AWARD NUMBER: W81XWH-14-1-0385

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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REPORT DATE: Oct 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE Oct 2019	2. REPORT TYPE Annual	3. DATES COVERED 30 Sept 2018 - 29 Sept 2019

4. TITLE AND SUBTITLE

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

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0385

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Stephen B. Baylin, M.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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.

15. SUBJECT TERMS – key words or phrases identifying major concepts Epigenetic therapy, advanced ovarian cancer, DNA demethylation, histone deactylases, immune checkpoint therapy, viral defense, immune cell attraction

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	72	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION

We continue to pursue our goal to develop eventual "epigenetic" therapy strategies, with relatively low toxicities, which can potentially robustly extend the life expectancy of women with advanced ovarian cancer (OC). Over the past two 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 last year of our award using our no cost extension funds, we will continue to concentrate on **Specific Aim 3:** *to study how epigenetic therapy may sensitize OC cells to subsequent immunotherapies which targets checkpoints which are driving immune tolerance*. Our funded research leaders including Cindy Zahnow, Dennis Slamon, Drew Pardoll, and Peter Jones continue key aspects to our progress for **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* advanced *OC, headed by Dennis Slamon. This now is undergoing a final clinical analysis not yet quite completed.* The results will determine whether a phase 2 portion of the trial will move forward. Critically, samples from the trial are now being compiled for shipment to our Hopkins lab for performance of a key goal of our funding, to perform 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. We have also published our progress in **Specific Aim 4:** *Develop new combinations of epigenetic drugs which may provide for the highest level of efficacy for management of OC and:* **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.*

2. KEYWORDS

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

3. ACCOMPLISHMENTS

What were the major goals and objectives of the project?

The overall goals remain identical to those outlined in the original proposal and last year's progress report. We will particularly outline what has transpired during the past year in terms of where have focused our work and how we will spend this last upcoming year using our no cost extension funds.

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*** by, first identifying 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 Dr. Dennis Slamon (Li et al, Oncotarget, 2014). Although this task will be an ongoing one in terms of ultimate value, for our group in the Teal and many others, we then made tremendous progress from 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 mode, (*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 ant-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 in Fig. 1**). 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 will apply to the tumor samples from the leveraged clinical trial led by Dr. Slamon and colleagues and that has now completed enrollment.

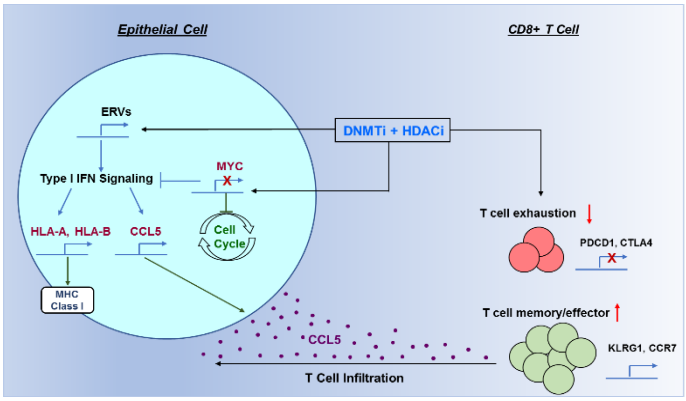

Fig.1. Summary of published data from the graphic for, Topper et al Cell, 2017 and as extrapolated to a model for OC (Stone et al Cell, 2017). The DNMTi plus HDACi treatment results in up-regulation of ERV's and type 1 interferon signaling to result in increased antigen presentation, upregulation of the cytokine, CCL5 accompanying T-cell attraction to tumors, and reversion of T-cell exhaustion to a cell effector state (up-regulation of genes like KLRG1 and CCR7 as shown). A key step is concomitant down-regulation of CMYC signaling which enhances reversion of tumor immune evasion.

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.*

As we have forward for addressing the specific tasks and studies in Specific Aim1, the findings have come to unequivocally become linked to synergize with the work in Specific Aim 2. Specifically findings in Aim1 now remarkably bolster the goal in this present aim to delineate *how epigenetic therapy may sensitize OC cells to subsequent chemo- and other therapies*. As later outlined below, and this same type of synergy has come to link findings in Specific Aim 1 to work in Specific Aim 3 with regards to sensitization by epigenetic therapy for OC cells to immune checkpoint therapy. A great portion for all of these links are fueled by a new finding over the past 1-2 years, 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 and with his leading efforts in a collaboration with the lab of Dr. Feyruz Rassool at the University of Maryland. For this present aim, the work 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. ***Our group has, excitingly translated these findings into a clinical trial due to start within the next 2-3 months, 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 in seen in the TNBC patients.***

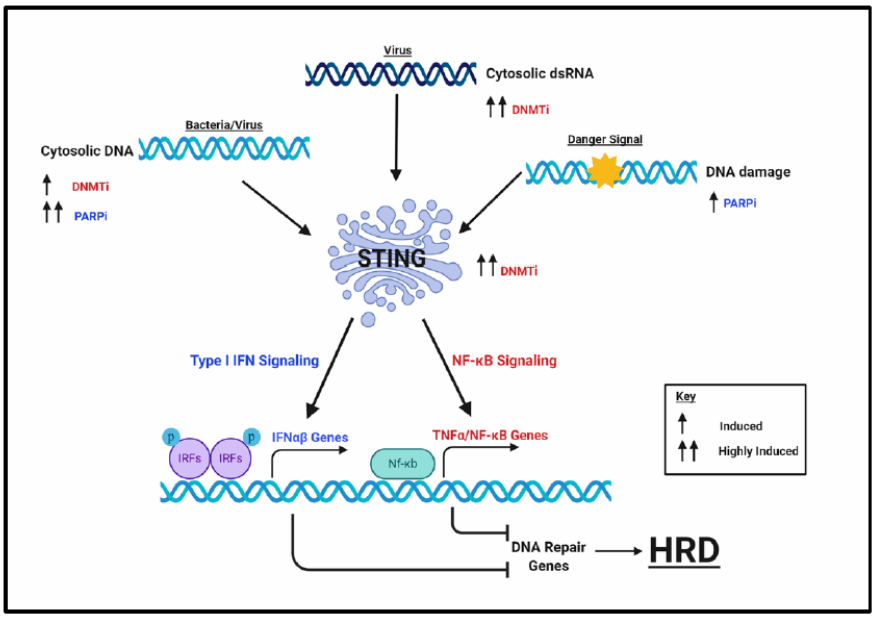

Fig. 2 Schematic of a multifaceted inflammatory response, which leads to HRD in TNBC and OC. In the proposed model for the combination therapy, the DNMTi induction of viral mimicry via cytosolic dsRNA combined with the PARPi increase of cytosolic dsDNA, converge to the activation of a DNMTi reconstituted STING signaling pathway. This activated response leads to a transcriptional increase IFNαβ and TNFα/NF-κB signaling, which facilitates the transcriptional repression of DNA repair associated genes in a STING dependent manner. The overall drug induced pathogen mimicry response create a BRCAness phenotype and thus enhances sensitivity to PARPi in the BRCA proficient setting.

