compared to about 75% in A2780 cells (Figure 6C-D). The combination treatment only slightly reduced the H3K9me1/2 levels compared to those after 5-aza-CdR treatment alone (Figure S13A-B). These data suggested that in OAW42 cells, 5-aza-CdR treatment is sufficient to remove most of the repressive H3K9me1/2 marks. The addition of G9Ai therefore might not be effective in further removing these marks globally. A search in our RNA seq data revealed that the A2780 and CAOV3 cells showed higher levels of G9A/GLP expression, while in the cells which did not respond synergistically, lower levels of G9A/GLP transcripts were found (Figure S13C). These data suggested that the synergy between G9Ai and 5-aza-CdR might be in part associated with G9A/GLP expression levels.

Combination treatment further decreases H3K9me2 levels at LTRs of ERVs in A2780 cells.

The RNA-seq data revealed that a cluster of ERVs could be further up-regulated by combination treatments making it likely that the LTRs of these ERVs were suppressed by H3K9me1/2 in addition to DNA methylation. Although our results demonstrated strong decreases in global H3K9me2 by G9Ai or combination treatment (Figure 6C-D), it was still not clear whether these changes also occur at ERV LTRs. We therefore performed chromatin immunoprecipitation assays followed by quantitative PCR (ChIP-qPCR) to detect the chromatin modification status at the LTRs of HERV-Fc1 and MLT1N2, as two examples in A2780 cells. There was enrichment of the repressive mark H3K9me2 at LTRs of these ERVs compared to the promoters of β -actin (ACTB) (Figure 6E). Indeed, the repressive H3K9me2 mark was dramatically reduced by G9Ai treatment alone. 5-Aza-CdR treatment resulted in increases, rather than decreases, of H3K9me2 levels at LTRs of these ERVs (Figure 6E), suggesting an “epigenetic switch” to silencing the ERVs by histone modifications upon loss of DNA methylation (49,51). H3K9me2 levels were further reduced by the combination treatment at LTRs of these ERVs, associated with an increase in active marks H3K4me3 and

H3K27ac (Figure 6E). Taken together, DNA demethylation by 5-aza-CdR and the additional chromatin alterations at ERV LTRs (decreasing H3K9me2 and increasing H3K4me3 and H3K27ac) induced by the combination treatment likely contributes to the synergistic up-regulation of ERVs.

DISCUSSION

Our study shows that inhibiting both DNA methylation and the histone methyltransferase G9A synergistically induces anti-tumor effects and enhances the “viral mimicry” response in ovarian cancer cells with high levels of G9A/GLP expression. Importantly, we have shown that G9A was overexpressed in primary ovarian tumors, and was the highest amongst 33 TCGA cancer types. In addition, over expression of G9A has been associated with poor prognosis of EOC (29). Therefore, a treatment strategy targeting both G9A and DNA methylation provides a promising treatment option since DNA methylation inhibitors have already been tested in clinical trials with promising outcomes (53,54). Our dual inhibition approach suggests a potentially new epigenetic therapy combination that may serve as a novel therapeutic strategy for ovarian cancer patients.

A key mechanism underlying the synergistic effects of G9Ai and 5-aza-CdR on ERV up-regulation may involve the “epigenetic switch” in which a group of ERVs were repressed by G9A upon loss of DNA methylation. This is in line with recent work from our laboratories that there is a gain in repressive marks such as H3K9me2/3 or H3K27me3 after DNA demethylation to maintain the silencing of some ERV LTRs (49–51). Since H3K9me2 levels at LTRs of HERV-Fc1 and MLT1N2 increased after 5-aza-CdR treatment, this histone mark may well be responsible for repressing these ERVs. This is different from the situation at promoters of protein coding genes where 5-aza-CdR effectively reduced H3K9me2 levels and depleted G9A (31,32). Therefore, the addition of G9Ai after 5-aza-CdR treatment could enhance the expression of these ERVs by further reducing the repressive H3K9me2 marks. It is worth noting that the “epigenetic switch” might be dependent on the expression levels of G9A/GLP or other silencing complexes such as the polycomb repressive complex 2 (PRC2) (51). In addition, different responses to DNA demethylation by 5-aza-CdR might also contribute to the variable synergistic effects observed in these cell lines after the combination treatment.

An interesting finding in this study is that G9A and DNA methylation appear to repress distinct sets of ERVs. This is consistent with our recent finding that DNA methylation tends to predominate in silencing the evolutionarily younger ERVs, which have higher CpG densities, while histone methyltransferases mainly target the middle-aged ERVs with less CpG density (49). Therefore, the combination treatment results in up-regulation of more ERVs that include those repressed by G9A or DNA methylation alone. For evolutionary “young” ERVs such as HERV-Fc1 and MLT1N1, DNA methylation plays a major silencing role, and demethylation by 5-aza-CdR can induce their expression while G9Ai treatment alone is not sufficient to up-regulate these ERVs. However, they were still repressed by G9A after 5-aza-CdR treatment (termed as the “epigenetic switch” mentioned above). Combination treatment simultaneously removed both repressive mechanisms, resulting in further up-regulation of these ERVs. In this way, combination treatment with the two

inhibitors synergistically strengthens the “viral mimicry” response and leads to increased anti-tumor effects.

Our findings also show that not all ERVs were equal with respect to their abilities to activate the viral defense pathway. In CAOV3 cells, where G9Ai treatment alone up-regulated equivalent numbers of ERVs as 5-aza-CdR (Figure 4A-C), the levels of induced viral defense genes were lower than those up-regulated by 5-aza-CdR treatment (Figure 3C). This observation reinforces our previous findings that the intermediate age ERVs mainly silenced by histone methyltransferases did not elicit a strong antiviral response (49). The strength of the viral mimicry response may be determined by the expression of evolutionary “younger” ERVs silenced by DNA methylation. Further studies are needed to characterize the effects of specific ERVs in inducing the antiviral response, which may be crucial for patient responses to the combination epigenetic therapy.

Our results also suggest that G9A plays different roles in various tissues or cell types. For example, it has been shown that G9A is essential in repressing developmental genes at euchromatic regions during early embryogenesis (55). In breast cancer cells, G9A has been shown to be responsible for aberrant silencing of tumor suppressor genes (30). In our study, G9Ai treatment alone did not induce up-regulation of tumor suppressor genes. In fact, very few genes were up-regulated, instead, a subset of ERVs were up-regulated by G9Ai treatment alone in some ovarian cancer cells, highlighting the important role of G9A in suppressing ERVs in ovarian cancer cells.

UNC0638 was designed based on the structure of G9Ai BIX01294 with improved *in vitro* potency and increased cell membrane permeability (46). UNC0638 exhibited high potency and specificity for inhibiting G9A and GLP with very low off-target toxicities (46). However, its potential for clinical application is limited by poor pharmacokinetics *in vivo*. The development of next generation G9Ai with comparable potency is needed to improve *in vivo* pharmacokinetics. Our current preclinical study has provided a rationale for the future clinical application using the combination of inhibitors for both G9A and DNMTs. We predict that this combination would be very helpful for those cancer types that exhibit overexpression of G9A or GLP proteins, especially for those cancers with poor prognosis correlated with higher G9A expression levels, such as ovarian cancer. Our results are limited to the four cell lines tested so that the generality of the results need to be replicated in a larger set. However, they are consistent with recent data we have obtained with knockdowns of histone methyltransferases, and point to the need for patient stratification based on the levels of G9A/GLP before combination treatment is initiated.

Conclusions:

DNA methylation and the histone modifications H3K9me1/2 are both important for silencing ERVs. Dual inhibition of these processes results in synergistic up-regulation of ERVs and induces an anti-viral response in some but not all cell lines, serving as a basis for exploring this novel combination treatment in the clinic especially for ovarian cancer patients with high levels of H3K9me1/2 histone methyltransferases G9A/GLP expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SIGNIFICANCE

Dual inhibition of DNA methylation and histone H3 lysine 9 di-methylation by 5-aza-CdR and G9Ai results in synergistic upregulation of ERV and induces an anti-viral response, serving as a basis for exploring this novel combination treatment in ovarian cancer patients.

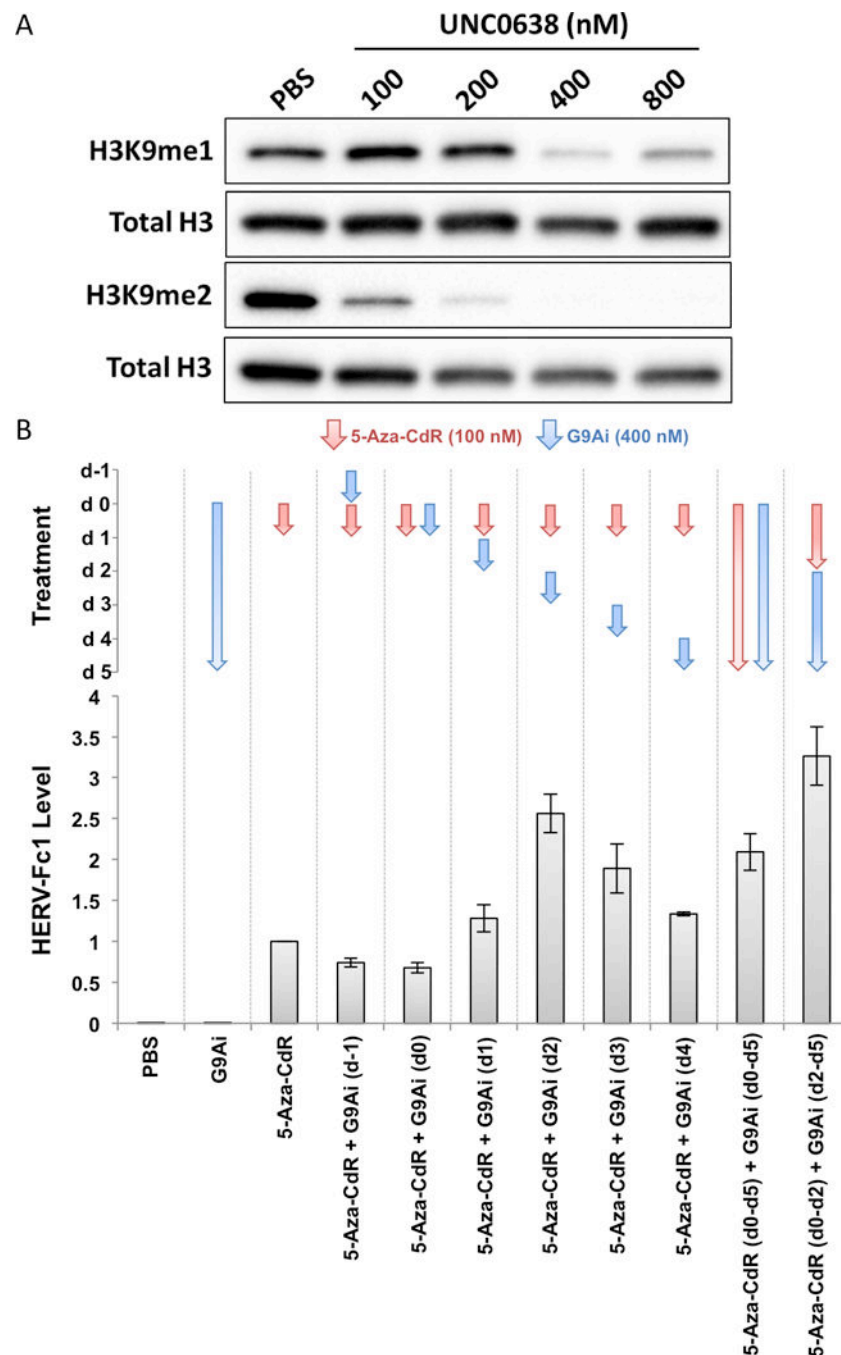


Figure 1. Optimization of treatment schedule by combination of G9Ai and 5-aza-CdR in A2780 cells. A) Western blot analysis of H3K9me1 and H3K9me2 levels in response to increasing concentrations of UNC0638 in A2780 cells 48 h after treatment. Total histone H3 was used as a loading control. Representative blots from 3 independent experiments were shown. (B) Effects of dosing schedule by combination treatment with G9Ai and 5-aza-CdR on HERV-Fc1 expression. A2780 cells (2.5×10^5) were seeded in 100 mm dishes at d -1, treated with 400 nM G9Ai, 100 nM 5-aza-CdR or their combinations according to the schedule shown at

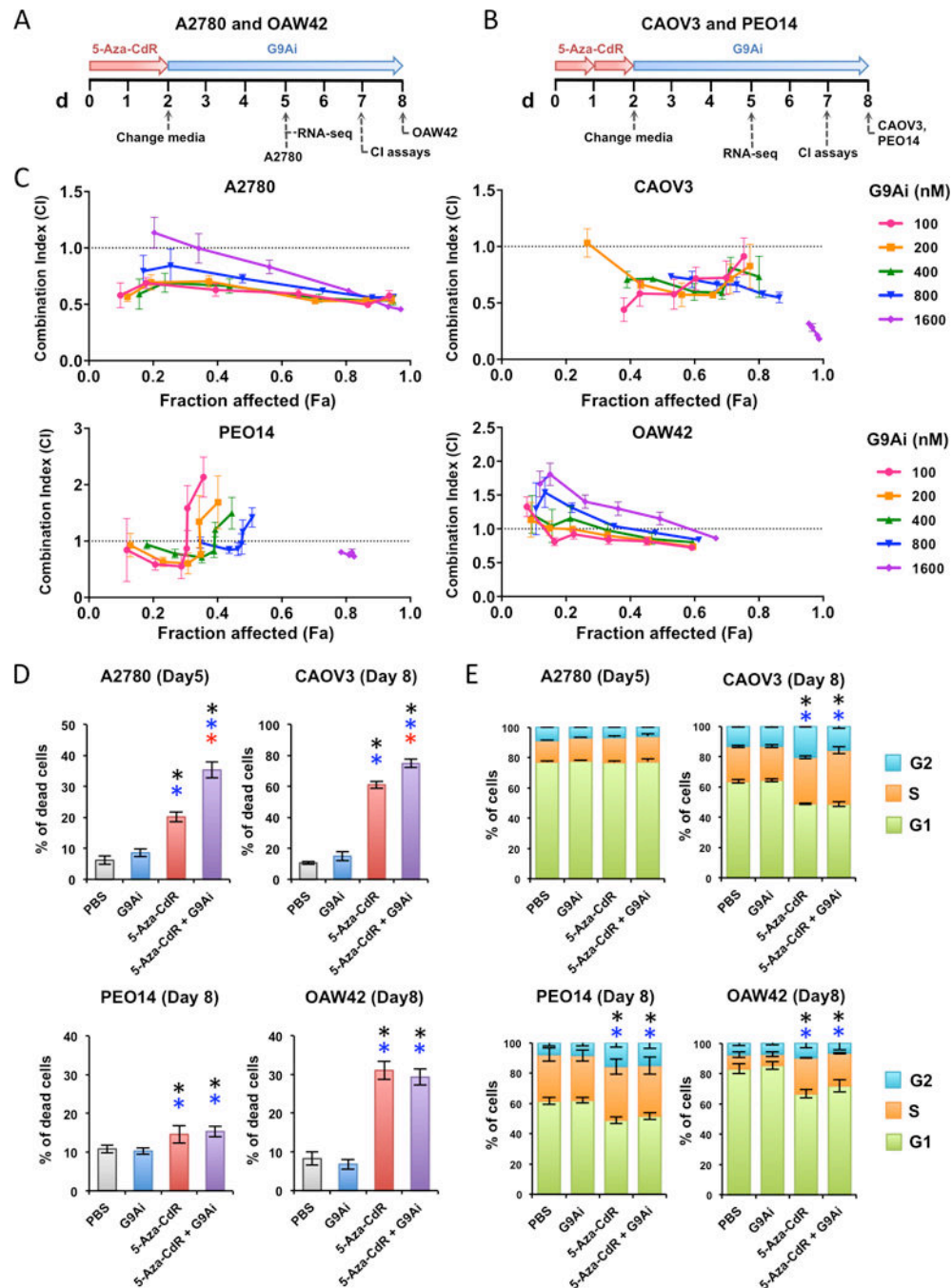
the top panel. Cells were harvested at d 5 after 5-aza-CdR treatment. HERV-Fc1 expression levels were then assayed by quantitative RT-PCR using the expression levels of TATA-binding protein (TBP) as a loading control, and normalized to the level of HERV-Fc1 expression after 5-aza-CdR treatment alone. Values are presented as mean $\pm$ SEM of three independent experiments. A one-way repeated measures ANOVA was used for statistical analysis. *P <0.05.

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**Figure 2.**

Effects of combination treatment with 5-aza-CdR and G9Ai on proliferation, cell death, and cell cycle in A2780, CAOV3, PEO14 and OAW42 cells. A) Dosing schedule for the combination treatment in A2780 and OAW42 cells. B) Dosing schedule for the combination treatment in CAOV3 and PEO14 cells. C) Chou-Talalay model of the effects of combination treatment with varying concentrations of G9Ai and 5-aza-CdR. The fraction affected (Fa) values were determined using CellTiter-Glo luminescent cell viability assay as shown in Figure S3. Combination index across the Fa values were calculated by CompuSyn software

(ComboSyn, Inc.). $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additive effect, and antagonism, respectively. Values are presented as mean $\pm$ SEM of three independent experiments. D) Amine-based viability assay using Ghost Dye Violet 450 (Tonbo Biosciences) analyzed by flow cytometry. Values are mean $\pm$ SEM of three independent experiments. One-way repeated measures ANOVA with Geisser-Greenhouse's epsilon correction was used for statistical analysis. * $P < 0.05$, stars in black, blue and red indicate comparison to untreated, G9Ai treated, and 5-aza-CdR treated samples, respectively. E) Bar graphs show the percentages for G1, S, and G2/M cells by propidium iodide (PI) staining and analyzed by flow cytometry. Values are mean with 95% CI from three independent experiments. Beta mixed-effects regression with a random intercept to account for experimental (batch) differences were used to compare the differences between cell phases and treatments. FDR multiple testing adjustments were performed to account for multiple testing. R v3.4.1 was used to fit these regressions (<https://cran.r-project.org/>). * $P < 0.05$ for all cell cycle phases. Stars in black and blue indicate comparison to untreated, and G9Ai treated samples, respectively.

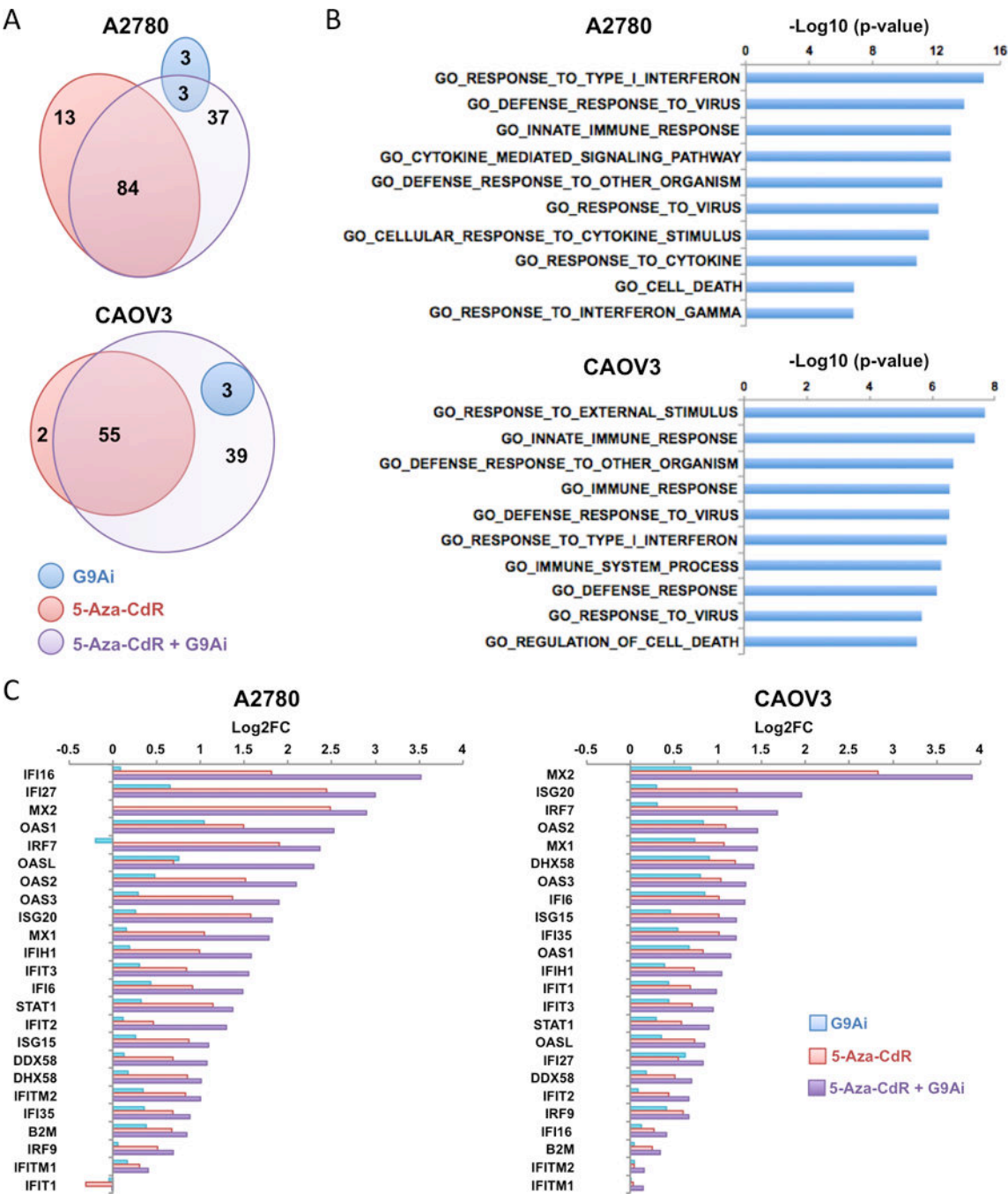


Figure 3. Combination treatments further up regulated viral defense genes in A2780 and CAOV3 cells. A) Venn diagrams show the up-regulated genes (greater than two-fold change, $p < 0.05$) after G9Ai, 5-aza-CdR, and combination treatment compared to untreated cells. B) Bar graphs show the top ten Gene Ontology Biological Processes (GOBP) terms of genes uniquely up-regulated by combination treatment (greater than two-fold change, $p < 0.05$). C) Bar graphs show the expression fold change (in log2 values) of 24 viral defense genes as

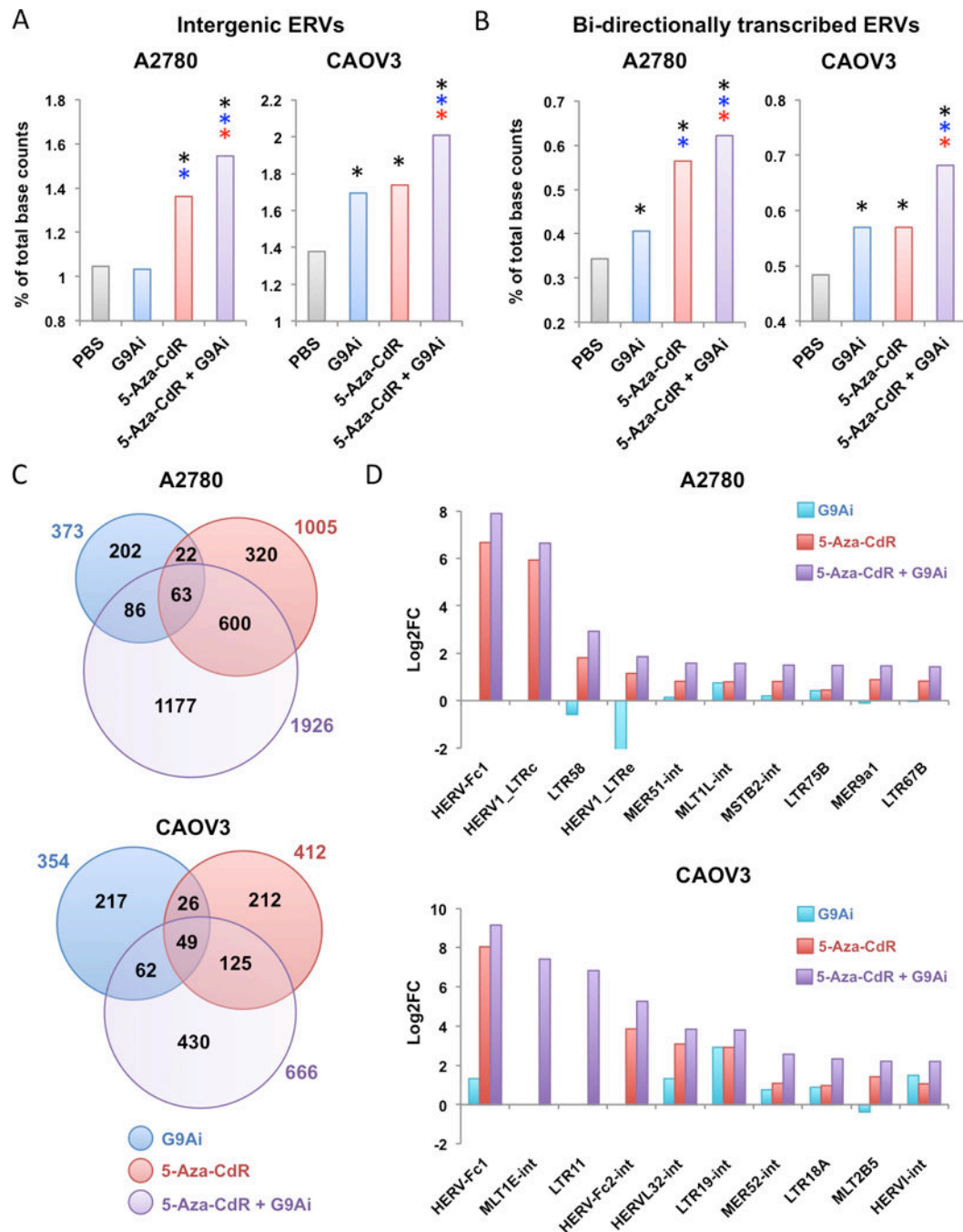
previously published (9,10) in A2780 and CAOV3 cells after G9Ai, 5-aza-CdR, and combination treatment compared to untreated cells.

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**Figure 4.**

Combination treatments further up regulated ERVs in A2780 and CAOV3 cells. A) Bar graphs show percentage of ERV base counts mapped uniquely to intergenic region relative to total base counts in two independent RNA sequencing experiments. * $P < 0.05$ by χ^2 test for equality of proportions without continuity correction. B) Bar graphs show percentage of bi-directionally transcribed ERV base counts mapped uniquely to intergenic region relative to total base counts in two independent RNA sequencing experiments. * $P < 0.05$ by χ^2 test for equality of proportions without continuity correction. Stars in black, blue, and red indicate

comparison to untreated, G9Ai treated, and 5-aza-CdR treated samples, respectively. C) Venn diagrams show the up-regulated ERVs (greater than two-fold change, $p < 0.05$) after G9Ai, 5-aza-CdR, and combination treatment compared to untreated cells. D) Top 10 most up-regulated ERV families in A2780 and CAOV3 cells. Values are log2 fold change of transcripts uniquely mapped to individual ERV family after G9Ai, 5-aza-CdR, and combination treatment compared to untreated cells.

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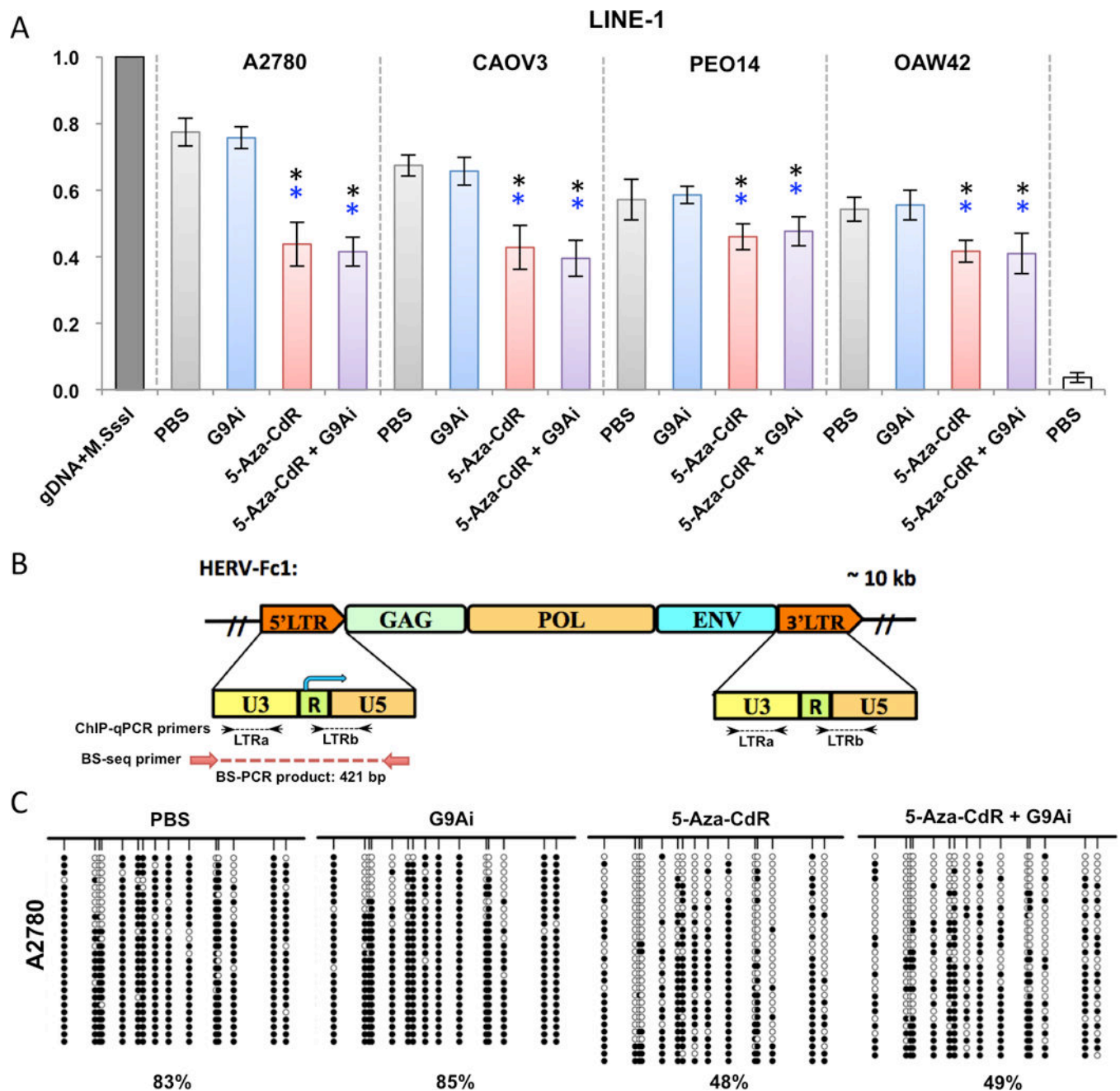
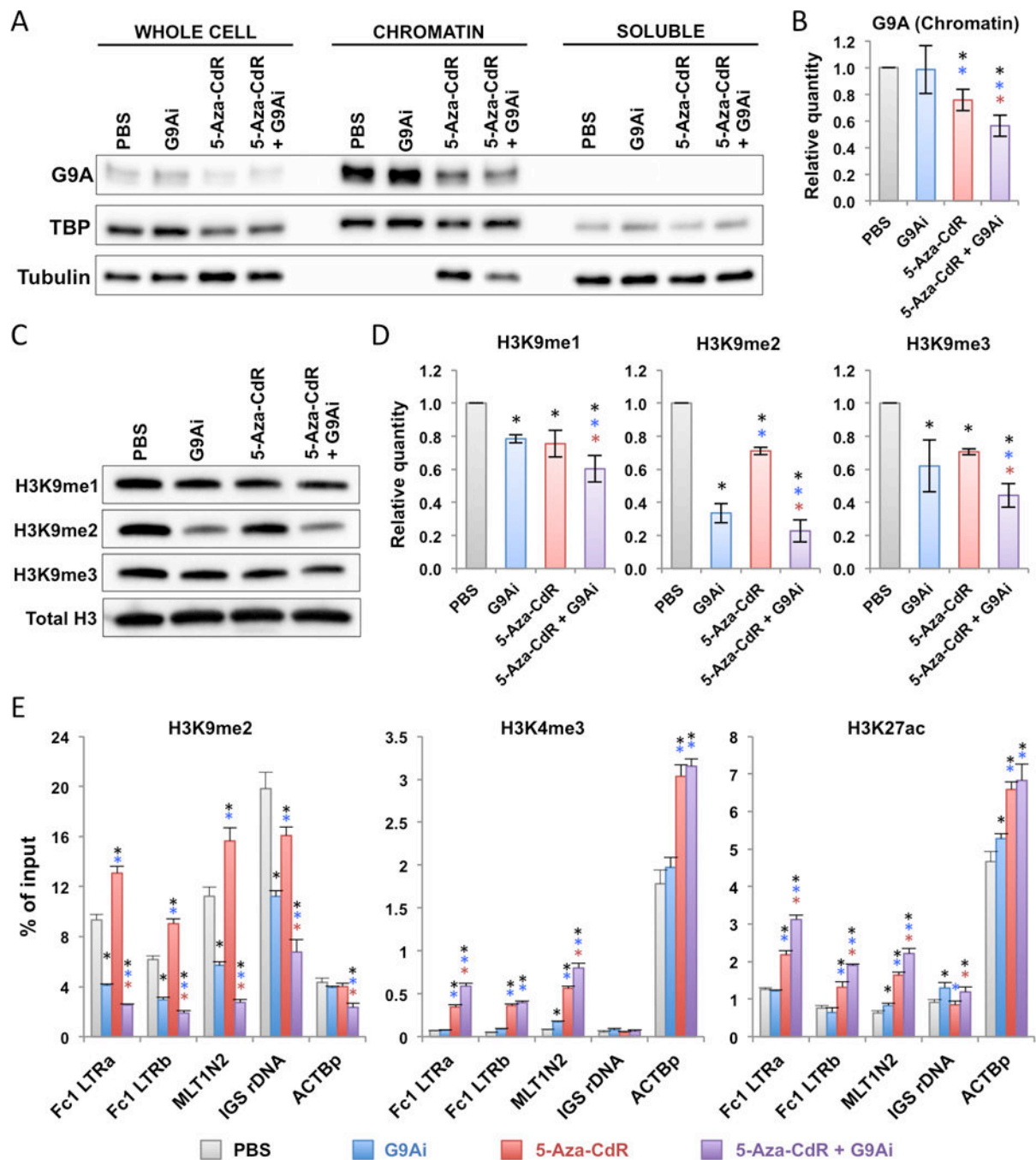


Figure 5.

DNA methylation levels at LINE-1 elements and the 5'LTR of HERV-Fc1 before and after treatments. A) MethyLight analysis of DNA methylation at LINE-1 elements in A2780, CAOV3, PEO14, and OAW42 cells before and after treatment. MethyLight assay was performed using primers against methylated LINE-1 elements in bisulfite-converted DNA from these cells, alongside genomic DNA from DKO1 cells as a negative control. Methylation levels were calculated in triplicate by $\Delta\Delta C_t$ method using unbiased Alu reactions as copy number controls for each sample and normalized to fully methylated reference DNA (M.SssI treated genomic DNA). Values are mean $\pm$ SEM, and one-way

repeated measures ANOVA was used for statistical analysis. *P <0.05, stars in black, blue, and red indicate comparison to untreated, G9Ai treated, and 5-aza-CdR treated samples, respectively. B) A schematic diagram of HERV-Fc1 showing the structure of LTRs and the locations of primers for bisulfite sequencing and ChIP-qPCR. C) Bisulfite sequencing of the 5'LTR of HERV-Fc1 at d 5 after treatments in A2780 cells. Bubble maps show the methylation status of cells before and after treatment, with each row representing a different read and each column representing a different CpG position. Filled and open circles indicate methylated and unmethylated CpG sites, respectively. The numbers below each bubble map show the respective CpG methylation percentages.

**Figure 6.**

Changes in G9A protein and histone modification levels in A2780 cells at d 5 of the treatment schedule. A) Western blot analysis of G9A protein levels in whole cell, chromatin, and soluble fractions. TATA-binding protein (TBP) and beta tubulin proteins were used as controls. Representative blots were shown from three independent experiments. B) Bar graph shows the quantification of the chromatin-associated G9A protein levels relative to TBP protein levels. The values were normalized to the levels of G9A protein before treatment and are represented as mean $\pm$ SEM in three independent experiments. One-way

repeated measures ANOVA with Geisser-Greenhouse's epsilon correction was used for statistical analysis. *P <0.05; stars in black, blue and red indicate comparison to untreated, G9Ai treated, and 5-aza-CdR treated samples, respectively. C) Western blot analysis of total H3K9me1/2/3 modification levels in A2780 cells after treatments. Representative blots were shown from three independent experiments. D) Bar graphs show the quantification of H3K9me1/2/3 modification levels relative to total H3 levels and normalized to the levels in untreated samples in three independent experiments. Statistical analysis was performed as in B). E) Quantification of H3K9me2, H3K4me3 and H3K27ac levels by ChIP-qPCR at LTR regions of HERV-Fc1 (Fc1 LTRa and b) and MLT1N2. The intergenic spacer region of the 35S ribosomal DNA genes (IGS rDNA) and the promoter region of beta-actin (ACTBp) were used as controls. Values are mean $\pm$ SEM (n=3). One-way repeated measures ANOVA was used for statistical analysis. *P <0.05; stars in black, blue and red indicate comparison to untreated, G9Ai treated, and 5-aza-CdR treated samples, respectively.



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The emerging role of epigenetic therapeutics in immuno-oncology

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Abstract

The past decade has seen the emergence of immunotherapy as a prime approach to cancer treatment, revolutionizing the management of many types of cancer. Despite the promise of immunotherapy, most patients do not have a response or become resistant to treatment. Thus, identifying combinations that potentiate current immunotherapeutic approaches will be crucial. The combination of immune-checkpoint inhibition with epigenetic therapy is one such strategy that is being tested in clinical trials, encompassing a variety of cancer types. Studies have revealed key roles of epigenetic processes in regulating immune cell function and mediating antitumour immunity. These interactions make combined epigenetic therapy and immunotherapy an attractive approach to circumvent the limitations of immunotherapy alone. In this Review, we highlight the basic dynamic mechanisms underlying the synergy between immunotherapy and epigenetic therapies and detail current efforts to translate this knowledge into clinical benefit for patients.