Specific Aim 3: *to study how epigenetic therapy may sensitize OC cells to subsequent immunotherapies which targets checkpoints which are driving immune tolerance*. As introduced in the Specific Aim 2 section above, synergy has come to link findings in Specific Aim 1 to work in this Specific Aim 3 with regards to sensitization by epigenetic therapy for OC cells to immune checkpoint therapy. Details are as follows as outlined in each Major Task.

Major Task 1: *to develop the in-vitro pre-clinical systems to outline the sensitivities and derive molecular signatures that track with these.* We completed by the start of 2018, former mentees Michael Topper, Ph.D. and e, Meredith Stone, Ph.D., led exciting studies (*Topper et al, Cell, 2017; Stone et al, PNAS, 2017)* indicating how our epigenetic therapy drugs could induce in cancer cells, including OC an immune response (work all summarized in **Fig. 1** earlier above).

This Task is then essentially complete with regards to TEAL funding *although the results have resulted in analyses that will be used in correlative science for a completed OC clinical trial as will be outlined later below.*

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 major findings, in published work again led by former mentees Michael Topper, Ph.D. and Meredith Stone, Ph.D., led exciting studies (*Topper et al, Cell, 2017; Stone et al, PNAS, 2017)* defined in Task 1 above, resulted in our derivation of in-vivo, mouse model systems for OC and non-small cell lung carcinoma (NSCLC), which produced discoveries providing key insight into how epigenetic therapy may help reverse immune evasion to help sensitize to immune checkpoint therapy for OC. This work also is providing a growing biomarker system for potentially predicting patient responses and monitoring therapy. The basic findings are, that combining our DNA demethylating agent (AZA) with the histone deactylase inhibitor HDACi), entinostat leads leading to interferon responses that accompany a strong anti-tumor response with recruitment of active CD8 T-cells, secretion of a key cytokine to recruit such cells, and diminishes tumor promoting macrophages. Simultaneously, the therapy suppresses the C-MYC and its target genes which we showed otherwise suppresses the above immune signaling and this loss of oncogenic function can contribute to the anti-tumor response as well (all above studies previously summarized in **Fig. 1** earlier above). All of these data, indeed accompanied a strong anti-tumor response and/or increased response to immune checkpoint therapy in the mouse models.

As for Task 1 in this aim, we have completed TASK2 with regards to TEAL funding. *Again, as also for TASK1, the results of this in-vivo work have especially resulted in approaches for correlative science for a completed OC clinical trial as will be outlined later below.*

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*: As reported previously, These studies, under the direction of Dr. Peter Jones at the Van Andel Research Institute (VARI), with close collaboration

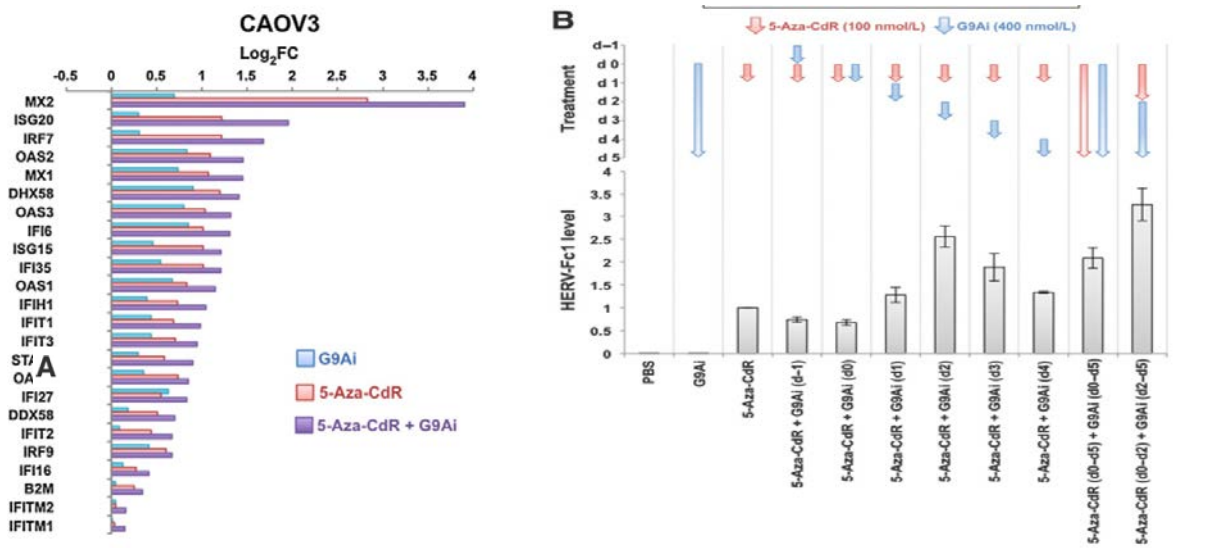

Figure 2. A. Combination treatments further upregulated viral defense genes in A2780 and CAOV3 cells. Bar graphs show the expression fold change (in log2 values) of 24 viral defense genes as per our previously published studies (Rouloix et al, Cell 2015; Chiappinelli et al, Cell, 2015) in CAOV3, ovarian cancer cells after G9Ai, 5-aza-CdR, and combination treatment

compared with untreated cells. B. Effects of dosing schedule by combination treatment with G9Ai and 5-aza-CdR on HERV-Fc1 expression. A2780 cells (2.5 x 10^5) were seeded in 100-mm dishes at day 1, then treated with 400 nmol/L G9Ai, 100 nmol/L 5-aza-CdR, or their combinations according to the schedule shown by arrows at the top. Cells were harvested at day 5 after 5-aza-CdR treatment. HERV-Fc1 expression levels were then assayed by quantitative RT-PCR using the expression levels of 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 +/- SEM of three independent experiments. A one-way repeated-measures ANOVA was used for statistical analysis.

from Dr. Baylin were completed and published in 2018 (*Liu et al, Cancer Res., 2018*). We have found 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 (ERV) (**Fig. 2**). 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 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.

The ongoing task as of now is to extrapolate the data to an in-vivo model 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 and for immune effects in Task 4 above, it is not soluble for in-vivo administration. However, we have now received, and are testing in mice a new drug from the company GBT. We have verified that this drug is a potent G9a inhibitor (G9ai) at nM doses and have embarked on the mouse experiments during the last year of regular funding and are continuing to use or no cost extension funds to complete this work. The studies will use both human OC tumors studied as xenografts in immune- compromised mice and will also our immune competent mouse model of OC as described in sections above. The treatments will test the GBT drug and AZA alone and in combination. Once we see if we have an anti-tumor results in the models, we will analyze pre- and post- treatment tumor samples for all the indices studied in the studies in our published papers by Stone et al (PNAS, 2017) for the OC work and by Topper et al (Cell, 2017) for lung cancer work (summaries for the finding in past progress reports and in **Fig. 1** of this report).

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 an instituted clinical trial of the DNA demethylating drug, guadecitabine from Astex and the immune checkpoint inhibitor az from Merck. The goal is to see whether the guadecitabine will provide efficacy of the immune checkpoint therapy in 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 from the no cost extension funds this next year to coordinate provision of key trial samples to the Baylin lab for correlative science studies not funded by the trial.