Immune-checkpoint inhibition was introduced as a novel clinical paradigm of cancer therapy in March 2011 with the FDA approval of the anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) antibody ipilimumab for the treatment of advanced-stage melanoma. This treatment paradigm was further established upon the approval, in late 2014, of the anti-

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Author contributions

M.J.T., M.V. and S.B.B. researched data for the article and wrote the manuscript. All authors made substantial contribution to discussions of content and reviewed and/or edited the manuscript before submission.

Competing interests

S.B.B. is an inventor of the methylation-specific PCR platform, which is licensed to MDxHealth in agreement with Johns Hopkins University; S.B.B. and Johns Hopkins University are entitled to royalty sales shares. S.B.B. is on the Scientific Advisory Board for Mirati Therapeutics. J.R.B. is on advisory board/consultant for Amgen, BMS (uncompensated), Celgene, Genentech, Janssen Oncology, Lilly, Merck and Syndax. J.R.B. receives grant research funding from AstraZeneca/MedImmune, BMS and Merck. K.A.M. is a consultant for AstraZeneca. All other authors declare no competing interests.

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programmed cell death 1 (PD-1) antibodies nivolumab and pembrolizumab for the same indication. Since then, inhibitors targeting the CTLA-4 and PD-1 immune checkpoints have revolutionized the management not only of melanoma but also of non-small-cell lung carcinoma (NSCLC), renal cell carcinoma (RCC) and Hodgkin lymphoma, among other malignancies, as evidenced by improved survival outcomes in these patient populations^{1–15}. Notably, the possibilities of immunotherapy as a cancer management strategy have long been recognized and pursued¹⁶. We are now in an age of renaissance of immunotherapy and immune-checkpoint inhibition is but one promising approach that has emerged; detailing aspects of the many other novel potentially efficacious immunotherapeutic strategies that are currently being explored is outside the scope of this Review, although examples include chimeric antigen receptor T cell therapy, vaccine-based approaches and natural killer (NK) cell-directed treatments^{17–24}. Despite the early excitement regarding the promise of immune-checkpoint inhibitors (ICI), the majority of patients with cancer fail to derive clinical benefit from or ultimately develop resistance to such treatment^{25–28}. Moreover, response rates vary between cancer types and are typically highest in patients with melanoma, urothelial cancer, NSCLC and colorectal cancers with microsatellite instability²⁹; certain cancers, such as those of the pancreas, breast or ovaries, seem to be intrinsically resistant to ICI^{29–32}, although patients with advanced-stage, programmed cell death 1 ligand 1 (PD-L1)-positive, triple-negative breast cancer have been shown to benefit from the addition of anti-PD-L1 antibodies to chemotherapy³³. The variability in responsiveness to immune-checkpoint inhibition among cancer types has been attributed to several factors, including tumour mutational burden (TMB), immune phenotype of the tumour microenvironment (TME) and mechanisms of tumour immune evasion. Thus, the development of combinatorial strategies with ICI is needed to maximize clinical benefit, with several approaches being tested in clinical trials. These include dual ICI (for example, pairing anti-CTLA-4 and anti-PD-1 antibodies) as well as immunotherapy combined with chemotherapy, radiotherapy or epigenetic therapy. Indeed, dual immune-checkpoint inhibition with ipilimumab plus nivolumab is the most established combinatorial approach; this combination has been reported to improve progression-free survival (PFS) outcomes in patients with advanced-stage RCC and metastatic melanoma, compared with those associated with sunitinib and ipilimumab monotherapy, respectively, and is approved for the first-line treatment of these cancers^{34,35}. The addition of pembrolizumab to chemotherapy has been shown to increase both PFS and overall survival (OS) in phase III trials involving patients with advanced-stage NSCLC, leading to FDA approval of this approach in the frontline setting^{36,37}. The pairing of radiotherapy with ICI is currently being tested in a variety of settings across a range of solid tumour types ([NCT02239900](#), [NCT03700905](#), [NCT03867175](#) and [NCT03693014](#)). Notably, patients with NSCLC receiving consolidation therapy with durvalumab (an anti-PD-L1 antibody) after chemoradiotherapy had a longer median PFS duration than those in a placebo group³⁸. Beyond NSCLC, case reports describing the potential benefit of combined radiotherapy plus ICI have been published across a variety of solid cancers^{39–41}.

In addition to the aforementioned combination regimens, the application of epigenetic therapy plus ICI is an emerging paradigm and an area of active clinical investigation (Supplementary Table S1). In this Review, we highlight the ‘roles’ of epigenetic regulation

in both tumour and immune cell populations and the implications of epigenetic drugs in the perturbation of these compartments. We also summarize the current state of preclinical and clinical development of epigenetic-immunotherapy.

Overview of the ICI paradigm

Principles of ICI

The advent of ICI is the product of many years of basic science research seeking to discern why anticancer immunotherapy was not reaching the promise suggested for over a century since the original seminal insights provided by William B. Coley¹⁶. This breakthrough was made possible through an increased understanding of immune tolerance of cancer and centres upon targeting checkpoints in T cell priming and activation^{42–44}, a concept that earned Allison and Honjo the 2018 Nobel Prize in Physiology or Medicine. Chronic interactions between tumour cells and subsets of immune cells induce this tolerance by rendering cytolytic CD8⁺ tumour-infiltrating lymphocytes ineffective in mounting antitumour responses^{45–49}. The basic constituents of this mechanism, which is defined as immune checkpoint activation, are interactions between receptors on T cells, most notably PD-1 and CTLA-4, and their respective ligands, PD-L1 and CD80 or CD86, present on tumour cells^{42,50,51} and/or antigen-presenting cells^{52–54}. Thus, the rationale for immune-checkpoint inhibition is the treatment with antibodies targeting PD-1, PD-L1 or CTLA-4 in order to reverse this inhibitory checkpoint action and facilitate antitumour effects^{1,51}. In addition to the aforementioned checkpoints, a number of other immune checkpoint pathways have been identified and studies are ongoing to determine the feasibility of the component receptors and ligands as therapeutic targets. These targets include lymphocyte activation gene 3 protein (LAG3), T cell immunoglobulin mucin receptor 3 (TIM3; also known as HAVCR-2), B and T lymphocyte attenuator (BTLA), NK cell receptor 2B4, T cell immunoreceptor with Ig and ITIM domains (TIGIT), V-type immunoglobulin domain-containing suppressor of T cell activation (VISTA), CD96 (also known as T cell surface protein tactile) and CD112 receptor (CD112R; also known as PVRIG), all of which are negative regulators of T cell activation⁵⁵. In addition, targeting of co-stimulatory immune checkpoint proteins, such as the tumour necrosis factor receptor superfamily members 4–1BB (CD137), CD40, OX40 and GITR, is another focus of immunotherapy drug development⁵⁵.

Prerequisite for a response to ICI

A clinical response to CTLA-4 and/or PD-1 or PD-L1 ICI is dependent on the immune status of the tumour in the following ways. First, antigen-specific CD8⁺ lymphocytes must be present within the TME^{56–58}. Second, the composition of resident immune cell populations must be polarized towards an immunopermissive state^{59–62}. Third, tumours must be functionally competent for MHC class I-mediated antigen presentation to be receptive to immune attack and be dependent upon the PD-1-PD-L1 axis as the dominant mechanism of immune escape (reflected in the requirement for tumoural PD-L1 expression as a criteria for treatment with PD-1 or PD-L1 inhibitors, in some approved indications)⁶³. A state of immune evasion arises if tumours lack these characteristics^{64–66}, which enables a cancer to live under the ‘radar’ of immune detection. This evasive state characterizes what

has been termed immune ‘cold’ tumours versus immune ‘hot’ tumours, which exhibit the defining characteristics detailed above^{58,67,68} (BOX 1; FIG. 1). Immune hot tumours often have higher mutational burdens than immune cold tumours and, relatedly, a greater number of neoantigens, which correlates with higher objective response rates to ICI across several common cancer types⁶⁹. The association between TMB and responsiveness to ICI is well established, although the implications with regard to T cell behaviours, such as tumour infiltration, are nebulous and perhaps context dependent. Analyses of The Cancer Genome Atlas melanoma specimens revealed a lack of correlation between a T cell inflammation gene-expression signature and nonsynonymous somatic mutation burden⁷⁰. By contrast, findings in RCC samples demonstrated a positive correlation between TMB and a T cell inflamed transcriptional signature⁷¹.

Therapeutic strategies aimed at converting immune cold tumours into immune hot tumours are currently being intensely investigated. The implications of epigenetic mechanisms in the control of these states and how epigenetic therapy can be used to optimize this transition are discussed in detail in a later section of this manuscript.

Epigenetic mechanisms and therapeutics

Basic principles of epigenetics

As extensively outlined in multiple reviews^{72–77}, epigenetics is the process by which changes mediating heritable patterns of gene expression are established without changing the sequence of DNA. Epigenetics can thus be viewed as a virtual ‘software package’ to control and utilize the information coded in the ‘hard drive’ of DNA. Thus, non-malignant cells and cancer cells have an epigenome’ constituted by regulation of the components of chromatin, which defines the interaction of DNA with proteins, principally histones. Nucleosomes, the 3D distribution of which throughout the genome essentially determines how DNA is packaged in a cell to regulate patterns of gene expression and chromosome structure, are the basic units of these interactions^{72,73,78–81}. This packaging process is fine-tuned by interactions mediated by methylation of genomic DNA at CpG sites and by covalent marks, principally acetylation and methylation, of amino acids on histones in the context of the nucleosomes^{72,75,76,80–82}.

Enzymes or ‘writers’ that establish DNA methylation (DNA methyltransferases (DNMTs)), histone acetylation (histone acetylases) and histone methylation (histone methyltransferases) control each of these processes. In turn, these epigenetic marks can be removed dynamically by enzymes referred to as ‘erasers’, which comprise the ten-eleven translocation enzymes that undo DNA methylation and histone deacetylases (HDACs) and histone demethylases that reverse histone acetylation and methylation, respectively^{83–85}. Histone marks can be activating for RNA transcription, for example, lysine acetylation and some methylation modifications, whereas others, such as lysine deacetylation and certain methylation marks, mediate repressive states of gene expression. Finally, the DNA methylation and histone modifications are recognized by regulatory proteins, or ‘readers’, that enable these chromatin processes throughout the genome to modulate transcriptional profiles^{84–87}.

Key specifics of the cancer epigenome

The cancer epigenome can be characterized by abnormalities in essentially every one of the epigenetic control features outlined in the preceding section^{73,75,84,85,88–90}; the most investigated aspects to date are cancer-specific alterations in DNA methylation and histone acetylation, as has been extensively reviewed elsewhere^{73,76,79,85,91}. The most common changes in DNA methylation found in cancer cells, as compared with their non-malignant counterparts, are global, genome-wide losses of methylation (hypomethylation) that could result in the upregulation of genes with pro-tumorigenic functions, accompanied by more focal, cancer-specific hypermethylation located at CpG rich sites or CpG islands in the promoter regions of hundreds of genes^{74,75,79,85}. These hypermethylated promoters can be associated with repression of expression or prevention of inducibility of involved genes, providing an alternative suppressive mechanism to genetic aberrations for the loss of function of key tumour suppressor genes and is a central feature of carcinogenesis^{75,76,92–96}. In addition, losses and gains of DNA methylation can involve other regulatory regions of the genome, such as gene enhancers, which often regulate networks of genes. The epigenetic alterations in these regulatory regions, which can be located distant from a given gene under their control, can influence cancer development^{97–101}.

Abnormalities of histone acetylation, most commonly losses at gene promoter regions that result from increased activity of HDACs, can either accompany alterations of DNA methylation in mediating important effects on the cancer epigenome or constitute independent controlling effects^{85,102–104}. Specifically, histone deacetylation, with or without coincident DNA methylation, can cause the repression of tumour suppressor genes^{105–108}. Conversely, increased histone acetylase-mediated histone acetylation can constitute a cancer abnormality associated with abnormal upregulation of gene expression¹⁰⁹. Thus, the targeting of DNA methylation and/or histone deacetylation (or acetylation), as extensively reviewed elsewhere^{104,110,111}, is a major focus of epigenetic therapy and is central to its combination with ICI, as discussed later in this Review.

Roles in immune cell differentiation

In the past decade, a number of elegant studies in the field of immunology have led to a much more cohesive view of the epigenetic regulation of normal physiological subsets of key immune cell lineages. These studies provide insights into the functions and interactions of these cell populations within the TME. The epigenetic regulation of differentiation has been studied across several major immune cell populations, including myeloid cells, CD8⁺ T cells and CD4⁺ T cells. The epigenetics of CD4⁺ T cell differentiation have been reviewed extensively^{112,113}, but remains an emergent paradigm; therefore, the following section is focused on the epigenetic regulation of CD8⁺ T cells and myeloid populations, the direct antitumour activities of which are potentially amenable to the actions of epigenetic drugs.

CD8⁺ T cell differentiation.—The activation and differentiation of CD8⁺ T cells are a result of stimulation following antigen presentation by professional antigen-presenting cells (Box 2). Epigenetic mechanisms have important roles in dictating the fate of T cells¹¹⁴ (FIG. 2). These mechanisms are essentially mediated by progressive, large-scale remodelling of chromatin, which modulates the accessibility of transcription factors to regulatory regions of

target genes involved in T cell development, maturation and lineage commitment^{114–116}. Transcription factor 7 (TCF7, also known as TCF1) has been identified as one of the key transcription factors involved in establishing the epigenetic identity of T cells during their differentiation and patterns the chromatin landscape, enabling T cell differentiation to evolve¹¹⁶. The pathways of naive CD8⁺ T cell differentiation to CD8⁺ effector T cells involve dynamic epigenetic changes in chromatin accessibility, with genome-wide gains and losses of DNA methylation and histone modifications observed during this process^{117–121} (FIG. 2). Similarly, epigenetic mechanisms also demonstrably regulate the dedifferentiation of CD8⁺ effector T cells to memory T cells^{118,122}. This phenotypic switch is accompanied by a reversal of epigenetic repression of naive T cell-associated genes but with maintenance of demethylation of key genes expressed in CD8⁺ effector T cells^{118,119} (FIG. 2). Thus, CD8⁺ memory T cells have distinct patterns of chromatin accessibility associated with the capacity for rapid re-induction of effector functions¹²³.

Another end point in the fate of effector T cells involves the acquisition of an ‘exhausted’ phenotype facilitated by chronic antigen stimulation. A hallmark of this cell state is the downregulation of effector functionality, as evidenced by a diminished capacity for induced production of tumour necrosis factor, IL-2 and IFN γ ¹²⁴. Exhausted T cells also have upregulation of inhibitory immune checkpoint molecules, such as PD-1, CTLA-4, LAG3 and TIM3, on the cell surface to levels exceeding those observed in effector T cells¹²⁵. In the context of immunotherapy, PD-1 is an important target, the expression of which is regulated by both DNA methylation¹²⁶ and alterations of chromatin accessibility¹²⁷. Notably, exhausted CD8⁺ T cells regain minimal effector and/or memory functions following PD-1 inhibition in mouse models of chronic lymphocytic choriomeningitis virus infection^{119,128}, thus suggesting the need to consider combination strategies to prevent the acquisition of or reverse the exhausted state. Crucially, when considering potential epigenetic-immunotherapy combinations, this finding was predominantly attributed to the epigenetic stability of these cells under PD-1 inhibition, in a state distinct from that of effector and memory T cells^{119,128}; however, this epigenetic state could be counteracted, and T cells reinvigorated, through sequential inhibition of DNMTs followed by PD-L1, with similar findings observed in an immune-checkpoint inhibition-refractory mouse TRAMP-C2 model¹¹⁹. These data highlight the importance of considering epigenetic plasticity in dictating the effects of current immune-checkpoint inhibitors on CD8⁺ T cells.

Myeloid differentiation.—Epigenetic modifications also regulate the fate of cells of the myeloid lineage by orchestrating their differentiation and activation. These changes mainly involve various histone modifications that regulate the binding of lineage-specific transcription factors to their target genes, largely by modulating chromatin accessibility^{129,130}. As discussed later in this manuscript, HDAC inhibitors, either alone or in combination with DNMT inhibitors, have proved to be effective in enhancing antitumour immunity across multiple preclinical models by depleting tumours of myeloid-derived suppressor cells (MDSCs) — a cell population known to induce peripheral T cell tolerance and to inhibit both T cell activation and proliferation^{131–133}. Epigenetic mechanisms have also been implicated in the regulation of macrophage polarization^{134,135}. Accordingly, in a mouse model of ovarian cancer, combination treatment with the DNMT inhibitor azacitidine

and the ornithine decarboxylase inhibitor α -difluoromethylornithine results in depletion of pro-tumorigenic M2-like macrophages from the TME and enrichment with inflammatory, antitumour, M1-like macrophages¹³⁶.

Targeting the cancer epigenome

In keeping with the crucial roles of epigenetic mechanisms in regulating the functions of non-malignant epithelial and immune cells as well as the epigenetic alterations associated with malignancy, strategies to target the cancer epigenome have proved effective in controlling tumour growth. The goal of such therapy is to reprogramme the epigenome of cancer cells in order to disrupt the self-renewal of stem-like cells, induce differentiation towards a non-malignant phenotype, block the invasive or metastatic behaviour of malignant cells, and/or sensitize tumours to other therapeutic interventions^{85,111,137–139}. Excitement surrounding these concepts is increasing now that the pharmaceutical industry has developed different drugs with which to target virtually all of the writer, eraser and reader functions outlined above (TABLE 1). Many of these agents are undergoing testing in phase I and/or II clinical trials and the demonstration of acceptable toxicity profiles and promising efficacy is anticipated. These studies might therefore facilitate the future use of epigenetic agents as monotherapies or in combinatorial strategies. The only epigenetic drugs currently approved by the FDA for use in patients are DNMT inhibitors for the treatment of myelodysplastic syndrome and acute myeloid leukaemia (AML), in combination with the BCL-2 inhibitor venetoclax for the latter disease, and HDAC inhibitors for the treatment of cutaneous or peripheral T cell lymphoma and relapsed multiple myeloma (TABLE 1). DNMT inhibitors include azacitidine, which can result in the demethylation both DNA and RNA, as well as DNA-specific demethylating agents, such as decitabine and its derivative with a longer half-life, guadecitabine¹⁴⁰. DNMT inhibitors and HDAC inhibitors are currently being studied alone and in combination with ICI across a variety of solid and haematological malignancies (TABLE 1). As the focus of this Review, we outline the promise of such combinations to enhance the efficacy of ICI and other immunotherapies in the following sections.

Rationale for epigenetic-immunotherapy

Epigenetic therapy modulates key regulatory features of both immune cells and tumour cells in ways that might overcome some of the current limitations of immunotherapy (FIGS 1; 2). For example, epigenetic drugs have the potential to reverse many processes that tumours engage to evade immune-mediated destruction (FIG. 1).

Epigenetic control of immune exhaustion

Much interest over the past several years has surrounded the state of immune cell ‘exhaustion’, which is a component of immune tolerance and evasion^{120,141}. In the scenario of T cell exhaustion associated with cancer, tumour-targeting CD8⁺ T cells adopt a unique differentiation state, in which they are unable to mount effector functions and thus their cytolytic activity against tumour cells is impeded¹²⁰. Importantly, as described above, this state is characterized by a complex programme of gene expression changes that are correlated with alterations in chromatin conformation and DNA methylation^{117,123}. In mouse models, epigenetic therapy can reverse these changes in chromatin conformation and

DNA methylation acquired during the transition to an exhausted T cell state, which is postulated to be induced by a DNMT3A-mediated de novo methylation programme¹¹⁹ (FIG. 2). Indeed, in these preclinical studies, prevention of the exhaustion state with DNMT inhibitors was associated with an increase in the efficacy of ICI with anti-PD-1 antibodies¹¹⁹.