During the first 2 years of our award, Dr. Slamon was successful in deriving the above leveraged trial and enrolling it after approximately the second half of year 5. The study completed accrual of the first 20 patients with pre-post treatment biopsies. The study team is now centrally collecting and double checking fresh frozen tissue pairs, as well as FFEE paired tissues that were collected per protocol. Confirmation once all tissues are at UCLA should be possible and likely two to three weeks. We requested that the study sites send them to UCLA.

What opportunities for training and professional development did the project provide?

The five years of funding from this grant have been exceptionally important for contributing mentoring to graduates students and fellow trainees throughout the entire period. The key examples are: **1) Kate Chiappinelli, Ph.D.**, a research fellow trainee with Dr. Baylin, now an Assistant Professor in the Cancer Center at George Washington University who contributed mightily for the early work for the proposal completing her much cited Cell paper in 2015 for which key data are in the Fig. 1 summary earlier above. She also played a key role in Dr. Topper's key paper in Cell at the end of 2017, and has contributed review articles to the field from TEAL work (**Chiappinelli et al, Cancer Research, 2016**) and beyond. She continues a career much focused on study of epigenetic changes in OC and the translational potential of her findings for therapy. **2) Meredith Stone, Ph.D.** obtained her graduate degree as a trainee working with co-investigator Dr. Cindy Zahnow. She led the work on the pivotal paper (Stone et al, PNAS, 2017) concerning our mouse model of OC outlined in Specific Aim 3 and has now gone on to a post-doctoral fellowship at the University of Pennsylvania continuing to pursue studies concerning the immunology of OC. **3) Michael Topper, Ph.D.,** obtained his graduate degree as a trainee working with Dr. Baylin. He led much of the work in Specific Aims 1-3 producing the pivotal paper for in-vitro and mouse model generating the concepts summarized in **Fig. 1** earlier (Topper **et al, Cell, 2017**) and with Dr. Rassool's and Baylin's lab which outlined in Specific Aims 2 outlining how epigenetic therapy can augment the efficacy of PARP inhibitors in OC and breast cancer. His findings have been pivotal to the leveraging design and implementation of two clinical trials referred to in the Specific Aims. He also was a major collaborator on the stone, PNAS paper mentioned above and has just first authored a major review on the use of epigenetic therapies to try and augment the efficacies of immune checkpoint therapy (**Topper et al, Nature Reviews Oncology, 2019**). After a year of postdoctoral fellowship work with Dr. Baylin, he has just been appointed to a faculty position as an Instructor at Hopkins.

Each of the above trainees has benefitted enormously from participation in all of the work outlined in the above sections and are this has resulted in there pursuing careers involving development of epigenetic therapies for OC and other cancers. They have the opportunities to become true leaders in this field.

How were the results disseminated to communities of interest?

As outlined last year, over the 4-5 years of studies, and as enumerated outlined below, we have published multiple of our key studies 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.

What do you plan to do during the next reporting period to accomplish the goals and objectives?

During the period of no cost extension, we will emphasize two key of our studies. ***First***, for Specific Aim 4, we will complete our studies in our mouse model of OC, as described in this Aim earlier, to conduct in-vivo mouse studies for treatment with the new G9a inhibitor from GBT. These are critical investigations as the role of G9a in stem cell function in cancer, as an over-expressed gene in several cancer types, and in chromatin controls of ERV's and other repeat sequence transcripts activated by our epigenetic therapies makes this protein a critical potential therapy target to pursue. This potential is partially fueled by the studies we have conducted in

specific Aim 4. Thus we will complete a thorough analysis of the in-vivo work to see, further, the anti-tumor potential of therapeutically engaging this target and further develop a new epigenetic treatment strategy for advanced OC.

Second, for Specific Aim 5, we will pursue the correlative science correlates to be conducted on tumor biopsies , and peripheral blood immune cells from the trial that Dr. Slamon has led, and which has enrolled to the point of interim clinical analysis. No matter what the interim results are for our this leveraged trial, which must see at least one formal tumor response in the 20 patients treated to justify considering expanding the patient enrollment numbers, the analyses of the above pre- and post-biopsy samples from the patients will provide important information for the field of trying to enhance immune checkpoint therapy for patients with advanced OC and other cancers. These studies will teach us how and whether our epigenetic therapy is getting to the tumors and activating the responses we have observed in all of our pre-clinical work throughout this TEAL funding.

4. IMPACT

***What was the impact on the development of the principal discipline(s) of the project*?**

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 for develop new means to treat this disease. These efforts are poised to continue far beyond just the years we have received funding from the TEAL award. Our seepening immersion in this field, the fact that each of our trainees are pursuing careers which continue to focus upon extending what they done in the TEAL award time all attests to what the grant has meant to all of us and hopefully, one day, to patients with OC and other major cancers. 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 our funding period, and so stated in every progress report, our great hope is that, at the end of the day, our studies will lead to new therapy regimens for OC and other major cancer types to the benefit of a great number of patients.

***What was the impact on other disciplines*?**

As outlined just above, and in previous progress reports, we truly believe our basic, pre-clinical studies of OC have had 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 documented whether our approaches, and scenarios for these form others can change the management for patients with OC and other cancers. Perhaps, at the least the promise has been kept alive and worthy of continued pursuit in the lab and the clinic. 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.

***What was the impact on technology transfer*?**

As outlined in each year's report, and again contributed to by the work over the past year, our viral defense signature, inclusive of the ERV transcripts has a patent applied for status as a biomarker system to predict and monitor the efficacy of applying epigenetic therapy to sensitize patients with advanced OC and all cancer types, to immune checkpoint therapy. It remains to be seen what aspects might be licensed for pursuit in the in the industrial sector. Our ongoing, leveraged OC and other clinical trials, and the final analyses of patient outcomes with a range of DNA demethylating agents plus immune checkpoint therapy will prove the final arbiter of where our work in the TEAL award actually impacts patient care.

***What was the impact on society beyond science and technology*?**

Hopefully, as outlined above, the biggest impact of our studies will be for patients. As also mentioned above, new trials are now testing our therapy paradigms for sensitizing to immune checkpoint therapy in patients with advanced NSCLC and bladder cancer 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, the thrust of our efforts are being assessed in the public arena. Just in the past year, for example Dr. Baylin has spoken twice to a Maryland based society for women's health and is scheduled to do so again, this time bring this conversation to Florida for a series of such talks. 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 we have established including in the clinic. For example we have applied our findings, as summarized in Figs. 1 and 2, to developmental of a new immune-histochemical (IHC) approach for tumor biopsies which could prove friendly to routine clinical assays for monitoring clinical trial results early on to predict long term patient survival following our therapy. Hopefully, this could have even more impact if our therapy approaches actually come to influence formal clinical management.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

At present, we do not anticipate any major changes to our work scope and directions as articulated in this report. We will continue to focus on Specific Aims 4 and 5 as defined earlier and trying to maximize our epigenetic therapies for OC including continued new work with the G9ai to develop in-vivo pre-clinical studies during the coming year. Additionally, and obviously if new indications warrant, we will try to extend these other aims within the limits possible via our no cost extension funding.

Actual or anticipated problems or delays and actions or plans to resolve them

None anticipated at this time. We have been moving at a rapid pace during the past year and hope to continue this.

Changes that had a significant impact on expenditures

None anticipated at this time.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None anticipated at this time. This category, pertinent to the TEAL award extends only to use of human tissues in the clinical trial or pre-clinical arena.