Epigenetic reversion of immunoediting

DNMT inhibitors and HDAC inhibitors are known to promote innate immune-related signalling in cancer cells, which could potentially also enhance recognition of these cells by adaptive immune cell populations^{142–147} (FIG. 1). Early examples of this concept include the induction of tumour antigens, termed cancer/testis antigens (CTAs), consisting largely of proteins usually expressed exclusively in embryonic or germ cells during development^{148–156}. The expression of CTA genes is controlled by transcriptional repression, including promoter DNA methylation and histone deacetylation in association with other histone modifications, thus making these genes likely targets of epigenetic therapy^{157–159}. In addition to CTA upregulation, epigenetic therapy can potentiate tumour cell immune recognition through restoration of the MHC class I antigen processing and presentation machinery — deficiencies which can be selected for during cancer immunoediting and, indeed, are one of the defining characteristics of immune hot tumours^{142–144,160–163}.

The list of actionable targets of epigenetic therapy has now expanded beyond CTAs, with a particular focus on DNMT inhibitor-mediated augmentation of signalling related to innate immunity and induction of inflammation-associated genes such as cytokines and chemokines^{142–144,146,147,164}. Intriguingly, these effects are typically predicated on potentiation of type I and III interferon signalling invoked by increased levels of cytoplasmic viral RNAs, a phenomenon termed viral mimicry^{146,147}. This response is largely centred on the transcriptional de-repression of endogenous retroviruses (ERVs)^{146,147}. ERVs have been incorporated into the human genome over millennia, such that they now account for ~8% of the genome¹⁶⁵, but are generally silenced in somatic cells through DNA methylation and repressive histone modifications^{166–169}. Thus, DNMT inhibitors induce demethylation of ERV sequences, which enables ERVs to be transcribed into RNAs that fold into double stranded RNA structures. Subsequent interactions between these viral double stranded RNAs and cognate cytoplasmic sensors triggers a viral defence response, including induction of type I interferon signalling.

Taken together, these effects emphasize the demonstrable potential of epigenetic therapy to facilitate immune recognition of tumour cells, not least through augmentation of antigen expression, processing and presentation (FIG. 1). The following sections summarize the evidence accumulated to date that epigenetic therapy can overcome barriers to clinical responses to immunotherapy.

Combined epigenetic therapy and ICI

Preliminary clinical observations

Observations made in an early phase I/II clinical trial of combination epigenetic therapy with the DNMT inhibitor azacitidine and the HDAC inhibitor entinostat¹⁷⁰ have helped to bring the concept of combined therapy with epigenetic drugs and ICI to the fore. These observations have guided the design of preclinical studies to explore the scientific underpinning for the promise of such approaches. Briefly, in this initial clinical trial involving 45 patients with advanced-stage refractory NSCLC¹⁷⁰, two patients exhibited very durable, Response Evaluation Criteria in Solid Tumors (RECIST)-defined objective responses to epigenetic therapy and survived for 3–4 years after treatment. Additionally, five patients who had disease progression during the trial were subsequently enrolled in the first trials of anti-PD-1 antibodies; three of these patients achieved RECIST objective responses whereas the remaining two patients had stable disease for 24 weeks before progression^{142–170}. Whilst these observations generated excitement, the underlying mechanism for the noted efficacy was not elucidated, thus spurring the initiation of multiple preclinical studies to evaluate the effects of epigenetic therapy on antitumour immune responses.

Promising preclinical data

DNMT inhibitor-based therapy.—Direct evidence of synergy between DNMT inhibition and immune-checkpoint inhibition has been established in the preclinical space across multiple model systems. In animal models of ovarian cancer or melanoma, the addition of a demethylating agent (decitabine and azacitidine, respectively) to anti-CTLA-4 antibody therapy increases the antitumour effect relative to that observed with immune-checkpoint inhibition alone, as evidenced by prolongation of survival^{146–171}, with an enhancement of cytolytic CD8⁺ T cell accumulation within the tumours noted in the ovarian cancer model¹⁷¹. Other studies provide evidence that the effectiveness of anti-PD-1 antibodies can also be potentiated with the use of DNMT inhibitors. In the MMTV-Neu mouse breast cancer model, treatment with guadecitabine augments both MHC class I expression and T cell chemotaxis via the CXC-chemokine ligand 9 (CXCL9)/CXCL10/CXCL11-CXC-chemokine receptor 3 (CXCR3) axis, which has been correlated with enhanced tumour infiltration of CD8⁺ T cells and subsequent potentiation of responses to anti-PD-1 antibodies¹⁷². Additionally, Yu et al.¹⁷³ delineated important decitabine-mediated immunological effects in a syngeneic mouse CT26 colon cancer model. These effects included mobilization of the antigen presentation machinery, intratumour accumulation of PD-1-positive CD8⁺ T cells and sensitization to anti-PD-1 antibody therapy. As mentioned above, in the mouse TRAMP-C2 model of immune-checkpoint inhibition-resistant prostate cancer, administration of decitabine was found to induce the sensitivity of CD8⁺ T cells to anti-PD-L1 antibodies through the prevention of the DNMT3A-mediated DNA methylation programme of exhausted T cells, thereby enhancing antitumour responses¹¹⁹. Together, these findings provide evidence supporting the effectiveness of combined DNMT inhibition and immune-checkpoint inhibition, which is mediated in part by epigenetic enhancements of the adaptive immune system.

HDAC inhibitor-based therapy.—The deployment of HDAC inhibitors in combination with immunotherapies has demonstrated efficacy across multiple animal models. The axes most amenable to perturbation by HDAC inhibitors are T cell chemoattraction gradients and myeloid cell populations, predominantly MDSCs. An early indication of the potential synergy between HDAC inhibition and immune-checkpoint inhibition was derived from a syngeneic mouse B16-F10 melanoma model¹⁷⁴. In this study¹⁷⁴, the concurrent application of the HDAC inhibitor panobinostat potentiated anti-PD-1 antibody therapy, thus resulting in slower tumour growth and longer survival than that observed with either treatment alone. The induction of PD-L1 after HDAC inhibition, mediated by a gain of *PDL1* gene promoter acetylation, was one notable in vitro observation¹⁷⁴. This induction of PD-L1 could constitute a possible resistance mechanism when HDAC inhibition is paired with ICI and should, therefore, be considered when evaluating PD-L1 tumour positivity in the context of epigenetic therapy. In a study using a subcutaneous mouse hepatocellular carcinoma model, administration of the HDAC inhibitor belinostat was demonstrated to enhance the efficacy of CTLA-4 inhibition, but not PD-1 inhibition, in association with increases in the abundance of M1-polarized tumour-associated macrophages and in IFN γ production by tumour-specific CD8⁺ T cells as well as decreased numbers of splenic regulatory T (T_{reg}) cells¹⁷⁵. Tumour antigen-presenting cells had upregulation of PD-L1 early after treatment with the HDAC and CTLA-4 inhibitors, with later upregulation of PD-1 on T cells also noted; simultaneous HDAC, PD-1 and CTLA-4 inhibition resulted in complete tumour rejection. Across multiple animal models of solid tumours, the HDAC inhibitor entinostat induced the depletion of MDSCs and enhanced the efficacy of anti-PD-1 therapy^{176,177}. Specifically, Orillion et al. found that the application of entinostat reduced the levels of both MDSC-associated chemoattractants and MDSC suppressive activity¹⁷⁷. Furthermore, evidence suggested that HDAC inhibition might promote the differentiation of this cell population¹⁷⁷.

In addition to MDSC-directed immune effects, HDAC inhibitors have been found to have a variety of effects on T cell responses. For example, the HDAC inhibition using romidepsin increased levels of T cell chemoattractants and tumour infiltration in multiple lung adenocarcinoma models, with a correlated sensitization to anti-PD-1 therapy¹¹⁷. Building on these preclinical studies, HDAC inhibition in combination with immune-checkpoint inhibition is currently being explored in multiple clinical trials (TABLE 1; Supplementary Table S1) and might be most efficacious against cancers with a type IV TME, a hallmark of which is a demonstrably high level of MDSC infiltration (BOX 1).

DNMT inhibitor and HDAC inhibitor combinatorial paradigm.—The aforementioned concepts based on the combination of DNMT or HDAC inhibitors with ICI have subsequently been extended to combination paradigms founded on both DNMT and HDAC inhibition, with robust antitumour effects observed in multiple preclinical solid tumour models^{144,176,178}. Importantly, in all cases, the efficacy of such combinations has been tightly tied to CD8⁺ T cell dependent mechanisms¹⁴⁴ and/or associated with sensitization to immune-checkpoint inhibition^{176,178}. In ovarian and lung cancer animal models, attraction of CD8⁺ T cells to the TME occurs in association with initiation of a type 1 T helper (T_H1) cell chemokine axis involving CC-chemokine ligand 5 (CCL5) and CXCL10 (REFS^{194,197}). These chemokines have the demonstrated ability to facilitate the

attraction of CD8⁺ T cells through interaction with CC-chemokine receptor 5 (CCR5) and CXCR3, respectively, on these cells^{179–182}. CCL5 seems of particular importance in patients with lung adenocarcinoma as this chemokine is an established hallmark of an active lymphocytic compartment in clinical samples and is associated with favourable survival outcomes¹⁸³. Additionally, in animal models of NSCLC, the application of epigenetic therapy prevents the aforementioned exhausted phenotype in tumour-associated CD8⁺ T cells¹¹⁹, with acquisition of effector and/or memory phenotypes noted¹⁴⁴.

In addition, preclinical studies in NSCLC models have identified MYC as a key target of combination DNMT plus HDAC inhibition (FIG. 1); suppression of MYC activity by such epigenetic therapy potentiates type I interferon signalling and the associated induction of CD8⁺ T cell-attracting chemokines, including CCL5 (REF¹⁴⁴). A similar pattern of immune effects emerged from studies by another group after genetic manipulation of MYC expression in a mouse model of *Kras*-mutated lung adenocarcinoma¹⁸⁴. In this study, MYC expression in tumour cells resulted in the production of CCL9 and IL-23; CCL9 was shown to mediate recruitment of PD-L1-positive macrophages and associated PD-L1-dependent expulsion of T and B cells, whereas IL-23 orchestrates exclusion of adaptive T cells and B cells and innate immune NK cells¹⁸⁴. The implications of the potential epigenetic regulation of MYC on the tumour immune microenvironment are not limited to the setting of NSCLC. Casey et al.¹⁸⁵ have established that MYC regulates the expression of both PD-L1 and CD47 in human T cell acute lymphoblastic leukaemia cells and mouse models of this disease. The regulation of these targets by MYC was found to be through direct binding and thus transcriptional induction, with MYC inactivation resulting in target downregulation and potentiation of antitumour responses noted in mouse models¹⁸⁵. Notably, CD47, termed the ‘do not eat me’ antigen, facilitates antagonization of macrophage-dependent immune surveillance through interaction with signal-regulatory protein α (SIRP α) on macrophages. Targeting of the CD47–SIRP α axis is an emerging paradigm in immunotherapy¹⁸⁶.

These preclinical studies establish, through a diverse set of mechanisms, the multifaceted potential utility of epigenetic therapy to enhance the efficacy of cancer immunotherapy (FIG. 1). These results have formed the basis of a growing number of clinical trials designed with the aim of translating the concept of epigenetic-immunotherapy into patient management.

Current clinical trials

The early clinical observations with combined DNMT and HDAC inhibition elucidated in the setting of advanced-stage, treatment-refractory NSCLC^{142,170}, together with data obtained from the preclinical studies discussed above¹⁴⁴, have prompted the initiation of two trials ([NCT01928576](#) and [NCT03220477](#); Supplementary Table S1). In these ongoing studies, DNMT plus HDAC inhibition is being combined with concurrent anti-PD-1 antibody therapy for patients with advanced-stage NSCLC. The protocols of these trials have been amended to focus on the potential of this combination in the treatment of both ICI-resistant and ICI-naïve patient populations. Additionally, multiple clinical trials involving DNMT and/or HDAC inhibition plus immune-checkpoint inhibition are ongoing across a variety of solid tumours and myelodysplastic syndrome and/or AML (Supplementary Table

S1). Notably, many of these trials include robust correlative studies, such as serial sampling of peripheral blood and tumour specimens for analyses of induced viral mimicry, interferon induction and T cell functional phenotypes (for example, [NCT01928576](#), [NCT03233724](#), [NCT03220477](#), [NCT03576963](#), [NCT02901899](#) and [NCT02397720](#)). Although objective responses, disease stabilization and encouraging OS outcomes have been observed in a previous clinical trial of such a combination in patients with AML^{186,187}, careful consideration of the optimal study populations and epigenetic agents are needed. This requirement is exemplified by results from a randomized, placebo controlled phase II trial of oral azacitidine (CC-486) added to pembrolizumab that failed to show a statistically significant difference in PFS (HR 1.374, 90% CI: 0.926–2.038; $P = 0.179$) or OS (HR 1.375, 90% CI: 0.830–2.276; $P = 0.297$) in patients with advanced-stage NSCLC¹⁸⁸. Of note, the combination treatment group in this study received a median of two fewer cycles of therapy than the placebo group, in association with increases in the proportions of patients that had treatment-related adverse events, dose reductions and treatment interruptions¹⁸⁸. This increased toxicity might be reflective, in particular, of the intestinal and haematological toxicities noted for the oral formulation of azacitidine, which have been associated with dose interruptions or reductions in 16% and 19% of patients (with myeloid neoplasms), respectively¹⁸⁹. These findings highlight the need for careful selection of epigenetic modifying agents in order to maximize the potential synergy with specific ICI whilst limiting treatment-related toxicities.

Currently, it is too early to know whether clinically significant efficacy will emerge from the ongoing trials of combined epigenetic therapy and immune-checkpoint inhibition, thus warranting movement of these combinatorial approaches further towards formal clinical use. As the results of these trials are reported in the coming years, a focus on biomarkers will be essential to allocating these therapies to the patients who are likely to derive the greatest benefit.

Emerging epigenetic partners for ICI

The aforementioned preclinical studies demonstrating immunological effects of DNMT inhibitors and HDAC inhibitors have fostered a growing number of reports that epigenetic drugs with different targets can enhance the efficacy of ICI. Indeed, many of the alternative combinations are undergoing testing in early phase clinical trials. Emerging preclinical findings suggest that the effectiveness of ICI might be further enhanced by future strategies incorporating single or multiple epigenetic drugs with diverse targets.

EZH2 inhibitors

Therapeutics of this class inhibit the activity of the enzyme enhancer of zeste homologue 2 (EZH2), which is the histone-lysine *N*-methyltransferase subunit of the polycomb repressive complex 2 (PRC2). PRC2, via the activity of EZH2, is responsible for placing the transcriptionally repressive histone modifications H3K37me2 and H3K27me3 (REFS^{190–193}). These forms of histone modifications are closely associated with genes vulnerable to cancer-specific DNA hypermethylation at gene promoter region CpG islands, which silences the expression or blocks the inducibility of the affected genes^{194–197}.

Multiple preclinical studies have demonstrated the potential of EZH2 inhibitors to augment the activity of immunotherapy or induce immunostimulatory effects. In mice harbouring tumours derived from patients with ovarian cancer together with adoptively transferred autologous CD8⁺ T cells derived from the same patients, the application of an EZH2 inhibitor in combination with a DNMT inhibitor led to the establishment a robust chemotactic gradient of the T_H1-type chemokines CXCL9 and CXCL10, thus facilitating the attraction of CXC3R⁺ CD8⁺ effector T cells, with subsequent sensitization to ICI with anti-PD-L1 antibodies¹⁹⁸. Moreover, Goswami et al.^{198,199} found that peripheral blood T cells from patients treated with ipilimumab had increased expression of EZH2; accordingly, they demonstrated that the use of an EZH2 inhibitor alone altered the phenotype and function of human T_{reg} cells and enhanced the cytotoxic activity of human CD8⁺ effector T cells as well as sensitizing syngeneic mouse MB49 bladder cancer and B16-F10 melanoma to anti-CTLA-4 ICI^{198,199}.

Additionally, EZH2 seems to have a specific role in mechanisms of adaptive resistance to immunotherapy (with anti-CTLA-4 antibodies or IL-2), whereby infiltrating tumour-reactive CD8⁺ T cells trigger induction of EZH2 in melanoma cells that leads to epigenetic silencing of antigen processing and presentation machinery as well as repression of T_H1 cell-associated chemokines. EZH2 inhibition can reverse this adaptive resistance programme and enhances the efficacy of anti-CTLA-4 antibody therapy in mouse melanoma models²⁰⁰. Several EZH2 inhibitors have entered clinical testing (TABLE 1). Together, these studies provide initial indications of synergy between EZH2 inhibition and immune-checkpoint inhibition and have provided the foundations for the initiation of phase I/II clinical trials of such combinations (Supplementary Table S1).

LSD1 inhibitors

Lysine-specific histone demethylase 1A (LSD1; also known as KDM1A) is the enzyme responsible for erasure of the key mono-methyl (me1) and di-methyl (me2) chromatin marks on histone H3, predominantly at lysines 4 and 9 (H3K4 and H3K9). This enzyme thereby functions as a transcriptional co-regulator in a context-dependent manner through demethylation of the repression-associated H3K9me1 and H3K9me2 marks or the activation-associated H3K4me1 and H3K4me2 marks²⁰¹. LSD1 can also demethylate a number of nonhistone substrates, including DNMT1, and the loss of LSD1 expression is correlated with a decrease in DNMT 1 levels owing to increased methylation and destabilization of this protein²⁰²; therefore, LSD1 inhibition could potentially result in decreased global DNA methylation. Of note, LSD1 is also overexpressed in a number of malignancies and, thus, LSD1 inhibitors might be promising potential therapeutic options in a variety of cancers^{203–205}. Moreover, The Cancer Genome Atlas data indicate that LSD1 expression is inversely correlated with CD8⁺ T cell infiltration into various cancers^{206,207}. Accordingly, LSD1 inhibitors have been shown in multiple mouse cancer models to induce the viral mimicry-like response, with remarkable similarity to the effect observed with DNMT inhibitors^{206,207}, which enhances the recruitment of T cells, increases antigen presentation and thereby sensitizes poorly immunogenic tumours, such as triple-negative breast cancers, to anti-PD-1 ICI^{206,207}. Thus, LSD1 inhibitors now take a place among future, potential combinatorial epigenetic therapy strategies to enhance the efficacy of ICI.

Pharmacological inhibitors are currently being tested in phase I/II clinical trials involving patients with various advanced-stage malignancies (TABLE 1), including in combination with ICI (Supplementary Table S1).

G9a inhibitors

The histone-lysine *N*-methyltransferase EHMT2 (also known as G9a) places the aforementioned repressive H3K9me2 mark in chromatin, including in the promoters of abnormally DNA hypermethylated genes (indeed, G9a and DNMT1 can function as part of a ternary complex)²⁰⁸. The gene encoding G9a can be overexpressed, with and without being amplified, in multiple tumour types, which has been associated with advanced-stage disease and an unfavourable prognosis^{209–211}. Of note, knockout studies in mice have revealed a role of G9a in the maintenance of stem cell self-renewal²¹². Specific inhibitors of G9a are available for preclinical experiments, although a compound suitable for use in clinical trials has not yet been developed. However, an important role for G9a inhibition as a means of inducing viral mimicry has emerged — when used in combination with a DNMT inhibitor, a G9a inhibitor reduces H3K9me2 levels within the long terminal repeat regions of ERVs and thus augments ERV transcription in ovarian cancer cell lines²¹³. Moreover, in cancer cells, repression of ERV sequences without DNA methylation is maintained, in part, by the presence of G9a and H3K9me2 at transcriptional start sites²¹⁴. Thus, inhibition of G9a is an intriguing future candidate strategy for the enhancement of the therapeutic activity of ICI.