6. PRODUCTS

Publications, conference papers and presentations

Publications

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.

Presentations

Stephen B. Baylin, M.D.

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

Cynthia A. Zahnow, Ph.D.

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

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on the project

Johns Hopkins University

Name:	Stephen B. Baylin, M.D.
Project Role:	PI (Senior/Key Personnel)
Research Identifier:	N/A
Nearest person month worked:	4
Contribution to Project:	Dr. Baylin oversees all studies and activities conducted under this proposal.
Funding Support:	See Other Support

Name:	Cynthia Zahnow, Ph.D.
Project Role:	Co-Investigator (Senior/Key Personnel)
Research Identifier:	N/A
Nearest person month worked:	2
Contribution to Project:	Dr. Zahnow collaborates with Dr. Baylin on all of the studies in the lab.
Funding Support:	See Other Support

Name:	Drew Pardoll, M.D., Ph.D.
Project Role:	Co-Investigator (Senior/Key Personnel)
Research Identifier:	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Pardoll works with the Baylin group for all of the studies on how epigenetic therapy can sensitize ovarian cancers to immune checkpoint therapy.
Funding Support:	See Other Support

Name:	Ray-Whay Yen
Project Role:	Research Associate
Research Identifier:	N/A
Nearest person month worked:	8
Contribution to Project:	Ms. Yen is responsible for working with the entire Hopkins group for all of the pre-clinical work on ovarian cancer.
Funding Support:	No change

Name:	Michael Topper, Ph.D.
Project Role:	Instructor / Teal Junior Scientist
Research Identifier:	N/A
Nearest person month worked:	9
Contribution to Project:	As outlined above, Dr. Topper continues to contribute enormously to our Teal work and former trainee Dr. Chiappinelli also continues collaborating with him. His academic growth is discussed in detail in Section 8, Special Reporting.
Funding Support:	Dr. Topper became an Instructor on 1/1/2020. He is supported 30% on the DOD Teal, 20% on an Emerson award, and 5% on a SU2C Seed Package.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. See next pages for Drs. Baylin, Zahnow and Pardoll's Other Support.

OTHER SUPPORT

BAYLIN, STEPHEN B.

ACTIVE

P30 CA006973 (PI: Nelson)
Title: Regional Oncology Research Center – Senior Leader
Time Commitment: 0.6 calendar
Supporting Agency: NIH/NCI
Procuring Contracting/Grants Officer: Precilla L. Belin
Address of Grants Officer: National Cancer Institute, Building 6116-700, 6116 Executive Blvd, Rockville, MD 20852
Performance Period: 5/7/1997-4/30/2022
Level of Funding: $12,836 (salary support only)
Project's Goal(s): CORE grant for the Johns Hopkins Oncology Center. Stephen Baylin receives salary support only for leadership and microarray core responsibilities.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

R01 ES011858 (PI: Baylin/Easwaran/Rassool)
Title: DNA Methyltransferase Gene Expression in Colon Cancer
Time Commitment: 1.44 calendar
Supporting Agency: NIH/NIEHS
Procuring Contracting/Grants Officer: Frederick Tyson
Address of Grants Officer: National Institute of Health, Keystone Park 3064, 615 Davis Dr, Durham, NC 27709
Performance Period: 4/1/1991-2/29/2024
Level of Funding: $464,830
Project's Goal(s): Understand, further, the role of altered regulation and patterns of DNA methylation in the progression of colon cancer.
Specific Aims: 1. To determine mechanisms by which SOX17 blocks Wnt activation in CRC evolution. 2. To develop mouse models for CRC evolution based on epigenetic loss of Hic1. 3. To explore specific stages of CRC tumorigenesis mediated by epigenetic silencing of stem/progenitor cell related genes. 4. To define molecular determinants which initiate and/or maintain gene promoter DNA hypermethylation and gene silencing in CRC evolution.
Project Overlap or Parallel: No scientific or budgetary overlap.

90046519 (PI: Baylin/Casero/Zahnow)
Title: Novel Therapies Targeting Epigenetic Silencing of Tumor Suppressors
Time Commitment: 0.05 calendar
Supporting Agency: Samuel Waxman Cancer Research Foundation
Procuring Contracting/Grants Officer: Carole Asher
Address of Grants Officer: 420 Lexington Ave., Suite 825, New York, NY 10170
Performance Period: 7/1/2011-6/30/2021
Level of Funding: $25,000
Project's Goal(s): The goals of this project are: Project 1: To examine newly identified lysine specific demethylase 1 (LSD1) inhibitors in order to advance the understanding of the functioning and targeting of LSD1 for clinical utility. Project 2: To show that epigenetic therapy at very low, non-toxic doses, can dramatically blunt the tumorigenic properties of subpopulations of leukemic and solid tumor populations of "stem-like" cells. Project 3: To demonstrate that low dose epigenetic therapy re-sensitizes drug tolerant breast cancer cells to conventional, single agent chemotherapeutics or targeted therapy.

Specific Aims: 1. To perform, in Kasumi AML cells, and other lines, genome-wide studies of DNA methylation, chromatin and, gene expression patterns, including pathway analyses, for activating and repressive marks in separated populations of tumorigenic CD34+/CD38- versus non-tumorigenic CD34- cells. 2. To examine changes in the above genome-wide patterns induced by low doses of DNA demethylating and histone deacetylation inhibiting drugs, already shown to inhibit the leukemic engraftment of the whole cell population, alone and together, on the above separated populations. 3. To derive markers for prediction and monitoring of epigenetic therapy from the above studies and which can be studied in primary tumor samples, and patient samples.
Project Overlap or Parallel: No scientific or budgetary overlap.

W81XWH-14-1-0385 (Baylin)
Title: A New Paradigm for the Treatment of Ovarian Cancer: The Use of Epigenetic Therapy to Sensitize Patients to Immunotherapy and Chemotherapy
Effort: 1.8 calendar
Supporting Agency: CDMRP
Name of Procuring Contracting/Grants Officer: Susan Dellinger, Grants Officer
Address of Funding Agency: 1077 Patchel St., Bldg 1077, Fort Detrick, MD 21702
Period of Performance: 09/30/2014-09/29/2020 (NCE)
Level of Funding: $496,554
Project's Goal: The major goal of this project is to robustly prolong the survival of patients with serous ovarian cancer (OC) through introducing epigenetic therapy paradigms
Specific Aims: 1) To uncover the mechanisms through which epigenetic therapy may, alone, achieve robust, durable responses in patients with advanced ovarian cancer (OC) 2) Study how epigenetic therapy may sensitize OC cells to subsequent chemotherapies 3) Study how epigenetic therapy may sensitize OC cells to subsequent immunotherapies which targets checkpoints which are driving immune tolerance 4) Develop new combinations of epigenetic drugs which may provide for the highest level of efficacy for management of OC 5) Bring all of the above studies to bear on leveraging clinical trials of epigenetic therapy on OC
Project Overlap or Parallel: No scientific or budgetary overlap.

90058334 (PI: Baylin)
Title: Clinical trials of epigenetic therapy sensitized patients with advanced non-small cell lung cancer to chemotherapy and immunotherapy
Time Commitment: 0.05 calendar
Supporting Agency: AACR – Jim Toth Sr. Breakthrough Prize in Lung Cancer
Procuring Contracting/Grants Officer: Susan Frank
Address of Grants Officer: 1900 Avenue of the Stars, 14th Floor, Los Angeles, CA 90067
Performance Period: 7/1/2014-6/30/2020
Level of Funding: $340,380
Project's Goal(s): Enroll the targeted number of patients and complete both clinical trials which, if efficacy emerges, could truly lead to a new, robust management of advanced NSCLC, the world's biggest cancer killer.
Specific Aims: Milestone 1: This phase 2 trial for patients with NSCLC to directly assess priming of epigenetic therapy to subsequent chemotherapy is now open and accruing. Milestone 2: This phase 2 trial for patients with NSCLC, to directly assess priming of epigenetic therapy for anti-PD1 immuno-therapy, is now open and accruing at Hopkins – and being sent to IRB's at USC and Memorial Sloan Kettering.
Project Overlap or Parallel: No scientific or budgetary overlap.

90061408 (PI: Baylin)
Title: Clinical trials of epigenetic therapy in non-small cell lung cancer
Time Commitment: 0.05 calendar

Supporting Agency: Rising Tide Foundation
Procuring Contracting/Grants Officer: Eveline Mumenthaler
Address of Grants Officer: Herrenacker 15, 8200 Schaffhausen, Switzerland
Performance Period: 1/1/2015-12/31/2021
Level of Funding: $331,360
Project's Goal(s): We are addressing the hypothesis that reversal of cancer-specific DNA methylation and chromatin abnormalities can potently change the management of NSCLC.
Specific Aims: The two new trials, scheduled to involve a total of some 315 patients, are deigned to formally test: 1) whether low dose epigenetic therapy with a DNA demethylating agent plus a histone deactylase inhibitor does sensitize patients with advanced NSCLC to subsequent, standard, chemotherapy; and 2) whether this treatment sensitizes the same patient population to immunotherapy targeting immune checkpoints which drive immune tolerance.
Project Overlap or Parallel: No scientific or budgetary overlap.

R01 HD082098 (PI: Zambidis)
Title: Functional vascular progenitors from naïve human iPSC
Time Commitment: 0.24 calendar
Supporting Agency: Natl Ins of Child Health & Human
Procuring Contracting/Grants Officer: Mahua Mukhopadhyay
Address of Grants Officer: P.O. Box 3006, Rockville, MD 20847
Performance Period: 5/1/2015-2/29/2020
Level of Funding: $186,750
Project's Goal(s): To develop novel gene targeting and regeneration approaches for treating pediatric and adult vascular disorders using a newly discovered class of human iPSC converted to a ground state of naïve pluripotency.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

90061607 (PI: Baylin)
Title: VARI-SU2C Epigenetics Dream Team
Time Commitment: 0.05 calendar
Supporting Agency: Van Andel Research Institute
Procuring Contracting/Grants Officer: Jerry Callahan
Address of Grants Officer: 333 Bostwick Avenue, NE, Grand Rapids, MI 49503
Performance Period: 10/1/2014-9/30/2020
Level of Funding: $45,455
Project's Goal(s): Our Dream Team unites scientists at major cancer research institutions who are poised to propel the early promise of epigenetic therapy in blood malignancies to the forefront of management for patients with breast, colon and lung cancer.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

R21 CA212495 (PI: Easwaran)
Title: High-efficiency microfluidic-assisted single-cell DNA methylome sequencing
Time Commitment: 0.24 calendar
Supporting Agency: NCI
Procuring Contracting/Grants Officer: Jerry Li
Address of Grants Officer: 9609 Medical Center Dr., Bethesda, MD 20892
Performance Period: 8/3/2017-7/30/2020
Level of Funding: $150,000
Project's Goal(s): The goal of this project is to devise a novel methodology for high-efficiency multiplexed profiling of DNA methylation in single cells by bisulfite sequencing.

Specific Aims: Aim-1: Develop a novel methodology that combines DNA isolation and bisulfite treatment with microfluidics-assisted modified genome amplification and library preparation from single cells. Aim-2: Validate methodology by performing WGBS on few cell numbers and single cells. Aim 3: Demonstrate 'utility of technology' by mapping progressive epigenetic changes in ex vivo organoid models of tumorigenesis.
Project Overlap or Parallel: No scientific or budgetary overlap.

90076072 (PI: Anagnostou)
Title: Genomic and Epigenetic Mechanisms of Response and Resistance to Epigenetic….
Time Commitment: 0.6 calendar
Supporting Agency: V Foundation for Cancer Research
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: 14600 Weston Parkway, Cary, NC 27513
Performance Period: 11/1/2017-11/1/2020
Level of Funding: $12,483 (salary only)
Project's Goal(s): We propose to pinpoint the mechanisms that mediate response and resistance to these therapies by looking at the genetic make-up of cancer cells as well as by studying the tumor microenvironment.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

90077253 (PI: Baylin)
Title: Effect of GBT G9a inhibitors in cancer cell lines and in xenograft models
Time Commitment: 0.24 calendar
Supporting Agency: Global Blood Therapeutics
Procuring Contracting/Grants Officer: Tony Peng
Address of Grants Officer: 171 Oyster Point Blvd, Ste. 300, South San Francisco, CA 94080
Performance Period: 2/26/2018-2/25/2020
Level of Funding: $75,000
Project's Goal(s): The ultimate goal is to translate all promising data into clinical trials for cancer management.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

900780913 (PI: Baylin)
Title: Janssen Initiative For Determining Colon Cancer Risk and Exploring means For Prevention and Interception
Time Commitment: 1.2 calendar
Supporting Agency: Janssen Research & Development LLC
Procuring Contracting/Grants Officer: Dashyant Dhanah
Address of Grants Officer: 1400 McKean Rd., Spring House, PA 19477
Performance Period: 4/10/2018-4/01/2021
Level of Funding: $232,558
Project's Goal(s): Our group proposes to participate in this important initiative by exploring how the epigenetic abnormalities in cancer, long the focus of our group, contribute to the risk for and initiation and early progression of colorectal cancer (CRC).
Specific Aims: Specific Aim 1: Continuing pre-clinical studies in colon organoids aimed at outlining the dynamics through which epigenetic abnormalities contribute to the risk, initiation, and progression of CRC. Specific Aim 2: Collaborative analysis with Janssen of bioinformatics data currently derived by Janssen and Hopkins scientists. Specific Aim 3: Developing from the studies in Aims 1 and 2, and testing in a prospective manner in key CRC risk and established disease cohorts, the efficacies of derived biomarker strategies.

Project Overlap or Parallel: No scientific or budgetary overlap.

90078135 (PI: Baylin)
Title: The Celgene Cancer Center Consortium: Targeting UHRF1 to Cancer Therapy
Time Commitment: 1.2 calendar
Supporting Agency: Celgene
Procuring Contracting/Grants Officer: Kevin Mello
Address of Grants Officer: 86 Morris Ave., Summit, NJ 07901
Performance Period: 3/13/2018-3/21/2021
Level of Funding: $918,275
Project's Goal(s): We anticipate this will be a collaborative effort. Our hope is that each institution provides the biology, translational tools and strategy with our input to these areas.
Specific Aims: Our aim is to collaboratively develop for cancer therapy, inhibitors of UHRF1, a protein essential in mammalian cells for targeting DNA methyltransferases (DNMT's) to DNA to maintain DNA methylation.
Project Overlap or Parallel: No scientific or budgetary overlap.