BET inhibitors

The bromodomain and extra-terminal (BET) family encompass a number of epigenetic readers, namely BRD2, BRD3, BRD4 and BRDT; these proteins generally recognize acetylated lysines in histones²¹⁵, which accompany the open chromatin structures associated with active transcription, as described earlier. BRD4 is the most intensely investigated BET family member and inhibitors of this protein can suppress aberrantly active transcription in cancer^{216–218}. Initially, this inhibition was thought to be focused specifically on targets of MYC oncogene activation, but other affected pathways have now also been identified²¹⁹. A number of different BET inhibitors are being tested, including in combination with ICI in multiple phase I/II trials (TABLE 1; Supplementary Table S1), predominantly for the treatment of haematopoietic malignancies; a full assessment of efficacy of such agents is awaited.

Preclinical studies indicate that JQ1, one of the original bromodomain-targeted BET inhibitors, synergizes with anti-PD-1 antibodies in a mouse model of NSCLC with activating *Kras* mutation and *Tp53* deletion²²⁰. As observed in studies of DNMT plus HDAC inhibition, JQ1 induced an increase in the abundance of activated, tumour-infiltrating T cells with a T_H1-type cytokine profile as well as depletion of tumour-infiltrating T_{reg} cells, and resulted in stronger, more-durable antitumour responses and improved survival compared with those observed with either agent alone²²⁰. Thus, use of BET inhibitors might provide yet another future combinatorial approach to enhancing the efficacy of ICI.

Conclusions

Epigenetic therapy has emerged as a promising combination partner for use with immunotherapy of advanced-stage malignancies. The potential of epigenetic therapy to enhance patient benefit when compared to immunotherapy alone is centred on its ability to overcome certain limitations of current immunotherapeutic strategies. The success of immunotherapy is dependent on the existence of a certain type of immune environment, principally the presence of tumour-infiltrating lymphocytes and PD-L1 expression in the TME. Epigenetic therapy has been shown to modulate various components of the TME, including augmentation of CTA expression and of antigen processing and presentation, increased attraction and infiltration of CD8⁺ T cells, and prevention or reversion of T cell exhaustion with a concurrent increase in the abundance of effector and/or memory T cells (FIG. 1). As focused upon in this Review, combining epigenetic therapy with ICI is, therefore, one of several possible combinatorial approaches to enhancing efficacy of the latter treatment strategy. In a growing number of clinical trials across multiple cancer types (Supplementary Table S1), emphasis has been placed upon testing the established epigenetic therapy agents, DNMT inhibitors and HDAC inhibitors (alone or in combination) together with ICI. These approaches are being investigated both in patients receiving their first line of immune-checkpoint inhibition and, more recently, in patients harbouring relapsed and/or refractory disease after prior immune-checkpoint inhibition with the aim of reversing resistance to the immunotherapy. The future adoption of these approaches as accepted cancer management strategies will be dependent on the observation of efficacy signals in these trials. The future will also see the development of novel approaches involving next-generation epigenetic drugs combined with emerging immunotherapy modalities, including vaccine-based and adoptive T cell therapies^{221,222}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- The past decade has witnessed the emergence of immune-checkpoint inhibition as the potential fourth pillar of anticancer therapy; however, combination therapeutic paradigms are needed to maximize benefits and overcome resistance to immune-checkpoint inhibition.
- Epigenetic therapy has the ability to modulate the tumour microenvironment, for example, by inducing both the accumulation and infiltration of CD8⁺ lymphocytes through interferon-dependent, chemokine-mediated chemotaxis.
- Epigenetic therapy can also prevent the emergence and/or acquisition of an epigenetic programme of T cell exhaustion and can facilitate the formation of CD8⁺ effector and/or memory T cells.
- Histone deacetylase inhibitors can affect the tumour myeloid compartment by causing myeloid-derived suppressor cell depletion, differentiation and functional antagonism.
- Epigenetic modulators can enhance tumour cell recognition and potentiate type I interferon responses through MYC and MYC-related target downregulation.
- The combination of epigenetic drugs and immunotherapy is emerging as a crucial therapeutic paradigm across a variety of malignancies.

Box 1 |**Types of tumour immune microenvironments**

The past decade has seen the emergence of immunotherapy as one of the most promising treatment strategies for advanced-stage cancers. The ability of tumours to adapt in order to overcome innate and acquired immune mechanisms that would normally lead to recognition and killing of the tumour cells is a crucial aspect of cancer initiation and progression^{47,223}. Four postulated states of immune landscapes have been observed in tumours and dictate the vulnerability of the tumours to different immunotherapeutic strategies^{224,225}. This categorization is largely based on the expression of programmed cell death 1 ligand 1 (PD-L1) in the tumour microenvironment (TME) and the occurrence and distribution of CD8⁺ tumour-infiltrating lymphocytes (TILs) at the tumour site²²⁴.

Type I (adaptive immune resistance)

In the presence of abundant T cell infiltrates, tumours can develop adaptive immune-resistance mechanisms that often involve upregulation of PD-L1 (REFS^{44,226–229}). Accordingly, these tumours are generally referred to as ‘hot tumours’ and are identified by the presence of CD8⁺ TILs along with expression of PD-L1 in the TME²²⁷. The expression of PD-L1 is a feedback response to IFN γ secreted by TILs that, via triggering of the T cell inhibitory receptor programmed cell death 1 (PD-1), diminishes the potential of those TILs to mount an antitumour response²²⁷. This TME is that most poised for clinical benefit from single-agent immune-checkpoint inhibition with anti-PD-1 or anti-PD-L1 antibodies, because this intervention can restore the cytolytic activity of CD8⁺ T cells²³⁰.

Type II (immunological ignorance)

Tumours with this immune microenvironment, generally referred to as ‘cold tumours’, are characterized by an absence of CD8⁺ TILs as well as a lack of expression of PD-L1 (REFS^{57,231}). Patients with such tumours typically do not benefit from single-agent immune-checkpoint inhibition. Combinatorial therapeutic approaches using dual immune-checkpoint inhibition (typically with antibodies targeting PD-1 or PD-L1 and cytotoxic T lymphocyte antigen 4 (CTLA-4)), cancer vaccines, chimeric antigen receptor T cells or agents such as epigenetic drugs, which aid in recruiting key immune cells to the TME prior to the application of immune-checkpoint inhibition, are likely to be the most effective treatment strategies for these tumours^{11,35,144,178,232–234}.

Type III (oncogenic pathway activation)

These tumours, in which expression of PD-L1 is often a result of constitutive oncogenic signalling, are termed innate immune-resistant tumours and include those that are PD-L1-positive in the absence of CD8⁺ T cells^{235–237}. Such tumours underscore the importance of considering the presence of TILs in the TME in conjunction with PD-L1 status in order to predict the likelihood of a response to PD-1 or PD-L1 inhibition. Patients with tumours of this type will probably benefit from similar combinatorial therapeutic approaches to recruit TILs as those with type II tumours.

Type iV (immunological tolerance)

These tumours contain TILs that are rendered incapable of mounting antitumour responses despite a lack of PD-L1 expression in the TME. The development of immunological tolerance can result from immunoediting, which might involve suppression of the antigen processing and presentation machinery²³⁸, ineffective TIL activation owing to a lack of co-stimulatory signals or T cell exhaustion¹²⁴. These tumours can also contain immunosuppressive cells such as regulatory T cells and myeloid-derived suppressor cells²³⁹. Therapeutic approaches for these tumours include targeting of immune checkpoint proteins other than PD-1 or PD-L1 or immunosuppressive pathways, such as immunometabolism (including the adenosine and indoleamine 2,3-dioxygenase pathways)^{240,241}, adoptive transfer of immune effectors and cancer vaccine strategies²⁴². Combination epigenetic therapy and immunotherapy approaches hold great promise in the treatment of these tumours, given the role of epigenetic events in regulating CD8⁺ T cell differentiation^{117,118,120,243} and the ability of epigenetic therapy to prevent CD8⁺ T cell exhaustion¹¹⁹ and to shift CD8⁺ TILs to an effector and/or memory phenotype¹⁴⁴.

Box 2 |**Antigen-specific T cell activation**

The activation of naive T cells to form fully functional effector T cells is a highly regulated process that requires the simultaneous application of three distinct stimuli from professional antigen-presenting cells (APCs) such as dendritic cells or macrophages²⁴⁴. Signal 1 results from the interaction between the T cell receptor (TCR) and a peptide–MHC class II complex present on the APC or MHC class I in the setting of dendritic cell cross presentation²⁴⁵. This interaction triggers mitogen-activated protein kinase and phospholipase C signalling downstream of the TCR, inducing nuclear factor- κ B and activator protein 1 activation, and culminating in transcription and expression of IL-2 (REF²⁴⁶). Although required for T cell activation, signal 1 alone is not sufficient to induce clonal expansion of these cells; TCR stimulation in the absence of a co-stimulatory signal can induce the formation of anergic T cells, leading to peripheral tolerance or defective effector T cell populations²⁴⁷. The co-stimulatory signal, or signal 2, can result from many potential receptor interactions, the most well-established of which is the CD28 receptor on T cells with B7 family ligands (CD80 or CD86) on APCs^{248,249}. A crucial effect of signal 2 is increased transcription and stabilization of *IL2* mRNA²⁵⁰. Signals 1 and 2 acting in concert drive the onset of T cell activation and proliferation, thus initiating an expansion phase. T cell responses must be fine-tuned to a particular function or immunological response and, therefore, a third signal provides the basis for cell polarization, optimal effector functionality and survival²⁵¹. Signal 3 results from cytokines and/or chemokines present in the microenvironment, usually derived directly from APCs²⁵¹. This signal has a potent effect on T cell differentiation, with factors such as IL-12 and IFN α , IFN β and IFN γ skewing T cell fate towards cytotoxic T lymphocyte or type 1 T helper-type responses, whereas retinoic acid and transforming growth factor- β promotes the generation of regulatory T cells^{252,253}. In the presence of sufficient antigen, proliferation of activated T cells will be initiated and sustained; upon clearance of antigen, T cell populations enter contraction, followed by a memory phase²⁴⁴. During this memory phase, stable numbers of long-lived, antigen-specific CD8⁺ T cells remain in the circulation; this cell population has a distinct phenotype that enables rapid reinduction of a robust cytotoxic activity upon antigen re-encounter^{118,123}.

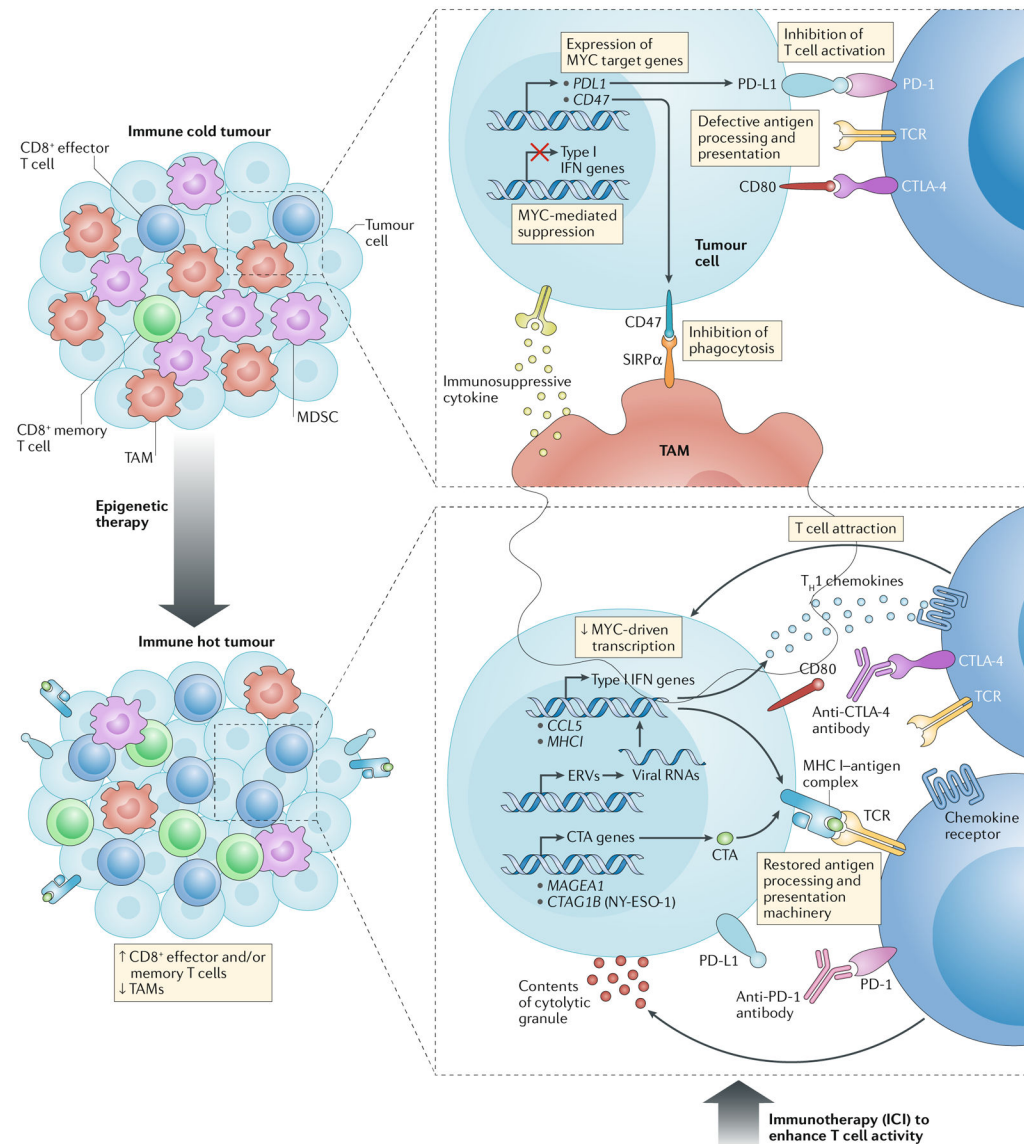


Fig. 1 | Effects of epigenetic therapy on the immune state of a tumour and rationale for the use of combination epigenetic and immunotherapy strategies in cancer.

Epigenetic therapy has the potential to convert a tumour from an immune repressive (immune cold) to an immune permissive (immune hot) state through effects on several factors of the tumour microenvironment that normally impede the therapeutic activity of immune-checkpoint inhibition. Immune cold tumours are characterized by the absence of tumour-infiltrating lymphocytes, the presence of immunosuppressive cell populations, such as tumour-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), and/or a lack of expression of programmed cell death 1 ligand 1 (PD-L1) by the tumour cells^{224,254}. Epigenetic agents can modulate the immune composition of the tumour microenvironment by decreasing the abundance of TAMs and MDSCs and increasing the numbers of CD8⁺ effector T cells and memory T cells^{144,178}. As well as having the potential to shift the differentiation of CD8⁺ tumour-infiltrating lymphocytes towards effector and/or memory phenotypes, epigenetic drugs can augment innate immune-related signalling and the

expression of inflammatory proteins, such as chemokines^{142–144,146,147,164}, which aid the recruitment of T cells to the tumour. In addition, epigenetic therapy can revert key aspects of cancer immunoediting via increased expression of tumour antigens, such as cancer/testis antigens (CTAs)^{148–151}, and restoration of the MHC class I (MHC I) antigen processing and presentation machinery (which is often dysregulated in tumour cells)^{142–144,160,161}, thus potentiating the immune recognition of tumours. Type I interferon (IFN) signalling is a major node of these immunological pathways and can be triggered in response to increased levels of cytoplasmic viral RNAs resulting from epigenetic de-repression of endogenous retroviruses (ERVs)^{146,147}. Epigenetic therapy can also induce the repression of MYC and MYC-related signalling, thus counteracting the immunosuppressive functions of this oncogenic transcription factor, which include downregulation of type I IFN-mediated gene expression, for example, of the gene encoding the T cell-attracting chemokine CC-chemokine ligand 5 (CCL5)¹⁴⁴; production of CCL9 that recruits immunosuppressive, PD-L1-positive macrophages to tumours and IL-23 that results in exclusion of T cells, natural killer cells and B cells (not shown); and upregulation of inhibitory immune-checkpoint proteins PD-L1 and CD47 in tumour cells, which suppress T cell activation and macrophage-mediated phagocytosis, respectively^{184,185}. All of the above contribute to the activity of epigenetic agents in converting immune cold tumours into immune hot tumours²²⁴, such that the tumours become amenable to immunotherapeutic interventions. For example, the effectiveness of immune-checkpoint inhibitors (ICI) in unleashing an effective T cell-mediated immune response is likely to be enhanced in the context of re-establishment of effective antigen-presentation mechanisms, upregulation of PD-L1, a decreased abundance of TAMs, and increases in the numbers of effector and/or memory T cells within the tumour microenvironment²²⁴. CTLA-4, cytotoxic T lymphocyte antigen 4; PD-1, programmed cell death 1; SIRP α , signal-regulatory protein α ; TCR, T cell receptor; T_H1 cell, type 1 T helper cell.

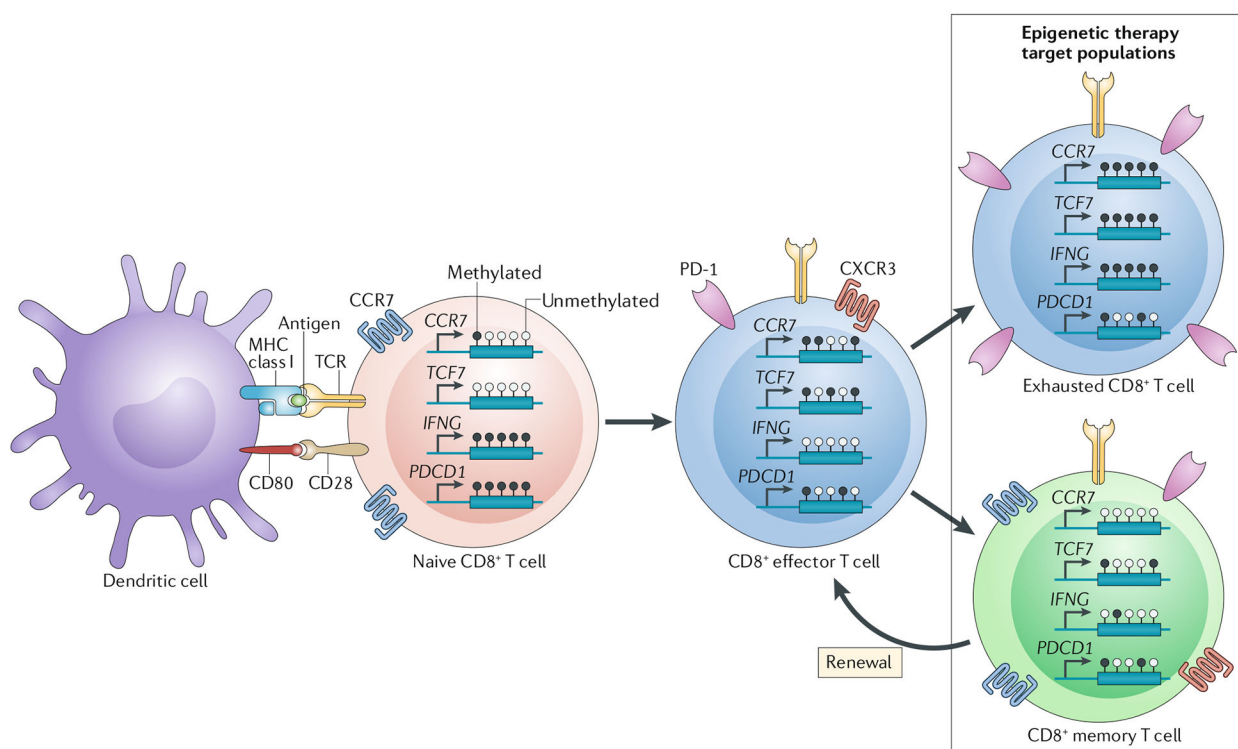


Fig. 2 |. Implications of DNA methylation-associated programmes on T cell differentiation.