90078268 (PI: Baylin)
Title: Developing a neo-antigen based anti-lung cancer vaccine for use with combination epigenetic-immunotherapy
Time Commitment: 0.24 calendar
Supporting Agency: Emerson
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: Unknown
Performance Period: 6/01/2018-5/31/2020
Level of Funding: $99,360
Project's Goal(s): We will mine exome- and RNA-seq data from pre-and post biopsies from our ongoing SU2C trial.
Specific Aims: Specific Aim #1: To determine the clonal expansion dynamics of memory and effector tumor associated CD8+ T cell populations in response to combination epigenetic-immunotherapy. Specific Aim#2: To identify therapy induced tumor antigen specific CD8+ T cells and develop a pooled peptide vaccination to deploy with combination epigenetic-immunotherapy.
Project Overlap or Parallel: No scientific or budgetary overlap.

R01 CA230995 (PI: Easwaran/Baylin)
Title: (PQ4) - Tools for simultaneous disruption of multiple epigenetically silenced genes for studying their roles in tumorigenesis using ex vivo human and mouse colon organoid and in vivo mouse models
Time Commitment: 1.08 calendar
Supporting Agency: NIH
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: Unknown
Performance Period: 9/1/2018-8/31/2023
Level of Funding: $228,750
Project's Goal(s): This proposal will develop generic tools for simultaneous manipulation of multiple genes in human cancer-relevant models that can be easily translated to other cancer models.
Specific Aims: Aim-1: Develop tools for inactivation of multiple gene combinations and identify effects on early tumor development using colon organoid model. Aim-2: Determine whether, and how, inactivation of multiple chosen genes promotes tumorigenesis by key colorectal cancer driver mutations. Aim-3: In vivo modeling to identify roles of cancer-specific epigenetically silenced genes in initiating in situ tumorigenesis.
Project Overlap or Parallel: No scientific or budgetary overlap.

AWARDED SINCE LAST SUBMISSION

U01 AG066101 (PI: Easwaran/Baylin)
Title: Characterizing age-associated epigenetic alterations and their roles in tumor development
Time Commitment: 0.24 calendar
Supporting Agency: NIH
Procuring Contracting/Grants Officer: Max Guo
Address of Grants Officer: 9609 Medical Center Dr., Bethesda, MD 20892
Performance Period: 5/15/2019-3/31/2021
Level of Funding: $75,000
Project's Goal(s): The goal of this proposal is to characterize in detail the age-associated epigenetic alterations and their functional roles in the age-related risk for cancer, so that we can devise approaches to either prevent or treat early neoplasms.
Specific Aims: Aim-1: Characterize age-associated epigenetic changes in bulk epithelial stem cells and "fitter" stem cell populations using mouse colon as a model organ/tissue. Aim-2: Determine tumorigenic potential of aged vs. young colon epithelium, and relate these findings to the ageing-related epigenetic changes.
Project Overlap or Parallel: No scientific or budgetary overlap.

R01 CA229240 (PI: Baylin/Easwaran)
Title: Organoid modeling to determine and reverse age-related epigenetic changes contributing to risk of colorectal cancer
Time Commitment: 1.08 calendar
Supporting Agency: NIH
Procuring Contracting/Grants Officer: Paul Okano
Address of Grants Officer: 9609 Medical Center Dr., Bethesda, MD 20892
Performance Period: 7/1/2019-5/31/2024
Level of Funding: $228,750
Project's Goal(s): We harness in this proposal, a novel model using mouse colon-derived organoids to identify determinants of aging as a key risk factor for colorectal cancer (CRC).
Specific Aims: Specific Aim 1: Dissect the acquisition of CIMP and its function in inducing the aging-like phenotype, and associated rapidity of oncogenic Braf-induced transformation. Specific Aim 2: Determine the direct relationship of in vivo age-associated methylation on cancer predisposition in mice and humans. Specific Aim 3: Determine whether and how age-related epigenetic changes may convey risk for the majority of CRC subtypes by studying effect on cancer initiation by mutant KRAS and APC.
Project Overlap or Parallel: No scientific or budgetary overlap.

90084134 (PI: Velculescu)
Title: Early detection of cancer in high-risk BRCA mutation carriers using liquid biopsies
Time Commitment: 0.24 calendar
Supporting Agency: Gray Foundation
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: Unknown
Performance Period: 7/1/2019-6/30/2024
Level of Funding: $693,635
Project's Goal(s): Develop and apply approaches for non-invasive early detection of cancer in high-risk BRCA mutation carriers.
Specific Aims: Unknown
Project Overlap or Parallel: No scientific or budgetary overlap.

90086203 (PI: Baylin)
Title: Bringing Epigenetic Therapy to the Management of Ovarian and Other Cancers

Time Commitment: 1.2 calendar
Supporting Agency: Miriam & Sheldon Adelson Medical Research Foundation
Procuring Contracting/Grants Officer: Joseph Bigley
Address of Grants Officer: OncoMethylome Sciences, 2505 Meridian Parkway, Suite 310, Durham, NC 27713
Performance Period: 10/1/2019-9/30/2022
Level of Funding: $457,010
Project's Goal(s): We are embarked on in-depth pre-clinical studies designed to directly bring "epigenetic" therapy, using existing DNA de-methylating agents and histone deactylase inhibitors (HDACi's), to the therapeutic management of advanced ovarian and other cancers.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

COMPLETED SINCE LAST SUBMISSION

90075114 (PI: Baylin)
Title: Bringing Epigenetic Therapy to the Management of Ovarian and Other Cancers
Time Commitment: 3 calendar
Supporting Agency: Miriam & Sheldon Adelson Medical Research Foundation
Procuring Contracting/Grants Officer: Marissa White
Address of Grants Officer: 300 First Avenue, Suite 330, Needham, MA 02494
Performance Period: 10/01/2014-9/30/2019
Level of Funding: $457,010
Project Goals: We are embarked on in-depth pre-clinical studies designed to directly bring "epigenetic" therapy, using existing DNA de-methylating agents and histone deactylase inhibitors (HDACi's), to the therapeutic management of advanced ovarian and other cancers.
Specific Aims: N/A
Justification: This grant has no overlap with the current proposal.
Project Overlap or Parallel: No scientific or budgetary overlap.

90075113 (PI: Velculescu)
Title: Ovarian Cancer Genome Analysis Platform
Time Commitment: 0.36 calendar
Supporting Agency: Miriam & Sheldon Adelson Medical Research Foundation
Procuring Contracting/Grants Officer: Joseph Bigley
Address of Grants Officer: OncoMethylome Sciences, 2505 Meridian Parkway, Suite 310, Durham, NC 27713
Performance Period: 10/1/2017-9/30/2019
Level of Funding: $1,638,535
Project's Goal(s): N/A
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

90067293 (PI: Baylin)
Title: Understanding the mechanisms underlying how epigenetic therapy may sensitize patients with multiple human cancer types to immune checkpoint therapy
Time Commitment: 0.12 calendar
Supporting Agency: Janssen Research & Development LLC
Procuring Contracting/Grants Officer: Dashyant Dhanah
Address of Grants Officer: 1400 McKean Rd., Spring House, PA 19477
Performance Period: 2/26/2016-2/26/2019
Level of Funding: $145,110

Project's Goal(s): A collaboration between Janssen Res & Dev and The Cancer Center at Johns Hopkins to study mechanisms underlying how epigenetic therapy may sensitize patients with multiple human cancer types to immune checkpoint therapy.
Specific Aims: To continue investigating mechanisms in tumor cells which may underlie epigenetic therapy priming to immune checkpoint therapy and provide biomarker strategies for prediction of efficacy.
Project Overlap or Parallel: No scientific or budgetary overlap.