T cell activation from the naive to an effector state is induced by interaction between the T cell receptor (TCR) and corresponding MHC class II–peptide complex on professional antigen-presenting cells or MHC class I in the setting of dendritic cell cross presentation (the context shown in the figure) in concert with co-stimulatory molecule interactions and inflammatory stimuli²⁵⁵. Bone marrow-derived antigen-presenting cells — predominantly dendritic cells but also macrophages or B cells — are sufficient to induce CD8⁺ T cell priming, whereas CD4⁺ T cells are unable to facilitate this process²⁴⁵. As elucidated in studies by Youngblood et al.¹¹⁸ and Ghoneim et al.¹¹⁹, among others, the methylation status of genes encoding several crucial mediators of T cell differentiation undergoes dynamic changes during the acquisition of major T cell phenotypes. For example, the transition from a naive to effector phenotype is characterized by the induction and repression of many distinguishing cell-surface markers, including the G protein-coupled chemokine receptors CXCR3 and CCR7 and the inhibitory immune-checkpoint receptor programmed cell death 1 (PD-1). CXCR3 expression has been shown to be epigenetically regulated in antigen-specific CD4⁺ T cells, although it remains unclear whether the same is true in CD8⁺ T cells, and renders effector T cells responsive to interferon-inducible, type 1 T helper (T_H1) cell-associated chemokines, such as CXCL9, CXCL10 and CXCL11, which tend to emanate from sites of inflammation²⁵⁶. Augmentation of PD-1 expression through demethylation of the *PDCD1* (PD-1) gene promoter and a regulatory region ~300 bp upstream of the transcription start site occurs rapidly following antigen stimulation of naive T cells as the direct result of activatory signalling from the TCR^{126,257}. PD-1 signalling acts as a negative feedback regulator of the inflammatory activity of T cells by inhibiting TCR-mediated

signalling²⁵⁸. Cell-surface expression of the homing receptor CCR7 is also dynamically regulated during the naive to effector phenotypic transition via DNA methylation and thus repression of the *CCR7* gene^{118,119}. CCR7 facilitates the recruitment of naive T cells from the bloodstream to lymphoid organs; therefore, downregulation of this receptor enables primed effector T cells to migrate from these organs to other tissues in surveillance of their cognate antigen²⁵⁹. In addition, effector T cells have an increase in methylation and thus repression of *TCF7*, which encodes transcription factor 7, as well as a loss of methylation and de-repression of *IFNG*, which encodes the inflammatory cytokine IFN γ ¹¹⁹. The post-effector fate of CD8⁺T cell generally involves the acquisition of either of two major phenotypes, namely exhausted or memory²⁶⁰. The exhausted state is characterized by whole-genome gains in DNA methylation, including sites in *TCF7*, *IFNG* and *CCR7* (REF¹¹⁹). These methylation gains result in reduced effector functionality in terms of both cytolytic activity and cell proliferation. In comparison with effector T cells, exhausted T cells have increased PD-1 expression and decreased CXCR3 expression, which act to sensitize T cells to inhibitory interactions with programmed cell death 1 ligand 1 and prevent chemotactic responses to (T_H1) cell-associated chemokines, respectively. The acquisition of the memory phenotype in effector T cells is correlated with the demethylation and thus re-expression of *CCR7* (REF.¹¹⁸), with retention of CXCR3 and PD-1 expression^{261–263}. Memory T cells demonstrate increased *IFNG* methylation compared with that associated with the effector state, but do not demonstrate the high methylation levels of this gene found in naive or exhausted CD8⁺ T cells^{118,119}. The T cell populations that seem to be most amenable to modulation with epigenetic therapies are those in the post-effector states of T cell differentiation (boxed area)^{119,144}.

Summary of therapeutics targeting epigenetic modifiers

Epigenetic agent	Stage of clinical development	Combination therapies
<i>DNMT inhibitors^a</i>		
Azacitidine	FDA approved: MDS and AML Phase I/II: various solid carcinomas, lymphomas and/or other haematological malignancies; Phase III: haematological malignancies	In combination with the BCL-2 antagonist venetoclax in AML HDAC inhibitors, immune-checkpoint inhibitors and/or chemotherapeutic agents
Decitabine or THU-DAC	FDA approved: MDS Phase I/II: various solid carcinomas, lymphomas and/or other haematological malignancies; Phase III: haematological malignancies and primary neoplasia of ovary	NA HDAC inhibitors, immunotherapies, PARP inhibitors and/or chemotherapeutic agents
Guadecitabine	Phase I/II: various solid carcinomas and/or haematological malignancies; Phase III: haematological malignancies	HDAC inhibitors, immunotherapies, PARP inhibitors and/or chemotherapeutic agents
<i>HDAC inhibitors^a</i>		
Entinostat	FDA breakthrough drug designation: advanced-stage breast cancer Phase I/II: various solid carcinomas, lymphomas and/or other haematological malignancies; Phase III: hormone receptor-positive breast cancer	Exemestane Immunotherapies
Vorinostat	FDA approved: CTCL Phase I/II: various solid carcinomas, lymphomas and/or other haematological malignancies; Phase III: CTCL, multiple myeloma and ALL	NA Immunotherapies, PARP inhibitors or chemotherapeutic agents
Romidepsin	FDA approved: CTCL Phase I/II: Hodgkin lymphoma, PTCL, multiple myeloma, NHL and/or solid carcinomas; Phase III: T cell lymphomas	NA Immunotherapies or chemotherapeutic agents
Panobinostat	FDA approved: multiple myeloma Phase I–III: various solid carcinomas, lymphomas and/or other haematological malignancies	Bortezomib (proteasome inhibitor) plus dexamethasone Immunotherapies or chemotherapeutic agents
Givinostat	Phase II: chronic myeloproliferative neoplasms	NA
Mocetinostat	Phase I/II: lymphoma, melanoma, NSCLC and/or other advanced-stage solid tumours	Guadecitabine and/or immune-checkpoint inhibitors, or brentuximab vedotin (anti-CD30 antibody–drug conjugate)
Valproic acid	Phase I–III: AML, MDS, various solid carcinomas and/or childhood ependymoma, virus-associated cancers	Azacitidine, chemotherapeutic agents or immune-checkpoint inhibitors
Belinostat	FDA approved: PTCL Phase I/II: T cell leukaemia or lymphoma, MDS or AML, glioblastoma or various other solid carcinomas and haematological malignancies	NA Zidovudine (±IFNα-2b), pevonedistat (NEDD8-activating enzyme inhibitor) or temozolomide (chemotherapy) plus radiotherapy
<i>HMT inhibitors</i>		
CPI-1205 (EZH2 inhibitor)	Phase Ib/II:mCRPC	Enzalutamide or abiraterone (anti-androgens) and prednisone

Epigenetic agent	Stage of clinical development	Combination therapies
	- Phase I/II: various advanced-stage solid tumours previously treated with a PD-1 or PD-L1 inhibitor - Phase I: B cell lymphoma	- Ipilimumab (anti-CTLA-4 antibody) - NA
Tazemetostat ^a (EZH2 inhibitor)	- Phase II: various advanced-stage NHLs and solid tumours, including <i>IVL</i>-negative tumours, tumours with <i>EZH2</i>, <i>SMARCB1</i> or <i>SMARCA4</i> mutation, or mesothelioma with <i>BAP1</i> loss of function, malignant rhabdoid tumours, mesothelioma and recurrent ovarian, primary peritoneal or endometrial cancer - Phase I/II: advanced stage urothelial carcinoma - Phase I/II: advanced-stage solid tumours and B cell lymphomas - Phase I: B cell NHLs	- NA - Pembrolizumab - Prednisolone (in those with DLBCL) - Atezolizumab (in those with follicular lymphoma or DLBCL)
Pinometostat (DOT1L inhibitor)	Phase Ib/II: <i>KMT2A</i> -rearranged AML	Standard chemotherapies
LSD1 inhibitors		
IMG-7289	Phase I: myelofibrosis	NA
Secldemstat (SP-2577)	- Phase I: Ewing sarcoma - Phase I: advanced-stage solid tumours	- NA - NA
INCB059872	- Phase I/II: advanced-stage solid tumours, including previously treated stage IIIB–IV NSCLC or stage IV microsatellite-stable colorectal cancer - Phase Ib: Ewing sarcoma - Phase I/II: advanced-stage malignancies, including AML or MDS, myelofibrosis, Ewing sarcoma, poorly differentiated neuroendocrine tumours or SCLC	- Pembrolizumab (anti-PD-1 antibody) plus epacadostat (IDO1 inhibitor) - NA - Azacitadine plus ATRA (for AML) or nivolumab (anti-PD-1 antibody; for SCLC)
BET inhibitors		
RO6870810	- Phase I: multiple myeloma - Phase I: DLBCL or high-grade B cell lymphoma with <i>MYC</i>, <i>BCL2</i> and/or <i>BCL6</i> rearrangement	- Daratumumab (anti-CD38 antibody) - Venetoclax and rituximab (anti-CD20 antibody)
INCB057643	- Phase I/II: advanced-stage malignancies - Phase I/II: advanced-stage solid tumours, including previously treated stage IIIB–IV NSCLC or stage IV microsatellite-stable colorectal cancer	- Standard-of-care agents - Pembrolizumab plus epacadostat
CPI-0610	- Phase II: myelofibrosis - Phase I: lymphoma	- Ruxolitinib (JAK inhibitor) - NA
Molibresib (GSK525762)	- Phase II: advanced-stage HER2-negative, hormone receptor-positive breast cancer - Phase II: haematological malignancies	Fulvestrant (selective oestrogen receptor degrader)

Epigenetic agent	Stage of clinical development	Combination therapies
	- Phase I: NUT midline carcinoma or other solid tumours - Phase I: castration-resistant prostate cancer - Phase I: advanced-stage and refractory solid tumours and lymphomas	- NA - NA - Abiraterone plus prednisone or enzalutamide - Entinostat
ZEN003694	- Phase II: triple-negative breast cancer - Phase I/II: mCRPC	- Talazoparib (PARP inhibitor) - Enzalutamide
BMS-986158	- Phase I/II: selected advanced-stage solid tumours or haematological malignancies - Phase I: paediatric cancers	- Nivolumab - NA
MK-8628	Phase I: AML (including de novo AML and AML secondary to MDS) or DLBCL	NA
AZD5153	- Phase I: Relapsed and/or refractory solid tumours and lymphomas - Phase I: NHL	- Olaparib (PARP inhibitor) - Acalabrutinib (BTK inhibitor)

ALL, acute lymphocytic leukemia; AML, acute myeloid leukaemia; ATRA, all-trans retinoic acid; BET, bromodomain and extra-terminal protein; BTK, Bruton tyrosine kinase; CTCL, cutaneous T cell lymphoma; CTLA-4, cytotoxic T lymphocyte antigen 4; DLBCL, diffuse large B cell lymphoma; DNMT, DNA methyltransferase; DOT1L, DOT1-like protein; EZH2, enhancer of zeste homologue 2; HDAC, histone deacetylase; HMT, histone methyltransferase; IDO1, indoleamine 2,3-dioxygenase 1; JAK, Janus kinase; LSD1, lysine-specific histone demethylase 1A; mCRPC, metastatic castration-resistant prostate cancer; MDS, myelodysplastic syndrome; NA, not applicable; NHL, non-Hodgkin lymphoma; NSCLC, non-small-cell lung cancer; PARP, poly(ADP-ribose) polymerase; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand 1; PTCL, peripheral T cell lymphoma; SCLC, small cell lung carcinoma; THU-DAC, tetrahydrouridine-decitabine.

^aPhase I-III studies of DNMT inhibitors and HDAC inhibitors, as well as phase II studies of tazemetostat monotherapy, are too numerous to list individually in separate bullet points and, for brevity, only broad descriptions of the disease setting and combination partners have been provided for these trials.



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DFMO and 5-azacytidine increase M1 macrophages in the tumor microenvironment of murine ovarian cancer.

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Abstract

Although ovarian cancer has a low incidence rate, it remains the most deadly gynecologic malignancy. Previous work has demonstrated that the DNMTi 5-Azacytidine (AZA) activates type I interferon signaling to increase IFN γ + T cells and NK cells and reduce the percentage of macrophages in the tumor microenvironment. To improve the efficacy of epigenetic therapy, we hypothesized that the addition of α -difluoromethylornithine (DFMO), an ornithine decarboxylase inhibitor, may further decrease immunosuppressive cell populations improving outcome. We tested this hypothesis in an immunocompetent mouse model for ovarian cancer and found that *in vivo*, AZA and DFMO, either alone or in combination, significantly increased survival, decreased tumor burden, and caused recruitment of activated (IFN γ +) CD4+ T cells, CD8+ T cells, and NK cells. The combination therapy had a striking increase in survival when compared to single agent treatment, despite a smaller difference in recruited lymphocytes. Instead, combination therapy led to a significant decrease in immunosuppressive cells such as M2 polarized macrophages and an increase in tumor-killing M1 macrophages. In this model, depletion of macrophages with a CSF1R-blocking antibody reduced the efficacy of AZA+DFMO treatment and resulted in fewer M1 macrophages in the tumor microenvironment. These observations suggest our novel combination therapy modifies macrophage polarization in the tumor microenvironment, recruiting M1 macrophages and prolonging survival.

INTRODUCTION

Although ovarian cancer has a low incidence rate of 1.3 percent nationally, it remains the most deadly gynecologic malignancy and the need for novel therapeutics is high (1). Cancer immunotherapy treatment options have grown rapidly in the last decade and have

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demonstrated considerable promise in multiple disease types; however, ovarian tumors have thus far not responded well to current therapies such as the immune checkpoint inhibitors α -PD-1 and α -PD-L1 (2–5). Low intra-tumoral CD8+ T cells and high immunosuppressive cell populations such as myeloid derived suppressor cells (MDSCs) and macrophages are associated with poor prognosis in ovarian cancer and could impact the efficacy of these immune therapies (6–9). Drug treatment strategies that alter the tumor and immune cell microenvironment could prolong survival for ovarian patients.

Macrophages demonstrate considerable plasticity in their development, responding to environmental signals such as cytokines and growth factors that dictate their phenotype (10). Classically polarized or M1 type macrophages are considered to be anti-tumorigenic, producing pro-inflammatory cytokines and promoting T cell immunity (10–12). In contrast, alternatively polarized or M2 type macrophages, normally involved in wound repair, are anti-inflammatory and can promote tumorigenesis (10–12). In order for immune checkpoint blockades such as α -PD-1 to be effective, there must be a robust T cell response in the tumor, and the relative proportions of these M1 and M2 macrophages has a significant impact on T cell immunity (10).

One treatment strategy that impacts immune cell populations in the tumor microenvironment is epigenetic therapies such as DNA methyl transferase inhibitors (DNMTIs) and histone deacetylase inhibitors (HDACIs) (13–19). 5-azacytidine (AZA) is a demethylating agent that incorporates into nucleic acids as a cytidine analog which cannot be methylated by DNA methyl transferases (DNMTs). AZA is FDA approved for myelodysplastic syndrome (MDS), and low nanomolar doses lead to decreased DNA promoter methylation and restored expression of hypermethylated genes in cancer (20). Additionally, AZA treatment induces the re-expression of hypermethylated, silenced endogenous retroviruses (ERVs) *in vitro*, which can elicit an anti-viral, interferon immune response that leads to T cell activation *in vivo* (15,18). Furthermore, AZA treatment of an ovarian cancer mouse model leads to increased immune cells in the tumor microenvironment, and combination AZA and HDACi sensitized tumors to α -PD-1 therapy (18). While first generation HDACIs combined with DNMTIs have demonstrated some promise in clinical trials for non-small cell lung cancer (21), there remains a need to discover novel treatment strategies that activate the immune system and provide long term remission for other solid tumors.

While the impact of epigenetic therapy on the immune system has been well established, emerging literature has shown that additional drug therapies can also regulate the immune system. In particular, polyamine-blocking therapy (PBT), the combined inhibition of polyamine biosynthesis and transport, significantly inhibits tumor growth in immunocompetent mice but not in athymic mice (22). Polyamines are naturally occurring, polycationic, alkyl amines that are absolute requirements for multiple cellular processes and are particularly important for tumor cell growth (23). 2-difluoromethylornithine (DFMO) is FDA approved for African sleeping sickness, and works as an inhibitor of ornithine decarboxylase (ODC), an essential enzyme that catalyzes a rate-limiting step of polyamine synthesis. DFMO treatment reduces intracellular polyamines and inhibits tumor cell growth in multiple model systems, but failed to demonstrate significant antitumor activity as a

single agent in advanced tumors. (24–26). It is currently being tested in clinical trials as a chemopreventive agent and for the treatment of neuroblastoma (27–32).

Similar to data obtained with PBT, DFMO treatment alters immune cell populations in the tumor microenvironment (33). Investigators demonstrated that DFMO treatment of immunocompetent mice, but not RAG1 knockout mice, inhibited tumor growth, decreased MDSC activity and increased infiltration of CD8+ T cells (33). The potential to combine epigenetic therapy with PBT is intriguing, as epigenetic therapies are known to activate a strong anti-viral, interferon response, while PBT attenuates immunosuppressive cells such as MDSCs.

We have therefore tested the hypothesis that the combination of AZA and DFMO would produce a more durable antitumor response due to immune related changes in the tumor microenvironment. In an immunosuppressive mouse model of aggressive high-grade serous ovarian cancer, we found that combination AZA and DFMO dramatically prolonged survival, and led to an increase in M1 versus M2 macrophages that may be important for the efficacy of this drug combination. Since both drugs are clinically approved and well-tolerated, there is potential to rapidly translate this combination to the clinic for treatment of ovarian cancer. Moreover, other solid tumors that are rich in macrophages could benefit from this treatment regimen as well, due to the impact this drug combination has on macrophage polarization.

MATERIALS AND METHODS

Drugs and reagents:

DFMO was kindly provided by Dr. Patrick Woster (Medical University of South Carolina). AZA was purchased from Sigma-Aldrich (Catalog No. 32067-2). α -PD-1 was kindly provided by the Michael Lim lab. α -CSF1R (BioXCell Clone AFS98) was generously provided by Janssen.

Animals:

Female C57BL/6NHsd wild-type (WT) mice (7–8 wk old) were purchased from Envigo International Holdings, Inc. (Indianapolis, IN). Mice were housed at the Johns Hopkins Kimmel Cancer Center Animal Resources Core and cared for in accordance with the policies of The Johns Hopkins University Animal Care and Use Committee and our approved animal protocol.

Syngeneic mouse model:

250,000 VEGF- β -Defensin ID8 (VDID8) syngeneic mouse ovarian surface epithelial (MOSE) cells were injected intraperitoneally into wild-type (WT) C57BL/6 mice. Cells were obtained from Dr. Chien-Fu Hung and tested for Mycoplasma every 6 months using MycoAlert PLUS (Lonza LT07–701) per manufacturer's instructions and as previously described (18). Dr. Katherine Roby developed the ID8 model via mild trypsinization of the ovarian surface epithelium, followed by long-term passage *in vitro* until the cells spontaneously immortalized (34). The parental ID8 clone has been further modified to

enhance its usefulness as a tool by overexpressing VEGF and β -defensin, making the tumor more aggressive and immunosuppressive (35). The VDiD8 cells are also positive for Luciferase and green fluorescent protein (GFP). While this model has proven to be an excellent research tool, it has limitations in representing high-grade serous ovarian cancer in humans because it is derived from mouse ovarian surface epithelium, not the fallopian tube, and is Trp53 wildtype. In mice however, ovarian cancer can arise from either fallopian tube epithelium (FTE) or ovarian surface epithelium (OSE) and ID8 is the most widely used MOSE model for immunotherapy studies in ovarian cancer.

Mice were treated with 0.5 mg/kg AZA/saline, Monday through Friday, every other week and continuous 2% DFMO in drinking water. 200ug of α -PD-1 or IgG was injected i.p. 4 times total on days 17, 20, 24, and 27 post i.p. injection of VDiD8 cells. 200 ug of α -CSF1R or IgG was injected i.p. twice weekly beginning two weeks prior to VDiD8 cell injection, and continuing throughout the duration of the experiment.

Ascites tissue harvest and processing:

When ascites fluid is collected from the mice, the cells obtained represent the tumor microenvironment and can be further analyzed to help illustrate the mixed population of cells surrounding the tumor. Ascites was collected, filtered, incubated in ACK buffer (Quality Biological) to lyse red blood cells, and washed. The mononuclear cells collected were then cultured for four hours in RPMI (Corning) with 10% FBS in the presence of phorbol 12-myristate 13-acetate (PMA) and ionomycin to stimulate cells, and brefeldin A and monensin (Invitrogen 004975-93) to cause aggregation of secreted proteins inside the cell.

Flow cytometry:

Cells were washed and blocked with FcR Blocking Reagent (Miltenyi Biotec 130092-575) and stained for cell-surface markers including Live/Dead (eBioscience 650865-14), CD45 (BD Biosciences 563891), CD3 (BD Biosciences 560527), CD4 (BD Biosciences 563331), CD8 (BD Biosciences 563152), NK1.1 (BD Biosciences 562921), F4/80 (BioLegend 123113), CD11b (BioLegend 101222), MHC II (isotype control 400627; BioLegend 107619), CD206 (BioLegend 141708), CD11c (BD Biosciences 564079), Ly6C (BD Biosciences 562728), Ly6G (BD Biosciences 563005), CD80 (BD Biosciences 553769), and CD86 (BD Biosciences 558703). Cells were permeabilized and stained for intracellular IFN γ (isotype control 554686; BD Biosciences 554413). Flow cytometry acquisition was performed on an LSR II cytometer (BD Biosciences), and data were analyzed using FlowJo software version 10.2.

Flow sorting:

Lysed and processed bulk ascites cells were blocked with FcR Blocking Reagent (Miltenyi Biotec 130092-575) and stained for cell-surface markers including Live/Dead (eBioscience 650865-14), CD45 (BD Biosciences 563891), F4/80 (BioLegend 123113), CD11b (BioLegend 101222), MHC II (isotype control 400627; BioLegend 107619), CD206 (BioLegend 141708), and CD11c (BD Biosciences 564079). Prepared cells were suspended in PBS and sorted immediately on a BSL-2 FACS Aria II. M1 macrophages were sorted on a

gate as follows: CD45+ L/D- F4/80+ CD11b+ MHC II+ CD206- CD11c-. M2 macrophages were sorted on a gate as follows: CD45+ L/D- F4/80+ CD11b+ MHC II- CD206+ CD11c-.

RNA isolation and quantitative reverse-transcriptase PCR:

Total RNA was isolated from sorted macrophages using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). 200 ng of RNA was used for cDNA synthesis using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD), followed by SYBR green-mediated real-time PCR (Universal SYBR Green Supermix, BioRad, Hercules, CA) using custom primers specific for Arg1, Fizz1, and iNOS2. (Arg1 F: CAGAAGAATGGAAGAGTCAG; Arg1 R: CAGATATGCAGGCAGGGAGTCACC; Fizz1 F: GGTCCCAGTGCATATGGATGAGACCA; Fizz1 R: CACCTCTTCACTCGAGGGACAGTTGG;

iNOS2 F: CCGAAGCAAACATCACATTCA; iNOS2 R: GGTCTAAAGGCTCCGGGCT). In each experiment, samples were performed in duplicate, normalized to β -actin as an internal control, and fold change in expression relative to M1 or M2 macrophage was determined using the $2^{-\Delta\Delta C_t}$ algorithm. Thermocycling was performed on a Bio-Rad iQ2 real-time PCR detection system and data collected using the iQ5 optical system software.

ELISA assays:

Bulk ascites fluid collected from individual treated mice was centrifuged at low speed (1000rpm) for 15 minutes, and 1,000uL of supernatant was collected and stored at -80C. Circulating CSF-1 levels in mice treated with IgG vs. CSF1R was detected using an ELISA kit (R&D Systems Kit #MMC00) according to instructions.

Polyamines:

Polyamines were analyzed via high-performance liquid chromatography (HPLC) as previously described (36).

Statistical analysis:

Data were graphed in GraphPad Prism 7.0 and tested for a Gaussian distribution using the Shapiro-Wilk test. Significance was determined for sets of data with more than two groups using the one-way ANOVA or Kruskal Wallis test dependent upon normality results from the Shapiro-Wilk test. If only two sets of data were compared, either the Mann-Whitney (non-parametric) or student's t test (parametric) were used dependent on normality results. Significances in survival data were determined by Mantel-Cox (log-rank) test. P values less than 0.05 were deemed significant. Outliers were removed from ascites volume datasets and ascites immune cell datasets using Peirce's criterion (37). Significances are shown as *P < 0.05, **P < 0.01, and ***P < 0.001 ****P < 0.0001.

RESULTS

Combination AZA and DFMO therapy reduces tumor burden and increases survival in an ovarian cancer mouse model.

To confirm that DFMO inhibits ODC in the model systems used, VDD8 cells were treated *in vitro* and *in vivo* and polyamine levels were determined (Fig S1a,b). *In vitro* treatment of VDD8 tumor cells led to a significant decrease in putrescine and spermidine with DFMO alone and when combined with AZA. However, AZA alone appeared to have a stimulatory effect on putrescine and spermidine synthesis (Fig S1a). In bulk ascites cells from treated animals, combination treatment led to a decrease in all three polyamines, including spermine (Fig S1b). No significant changes to the polyamine pools were observed with AZA treatment alone, but putrescine and spermidine were decreased (although not significantly) by DFMO treatment (Fig S1b).

To test the hypothesis that addition of DFMO to therapy using the DNMTi AZA would reduce tumor burden and improve overall survival in a mouse model of ovarian cancer, immunocompetent C57BL/6 mice were injected intraperitoneally (i.p.) with 250,000 VDD8 syngeneic MOSE cells. Mice were treated IP with AZA (0.5 mg/kg) or saline vehicle, DFMO (2% in water), or combination AZA and DFMO beginning three days post tumor injection (Fig 1a). Hemorrhagic ascites fluid consistently develops at approximately 4–5 weeks post VDD8 injection and is an accurate measurement of tumor burden in mice, allowing observation of tumor growth in real time (35,38). After draining hemorrhagic ascites fluid from mice for the second time (typically week 5 post tumor injection), mice treated with single agent AZA or DFMO present with higher tumor burden than mice treated with combination therapy (Fig 1b). Mice treated with combination therapy also exhibited the largest increase in overall survival with a median survival of 59 days compared to that of single agent AZA or DFMO of approximately 44 days (Fig 1c). Although total numbers of lymphocytes are significantly increased by single agent AZA or DFMO compared to vehicle, these numbers are not further enhanced with combination AZA+DFMO treatment (Fig 1d,e).

AZA and DFMO combination treatment significantly increases IFN γ + NK cells.

To pursue further whether changes in lymphocyte populations might account for the dramatic increase in survival observed with AZA+DFMO combination therapy, the numbers and activity of specific lymphocyte subpopulations in hemorrhagic ascites fluid at week 5 post tumor injection were analyzed. Single agent AZA or DFMO led to significant increases in T cell, NK cell, and IFN γ + lymphocyte populations examined in the tumor microenvironment (Fig 2a–g). In most cases, combination therapy did not alter immune populations over what was observed with single agents (Fig 2a–f). The exception however, was a significant increase in IFN γ + NK cells observed in combination treated mice versus AZA or DFMO alone (Fig 2g). It was hypothesized that the observed increase in IFN γ + cells in the model could lead to an increase in PD-L1 expression on the surface of tumor cells, possibly sensitizing the tumor to α -PD-1 therapy. Surface PD-1 expression on T cells is a signature of immune tolerance, and when engaged with its ligand PD-L1 on tumor cells, can limit the T cell's ability to proliferate and perform its effector functions (39,40).

Addition of α -PD-1 to the combination of DFMO and AZA treatment did not further decrease tumor burden in the mice, nor did it increase survival (Fig S2a–f). No changes were observed in the number of PD-1 expressing cells with single agent or combination treatment on either CD4+ or CD8+ T cells (Fig S2g,h). The lack of response to α -PD-1 therapy suggests that a T cell response may not be the primary mechanism of action in this combination drug therapy. Although AZA and DFMO treatment led to elevated IFN γ + NK cells and modest increases in T cells, it does not appear that the differences between combination treatment and single agents AZA or DFMO were significant enough to explain the dramatic increase in survival seen with combination treatment (Fig 1c).

Combination AZA + DFMO treatment results in a significant decrease in macrophages.

The myeloid immune cell populations were next examined to determine whether a decrease in immunosuppression may account for the striking differences in survival. MDSCs are suppressive immune cells sometimes present in the tumor microenvironment, high levels of which are associated with a poor prognosis in ovarian cancer (7). No significant decrease in non-lymphocyte or MDSC populations was observed after treatment with AZA and DFMO (Fig 3a,b). Instead, total macrophage populations in the tumor microenvironment were consistently decreased with AZA treatment, and decreased even further with the addition of DFMO (Fig 3c). Macrophages are professional antigen presenting cells capable of activating T cells. Surface expression of MHC II is essential for interaction with T cells, and the number of MHC II positive cells was increased with AZA, DFMO, and AZA+DFMO treatment compared to vehicle (Fig 3d,e,S3a,b). Importantly, MHC II expressing cells were increased significantly with combination treatment compared to single agent AZA, suggesting a possible explanation for the dramatic increase in survival (Fig 3d, 1c). In contrast, untreated mice had high populations of macrophages negative for the MHC II surface protein. These data suggest that macrophages may play an important role in tumor response to the combination drug therapy.

Combination AZA plus DFMO treatment leads to an increased ratio of M1 macrophages to M2 macrophages in the tumor microenvironment.

Next, surface markers were examined to distinguish between classical (M1) and alternative (M2) polarized macrophages. High populations of M2 macrophages are associated with a poor prognosis due to their ability to promote tumor growth (8–10). Because the surface marker CD206 is upregulated on M2 macrophages, flow cytometry was used to analyze macrophages high for CD206 and low for MHC II—a surface marker for M1 macrophages. Although total macrophages were decreased by the treatments, an increase in M1 macrophages was observed in the remaining macrophage population for all treatment groups (Fig 4a), as well as a decrease in M2 macrophages (Fig 4b, S4a). MHC II- CD206+ and MHC II+ CD206- macrophages were then sorted via flow cytometry, and RNA was isolated to perform RT-PCR on M1- and M2-specific genes (41–44). As expected, CD206+ macrophages demonstrated increased expression of Arg1 and Fizz1 compared to CD206- macrophages (Fig 4c,d), and MHC II+ macrophages had increased expression of iNOS2 compared to MHC II- macrophages (Fig 4e). These data confirm that macrophages expressing high levels of CD206 in our model also retain gene expression patterns that are characteristic of alternatively polarized M2 macrophages.

Interestingly, the decrease in M2 macrophages observed in AZA+DFMO treated mice was not a durable response, and as tumor burden increased in these mice, the relative proportion of M2 macrophages increased as well (Fig S5a,b). Macrophages in vehicle treated mice were therefore assessed at three different time points to determine whether M2 macrophages increase as the disease progresses. Indeed, relative levels of M2 macrophages increased as tumor burden increased in these mice, suggesting the importance of macrophages in disease progression of this ovarian cancer model (Fig 4f).

Blocking macrophages with CSF1R antibody diminishes the AZA plus DFMO response in the ovarian cancer mouse model.

To test whether the increase in M1 macrophages was important in the response to AZA and DFMO treatment, macrophages were blocked in the ovarian cancer mouse model using an antibody to CSF1R (45) (Fig 5a). Treatment with α -CSF1R resulted in decreased macrophages in the tumor microenvironment (Fig 5b) and a consequential increase in M-CSF levels in ascites fluid as measured by ELISA (Fig 5c). Increased M-CSF indicates that the α -CSF1 receptor block antibody is functional, as more ligand (M-CSF) is free, and less ligand is engaged with its receptor (45). Initially, the AZA+DFMO combination treatment still resulted in decreased tumor burden in mice, even with the observed decrease in macrophages; however, over time, tumor burden increased more rapidly in AZA+DFMO mice receiving α -CSF1R (Fig 5d,e). This decrease in macrophages also led to a decrease in overall survival, compared with AZA+DFMO mice that received IgG control (Fig 5f). Analysis via flow cytometry of M1 and M2 surface markers showed that with IgG control, AZA+DFMO mice had increased M1 macrophages and decreased M2 macrophages compared to vehicle, as was previously seen (Fig 5g,h; Fig 4a,b). Interestingly, while AZA +DFMO mice maintained low M2 macrophages in the presence of α -CSF1R (consistent with the action of α -CSF1R, Fig 5b), M1 macrophages were significantly decreased compared to AZA+DFMO mice receiving IgG control (Fig 5g,h). These results indicate that the presence of M1 macrophages is important for the mechanism of action of this combination drug therapy, as AZA+DFMO treated mice receiving α -CSF1R had decreased survival and increased tumor burden compared to IgG control.

DISCUSSION

Combination epigenetic and polyamine reducing therapy is an effective treatment strategy for ovarian cancer in immunocompetent mice, prolonging survival and decreasing tumor burden significantly. This treatment regimen represents the first combination of these two drug therapies in mice, and the first use of DFMO in an immunocompetent mouse model for ovarian cancer (46). Treatment with AZA alone led to an increase in IFN γ + NK cells, CD4+ T cells, and CD8+ T cells, as has been demonstrated before (14,18,19). Signaling of IFN γ via its receptor IFNGR1 on tumor cells can lead to increased expression of PD-L1 on tumor cells, thereby making this increase in IFN γ an attractive candidate for α -PD-1 therapy. However, α -PD-1 therapy had no significant impact on survival in this model when added to the combination AZA and DFMO. These results are in contrast to previous studies using AZA and HDACi where the addition of α -PD-1 produced a significant therapeutic response (18). Histone acetylation is essential for transcription of IFN γ , therefore the use of an

HDACi may explain the sensitization to α -PD-1 therapy previously seen, as increasing histone acetylation even further increased IFN γ levels in lymphocytes (47).

Analysis of the tumor microenvironment after the combination treatment with AZA and DFMO indicated that the impacts on macrophage polarization are critically important in this model. AZA treatment has been shown to decrease macrophages in the tumor microenvironment, though previously no distinction was made as to the polarization status of these macrophages (18,19). As the understanding of macrophages deepens, research has discovered that these cells once thought of as permanent, differentiated cells, are in fact quite plastic and able to respond to multiple signals including cytokines and chemokines that direct their behavior and alter their phenotype. Classically polarized M1 macrophages, induced by cytokines such as IFN γ and IL-12, upregulate expression of MHC II and can have tumoricidal functions. M1 macrophages metabolize arginine via iNOS to nitric oxide (NO), creating an oxidizing environment that is damaging to surrounding cells. DFMO treatment has been found to potentiate NO production in LPS-stimulated macrophages *in vitro* (48). Additionally, DFMO, via product inhibition through the increase in ODC substrate, ornithine, inhibits the enzyme arginase I, which is essential for function of alternatively polarized M2 macrophages (23,41). Inhibition of arginase I could lead to increased amounts of its substrate arginine, potentially providing more of the metabolite for use by M1 macrophages and iNOS (41,49). Treatment with DFMO may therefore increase M1 macrophages by making more of its essential metabolite arginine available, while AZA may help increase M1 macrophages via its interferon response and production of IFN γ , a cytokine which drives M1 polarization (15,18,19,49).

Depletion of macrophages in the tumor microenvironment using a CSF1R antibody significantly diminished the efficacy of combination AZA and DFMO, and decreased the levels of M1 macrophages. Tumor burden recurred more rapidly and survival was diminished in mice with fewer macrophages, suggesting that these M1 macrophages could have a tumoricidal role in ovarian tumors. This work represents the first combination of these two distinct treatment strategies in any cancer. The impact of AZA and DFMO on macrophages in the tumor microenvironment may not be specific to ovarian cancer, and could therefore possibly translate to other macrophage-rich tumors. Furthermore, the use of two well-tolerated and clinically approved drugs offers potential to test a third drug in combination to further prolong survival. Exploration of additional drugs that potentiate M1 macrophages is important, as these tumoricidal cells have potential to decrease tumor burden and help activate the immune system against cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance:

Combined epigenetic and polyamine-reducing therapy stimulates M1 macrophage polarization in the tumor microenvironment of an ovarian cancer mouse model, resulting in decreased tumor burden and prolonged survival.