90074377 (PI: Baylin)
Title: Developing a lab model for lung cancer initiation, risk and prevention
Time Commitment: 0.36 calendar
Supporting Agency: Am Lung Association
Procuring Contracting/Grants Officer: Alexandra Sierra
Address of Grants Officer: 55 West Wacker Dr., Suite 1150, Chicago, IL 60601
Performance Period: 7/1/2017-6/30/2019
Level of Funding: $100,000
Project's Goal(s): Our preliminary data and proposed studies for this proposal aim to use a new approach to study, in human lung epithelial cells *in-vitro*, the consequences of chronic exposure to low doses of cigarette smoke extract (CSC).
Specific Aims: Specific Aim 1: Determine effects of CSC exposure on epigenetic alterations and their ability to promote subsequent sensitization to transformation in cultured primary human bronchial epithelial cells and normal lung organoid cultures. Specific Aim 2: To determine cancer-driver roles for genes with CSC-induced abnormal epigenetic events using the CRISPR-based gene manipulation approach.
Project Overlap or Parallel: No scientific or budgetary overlap.

OTHER SUPPORT

ZAHNOW, CYNTHIA A.

ACTIVE

90046519 (PI: Casero/Baylin/Zahnow)
Title: Novel therapies targeting epigenetic silencing of tumor suppressors
Time Commitment: .12 calendar
Supporting Agency: Samuel Waxman Cancer Research Foundation
Procuring Contracting/Grants Officer: Carole Asher
Address of Grants Officer: 420 Lexington Ave., Suite 825, New York, NY 10170
Performance Period: 7/1/2011-6/30/2021
Level of Funding: $25,000
Project's Goal(s): The goals of Dr. Zahnow's project within this Collaborative Grant is to demonstrate that low dose epigenetic therapy re-sensitizes drug tolerant breast cancer cells to conventional, single agent chemotherapeutics or targeted therapy.
Specific Aims: 1. To test whether Azacytidine can sensitize endocrine-resistant breast cancers to anti-estrogen therapy. 2. To continue our investigation of the role of the immune system in the anti-tumorigenic response of breast cancer cells to epigenetic therapy with a special focus on interferon signaling and activation. **Justification:** This grant has no overlap with the current proposal.
Project Overlap or Parallel: No scientific or budgetary overlap.

P30 CA006973 (PI: Nelson)
Title: Regional Oncology Research Center – Resource Director
Time Commitment: 4.5 calendar
Supporting Agency: NIH/NCI
Procuring Contracting/Grants Officer: Devi Vembu
Address of Grants Officer: National Cancer Institute, Building 6116-700, 6116 Executive Blvd, Rockville, MD 20852
Performance Period: 5/7/1997-4/30/2022
Level of Funding: $67,385 (salary support only)
Project's Goal(s): CORE grant for the Johns Hopkins Oncology Center. Dr. Zahnow receives salary support only for serving as the Director of the Animal Facility and administrative duties to the Oncology Center.
Specific Aims: N/A
Justification: This grant has no overlap with the current proposal.
Project Overlap or Parallel: No scientific or budgetary overlap.

Award ID: W81XWH-14-1-0385(Baylin)
Title: A New Paradigm for the Treatment of Ovarian Cancer: The Use of Epigenetic Therapy to Sensitize Patients to Immunotherapy and Chemotherapy
Effort: 1.8 calendar
Supporting Agency: CDMRP
Name of Procuring Contracting/Grants Officer: Susan Dellinger, Grants Officer
Address of Funding Agency: 1077 Patchel St., Bldg 1077, Fort Detrick, MD 21702
Period of Performance: 09/30/2014-09/29/2020 (NCE)
Level of Funding: $496,554 annual direct costs
Project's Goal: The major goal of this project is to robustly prolong the survival of patients with serous ovarian cancer (OC) through introducing epigenetic therapy paradigms
Specific Aims: 1) To uncover the mechanisms through which epigenetic therapy may, alone, achieve robust, durable responses in patients with advanced ovarian cancer (OC), 2) Study

how epigenetic therapy may sensitize OC cells to subsequent chemotherapies, 3) Study how epigenetic therapy may sensitize OC cells to subsequent immunotherapies which targets checkpoints which are driving immune tolerance, 4) Develop new combinations of epigenetic drugs which may provide for the highest level of efficacy for management of OC, 5) Bring all of the above studies to bear on leveraging clinical trials of epigenetic therapy on OC.
Role: PI
Overlap: None

R01 CA204555 (PI: Sharma)
Title: Evaluation of molecular determinants of racial disparity in triple-negative breast cancer
Time Commitment: 0.48 calendar
Supporting Agency: NIH/NCI
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: Unknown
Performance Period: 4/1/2017-3/31/2022
Level of Funding: $228,750
Project's Goal(s): Our studies focus on examining the key molecules involved in racial disparity in triple negative breast cancer focusing on the role and importance of loss of tumor suppressor genes and resulting 'oncogene addiction' in triple negative breast cancer growth and progression.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

AWARDED SINCE LAST SUBMISSION

P50 CA228991 (PI: Shih)
Title: Regulation of the M1/M2 macrophage ratio in ovarian cancer
Time Commitment: 0.12 calendar
Supporting Agency: Ovarian Cancer SPORE – Developmental Research Award
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: Unknown
Performance Period: 8/1/2019-7/31/2020
Level of Funding: $30,000
Project's Goal(s): To target tumor–associated macrophages and activate the tumor immune response in ovarian cancer using AZA/DFMO in combination with a glutamine antagonist or an inhibitor for lysine specific histone demethylase 1 (LSD1).
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

90084423 (PI: Nelson)
Title: Regulation of the M1/M2 macrophage ratio in ovarian cancer
Time Commitment: 0.12 calendar
Supporting Agency: Ovarian Cancer SPORE – Developmental Research Award
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: Unknown
Performance Period: 8/1/2019-7/31/2020
Level of Funding: $30,000
Project's Goal(s): To target tumor–associated macrophages and activate the tumor immune response in ovarian cancer using AZA/DFMO in combination with a glutamine antagonist or an inhibitor for lysine specific histone demethylase 1 (LSD1).
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

90086203 (PI: Baylin)
Title: Bringing Epigenetic Therapy to the Management of Ovarian and Other Cancers

Time Commitment: 3 calendar
Supporting Agency: Miriam & Sheldon Adelson Medical Research Foundation
Procuring Contracting/Grants Officer: Marissa White
Address of Grants Officer: 300 First Avenue, Suite 330, Needham, MA 02494
Performance Period: 10/01/2019-9/30/2022
Level of Funding: $457,010
Project Goals: We are embarked on in-depth pre-clinical studies designed to directly bring "epigenetic" therapy, using existing DNA de-methylating agents and histone deactylase inhibitors (HDACi's), to the therapeutic management of advanced ovarian and other cancers.
Specific Aims: N/A
Justification: This grant has no overlap with the current proposal.
Project Overlap or Parallel: No scientific or budgetary overlap.