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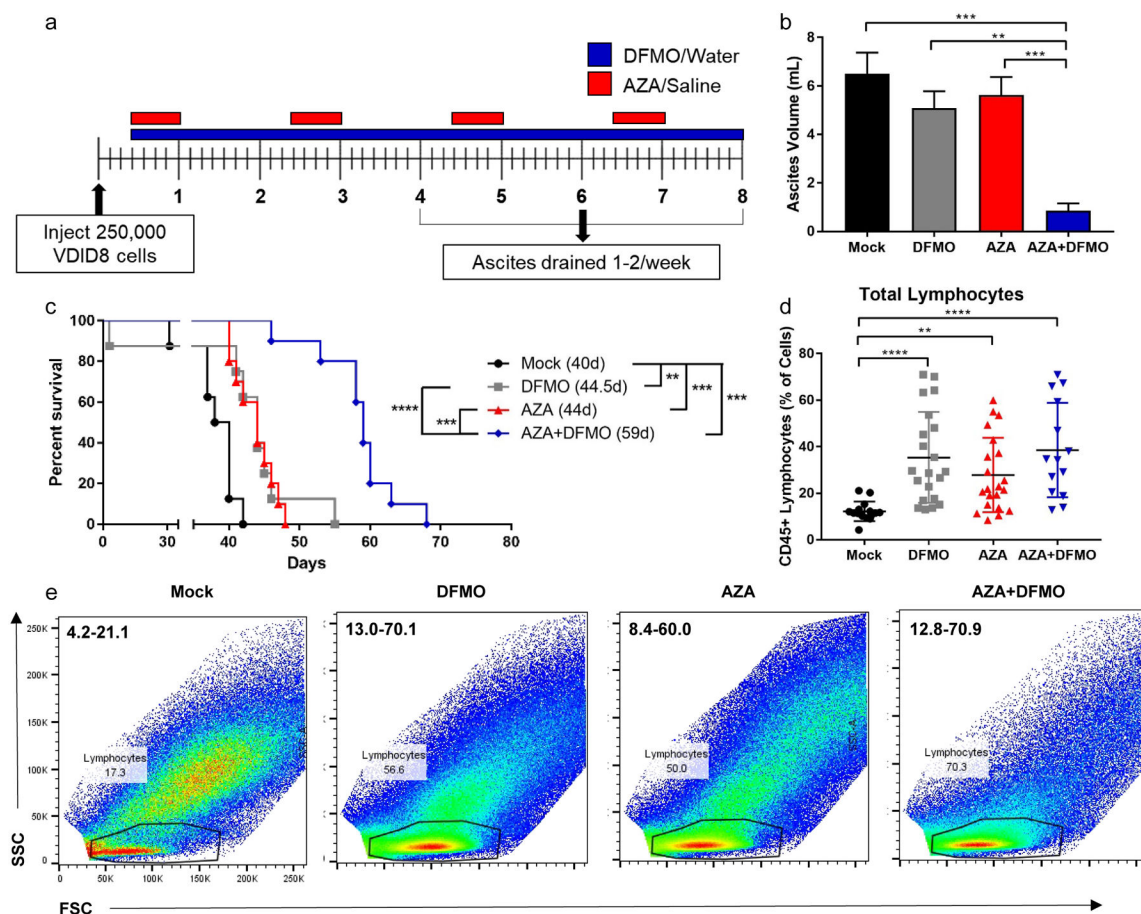


Figure 1. Combination AZA+DFMO reduces tumor burden and increases survival in an ovarian cancer mouse model.

a). Tumor cell injection and treatment schematic. Mice were injected i.p. with 250,000 VEGF-DEFB ID8 MOSE cells (VDID8). 0.5 mg/kg of AZA was given i.p. 5 days a week, every other week. 2% DFMO was provided in water bottles. Mice were treated throughout the duration of the experiment. Upon 25–30% weight gain, ascites fluid was drained from mice and processed for analysis of the tumor microenvironment. b) Tumor burden, represented by ascites volume, 5 weeks post tumor injection. Data is from the second ascites drain procedure, the first was 4 weeks post tumor injection. Representative data (mean $\pm$ SEM shown), $n = 10$; four biological replicates. Data were tested for a Gaussian distribution using Shapiro-Wilk test and found not to be normal. Significance was determined using Kruskal Wallis test; statistical outliers removed using Peirce's criterion. c) Representative survival curve (median survival in days); $n = 10$; four biological replicates. Significance determined using log-rank Mantel-Cox test. d) Total lymphocyte populations in week 5 bulk ascites fluid of mice; $n = 14$ –21. Data were tested for a Gaussian distribution using Shapiro-Wilk test and found to be normal after log transformation. Significance was determined using one way ANOVA. e) Flow cytometry plots of SSC vs. FSC demonstrating an increase in lymphocyte populations in ascites fluid at week 5 post tumor injection with AZA, DFMO, and AZA+DFMO treatment. Range of total lymphocyte population percentages are included

in the upper left hand corner for each plot. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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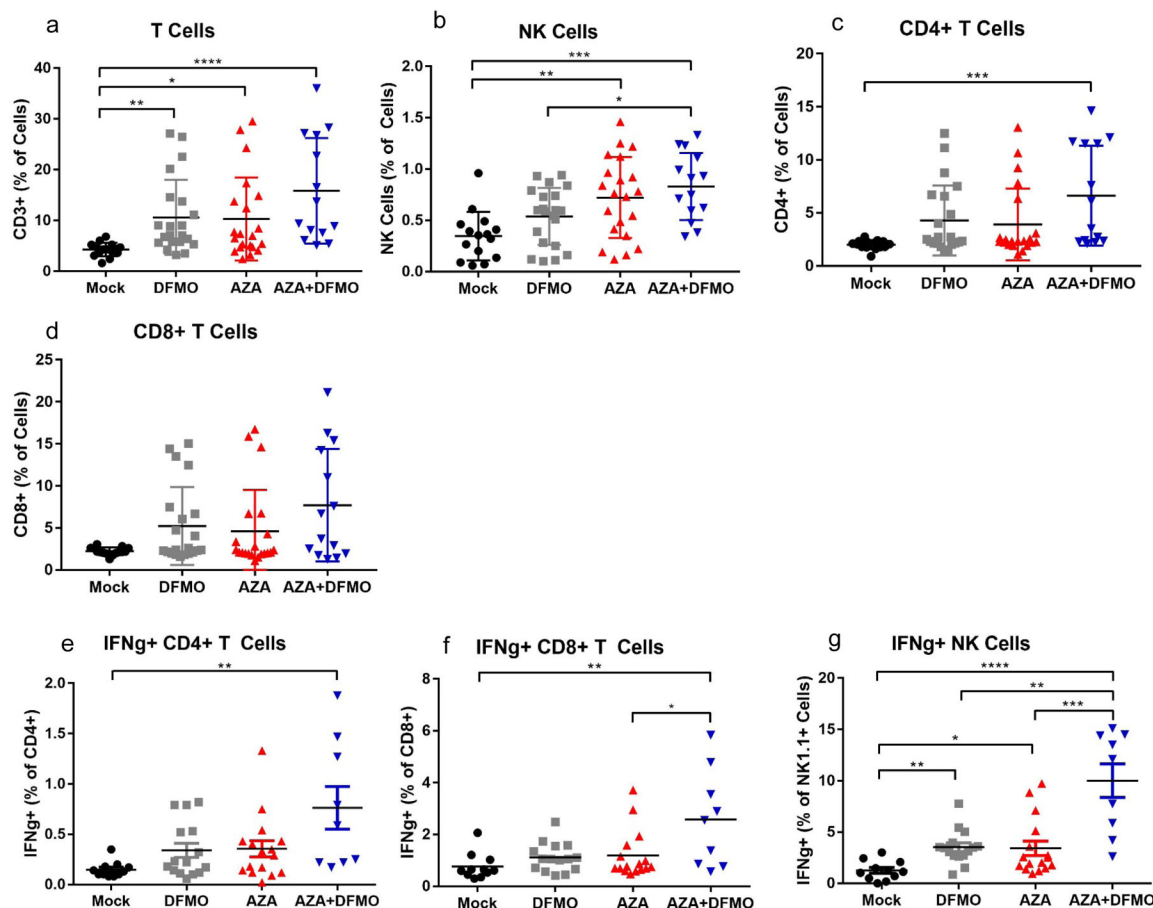


Figure 2. Combination AZA+DFMO elevates lymphocyte populations and IFN γ + lymphocytes in tumor associated ascites.

Ascites fluid was collected from treated mice and the cellular fraction was processed for FACS analysis. FACS analysis of cellular populations isolated from ascites at week 5 post injection demonstrate that combination treatment of 0.5mg/kg AZA and 2% DFMO was the most effective at significantly elevating total T cells (a), NK Cells (b), and CD4+ T cells (c). An upward trend in total CD8+ T cells (d) was observed as well. Both CD4+ and CD8+ T cells (e,f) and NK cells (g) showed an increase in IFN γ + cells with combination treatment. IFN γ + NK cells were significantly increased with combination AZA+DFMO compared to both single agent AZA or DFMO alone. Each data point represents cells harvested from 1 mouse. n = 14–21. All data were tested for a Gaussian distribution using Shapiro-Wilk test. Significance was determined using one way ANOVA (a,b, e-g) or Kruskal Wallis test (c,d) dependent upon normality results from Shapiro-Wilk test.

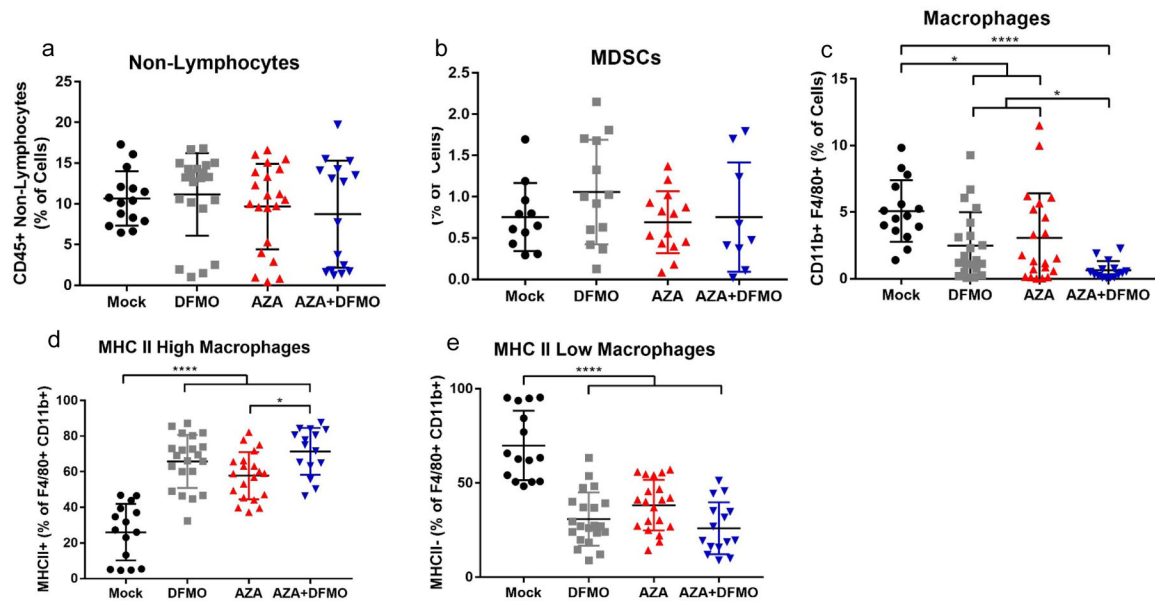


Figure 3. AZA-DFMO combination therapy decreases macrophages and alters the ratio of MHC II high to MHC II low macrophages.

Ascites fluid was collected from treated mice and the cellular fraction was processed for FACS analysis. No changes were observed between any of the treatment arms for non-lymphocytes (a) or MDSCs (b). A significant decrease in total macrophages was observed in combination treated mice compared to vehicle, as well as a significant decrease compared to AZA alone (c). Further analysis of macrophage populations revealed that the MHCII low (M2-like) population was decreased in all treatment arms (d), while the MHCII Hi (M1-like) population was increased across treatment arms (e). All data were tested for a Gaussian distribution using Shapiro-Wilk test. Significance was determined using one way ANOVA (b-e) or Kruskal Wallis test (a) dependent upon normality results from Shapiro-Wilk test.

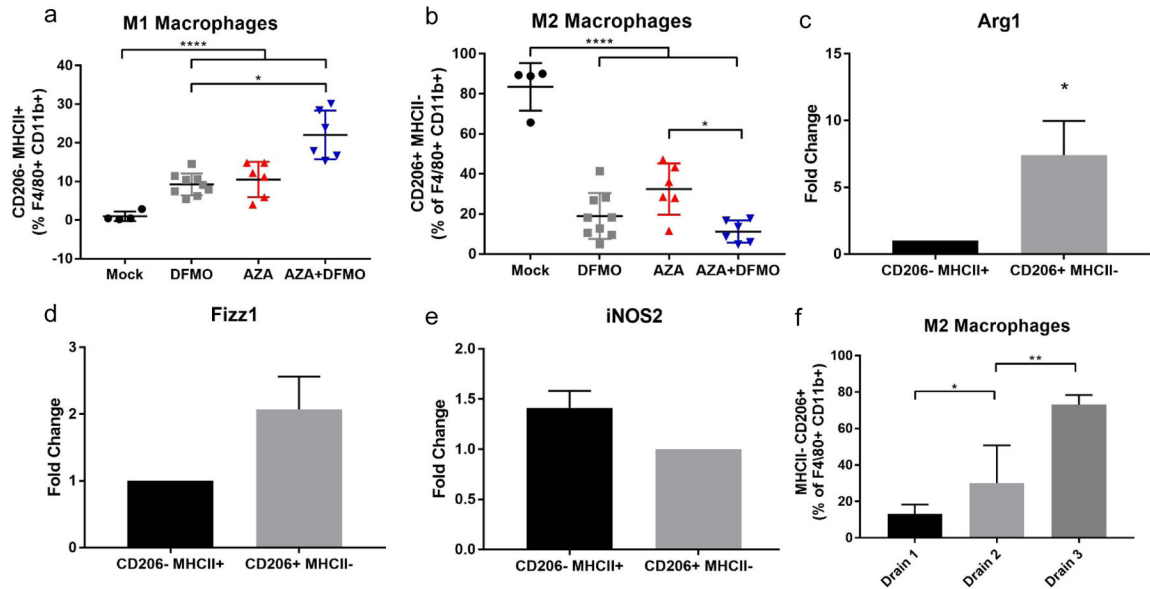


Figure 4. AZA+DFMO treatment reduces M2 polarization and increases M1 polarized macrophages in the tumor microenvironment.

a) Percentage of M1 macrophages (MHCII+ CD206-) were increased with DFMO and AZA treatment, and further increased with combination AZA+DFMO treatment. b) Percentage of M2 macrophages (MHC II- CD206+) were reduced in all treatment arms, with the greatest reduction observed in combination AZA+DFMO treatment. Macrophages were sorted from bulk ascites fluid collected from mice at week 5 post tumor injection. qRT-PCR for Arg1 (c) and Fizz1 (d) was performed on sorted macrophages (M2 macrophages = CD45+ L/D- F4/80+ CD11b+ MHC II- CD206+; M1 macrophages = CD45+ L/D- F4/80+ CD11b+ MHC II+ CD206-). Data confirms that MHC II- CD206+ macrophages exhibited gene expression signatures typical of M2 polarization. e) qRT-PCR of iNOS2 in M1 macrophages vs. M2 macrophages confirming that MHC II+ CD206- macrophages exhibited gene expression signatures typical of M1 polarization. f) Percentage of M2 macrophages (MHCII- CD206+) increase with tumor burden in vehicle treated mice. Drain 1 was performed at week 4 post tumor cell injection; drain 2 at week 5 and drain 3 at week 6. All data were tested for a Gaussian distribution and found to be normal using Shapiro-Wilk test. Significance was determined using one way ANOVA (a,b) or t test (c-e).

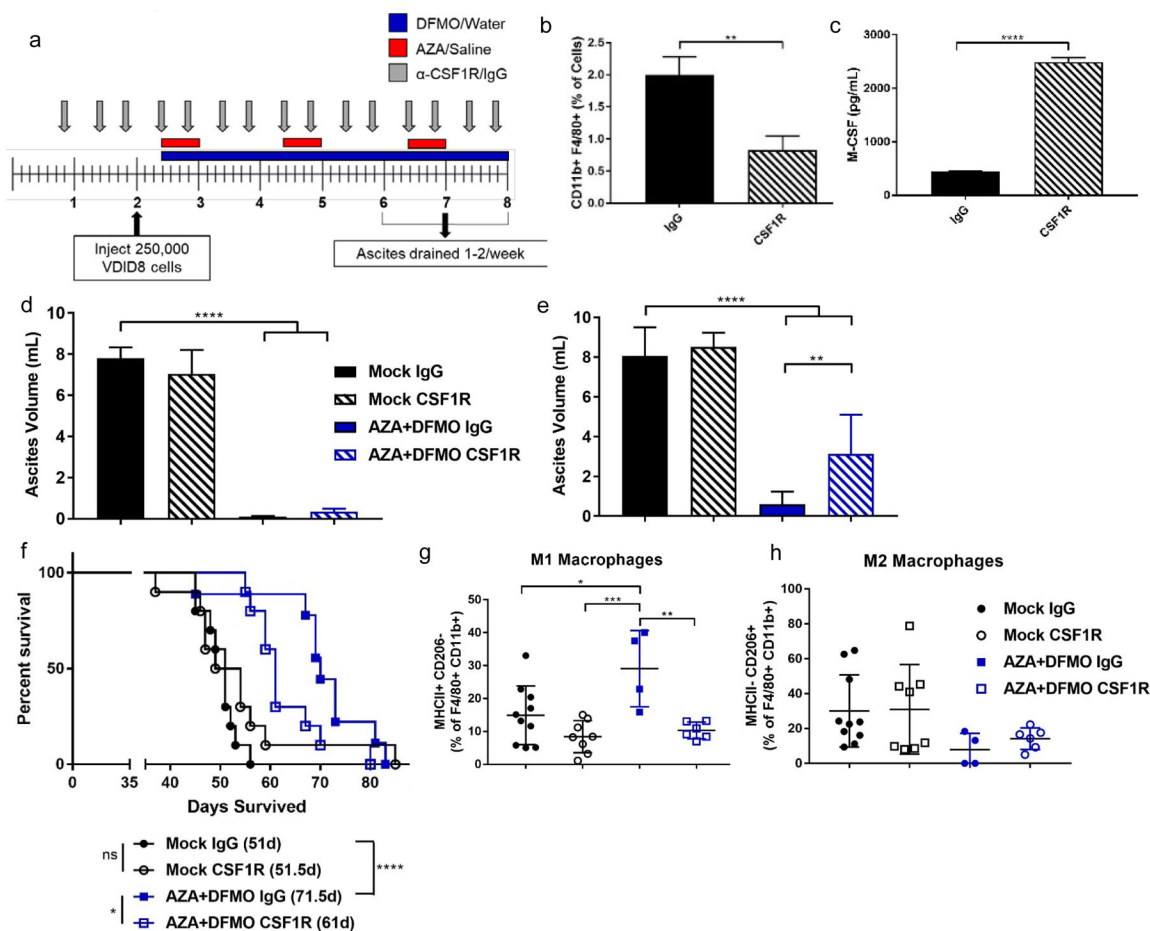


Figure 5. Increased M1 macrophages are essential to the efficacy of combined AZA+DFMO treatment in an ovarian cancer mouse model.

a) Treatment schematic for dosing with macrophage block antibody α -CSF1R. All mice are drained during each ascites draining procedure beginning at week 7. b) Reduction in total macrophages observed at the first drain (week 7 on schematic). c) ELISA of CSF-1 levels demonstrating an increase in circulating CSF-1 in the presence of receptor block CSF1R. d) Tumor burden represented by ascites volume in mice treated with AZA+DFMO in presence of CSF1R antibody or IgG control during the second drain (week 8 on schematic in a). e) Tumor burden during the third drain (week 9 on schematic in a) demonstrating an increase in tumor burden in AZA+DFMO mice receiving CSF1R. f) Survival curve of AZA + DFMO treated mice receiving CSF1R antibody. Mice with decreased macrophages due to the antibody demonstrated a decrease in survival compared to AZA+DFMO mice receiving IgG. g) M1 macrophages (MHC II⁺ CD206⁻) analyzed via flow cytometry. AZA+DFMO treated mice receiving CSF1R show no increase in M1 macrophages. h) M2 macrophages (MHC II- CD206⁺) analyzed via flow cytometry. M2 macrophages were reduced in both AZA+DFMO treatment arms, compared to mock treated mice. All data were tested for a Gaussian distribution and found to be normal using Shapiro-Wilk test. Significance was determined using a t test (b,c) or one way ANOVA (d,e,g,h).