COMPLETED SINCE LAST SUBMISSION

90067293 (PI: Baylin)
Title: Understanding the mechanisms underlying how epigenetic therapy may sensitize patients with multiple human cancer types to immune checkpoint therapy
Time Commitment: .12 calendar
Supporting Agency: Janssen Research & Development LLC
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: Unknown
Performance Period: 2/26/2016-2/25/2019
Level of Funding: $145,110
Project's Goal(s): A collaboration between Janssen Res & Dev and The Cancer Center at Johns Hopkins to study mechanisms underlying how epigenetic therapy may sensitize patients with multiple human cancer types to immune checkpoint therapy.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

90075114 (PI: Baylin)
Title: Bringing Epigenetic Therapy to the Management of Ovarian and Other Cancers
Time Commitment: 3 calendar
Supporting Agency: Miriam & Sheldon Adelson Medical Research Foundation
Procuring Contracting/Grants Officer: Marissa White
Address of Grants Officer: 300 First Avenue, Suite 330, Needham, MA 02494
Performance Period: 10/01/2014-9/30/2019
Level of Funding: $457,010
Project Goals: We are embarked on in-depth pre-clinical studies designed to directly bring "epigenetic" therapy, using existing DNA de-methylating agents and histone deactylase inhibitors (HDACi's), to the therapeutic management of advanced ovarian and other cancers.
Specific Aims: N/A
Justification: This grant has no overlap with the current proposal.
Project Overlap or Parallel: No scientific or budgetary overlap.

(PI: Baylin)
Title: Clinical trials of epigenetic therapy in non-small cell lung cancer
Time Commitment: 0.3 calendar
Supporting Agency: Rising Tide Foundation
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: Unknown
Performance Period: 1/1/2015-12/31/2019
Level of Funding: $331,360

Project's Goal(s): We are addressing the hypothesis that reversal of cancer-specific DNA methylation and chromatin abnormalities can potently change the management of NSCLC.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

(PI: Shih)
Title: Development of Targeted Therapies for Recurrent Ovarian Cancer
Time Commitment: 0.6 calendar
Supporting Agency: Ovarian Cancer Research Fund
Procuring Contracting/Grants Officer: Unknown
Address of Grants Officer: Unknown
Performance Period: 1/1/2017-12/31/2019
Level of Funding: $272,727
Project's Goal(s): Unknown
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

OTHER SUPPORT

PARDOLL, DREW M.

ACTIVE

Award ID: R01CA142779-06A1 (Pardoll/Topalian/Taube)
Title: B7-H1/PD1 modulation in cancer therapy
Effort: .96 Calendar months
Supporting Agency: NIH/NCI
Name of Procuring Contracting/Grants Officer: Jacquelyn Saval
Address of Grants Officer: 9609 Medical Center Drive, Rockville, MD 20850
Period of Performance: 12/01/2015-11/30/2020
Level of Funding: $99,494 annual direct costs
Project's Goal: The major goals of this multi-PI project are to define mechanisms regulating the expression of B7-H1 (PD-L1) by tumor cells and PD-1 by tumor-specific T cells, and to explore the molecular and immunological mechanisms contributing to the clinical effects of B7-H1/PD-1 blockade in therapeutic trials for patients with advanced metastatic cancers
Specific Aims: 1) Define mechanisms regulating PD-L1 expression by tumor cells and other cell types in the tumor microenvironment 2) Characterize factors influencing PD-1 expression by T cells. 3) Characterize immunological mechanisms underlying the clinical effects of PD-L1/PD-1 blockade in cancer therapy, including the co-expression of multiple checkpoint pathways that might provide resistance pathways to therapy
Overlap: None
Role: MPI (Contact PI)

Award ID: P30CA06973 (Nelson)
Title: Regional Oncology Research Center
Effort: 0.36 calendar months
Supporting Agency: NIH/NCI
Name of Procuring Contracting/Grants Officer: Jason Gill
Address of funding agency: 9609 Medical Center Drive, Rockville, MD 20850
Performance Period: 08/09/2012-04/30/2022
Level of Funding: $12,481* annual direct costs (*salary support only)
Project's Goal: The major goal of this project is to support research programs and shared resources at the National Cancer Institute Designated Cancer Center. The central goal of the Cancer Immunology program is the development of new effective cancer immunotherapies that are based on understanding the molecular recognition and regulation.
Specific Aims: N/A
Role: Co-Program Leader for Cancer Immunology
Overlap: None

Award ID: W81XWH-14-1-0385 (Baylin)
Title: A New Paradigm for the Treatment of Ovarian Cancer: The Use of Epigenetic Therapy to Sensitize Patients to Immunotherapy and Chemotherapy
Effort: .12 cal months
Supporting Agency: CDMRP
Name of Procuring Contracting/Grants Officer: Susan Dellinger, Grants Officer
Address of Funding Agency: 1077 Patchel St., Bldg 1077, Fort Detrick, MD 21702
Period of Performance: 09/30/2014-09/29/2020
Level of Funding: $ 434,072 annual direct costs
Project's Goal: The major goal of this project is to robustly prolong the survival of patients with serous ovarian cancer (OC) through introducing epigenetic therapy paradigms

Specific Aims: 1) To uncover the mechanisms through which epigenetic therapy may, alone, achieve robust, durable responses in patients with advanced ovarian cancer (OC) 2) Study how epigenetic therapy may sensitize OC cells to subsequent chemotherapies 3) Study how epigenetic therapy may sensitize OC cells to subsequent immunotherapies which targets checkpoints which are driving immune tolerance 4) Develop new combinations of epigenetic drugs which may provide for the highest level of efficacy for management of OC
5) Bring all of the above studies to bear on leveraging clinical trials of epigenetic therapy on OC
Role: Co-Investigator
Overlap: None

Award ID: N/A (Pardoll)
Title: The Johns Hopkins University Bloomberg-Kimmel Institute for Cancer Immunology
Effort: 1.2 calendar months
Supporting agency: Bloomberg Philanthropies
Procuring Contracting/Grants Officer: Patricia Harris
Address of Grants Officer: 25 E. 78th St, New York, NY 10075
Performance period: 01/01/2016-12/31/2020
Level of funding: $10,000,000 annual direct costs
Project's Goal(s): The goal of the Institute is to develop, within 10 years, immunotherapies that can place 50% of people with inoperable cancer into lifelong remission. The institute funds multiple immunotherapy related programs, cores, and pilot projects.
Specific Aims: N/A
Role: Institute Director
Overlap: None

Award ID: BMSC1259 (Pardoll/Topalian)
Title: Strategic Research Collaboration Agreement
Effort: 1.2 calendar months
Supporting agency: Bristol Myers Squibb
Procuring Contracting/Grants Officer: Fouad Namoun
Address of Grants Officer: 345 Park Avenue, New York, New York 10154
Performance period: 01/01/2017-12/31/2022
Level of funding: $3,441,044 annual direct costs
Project's Goal(s): This overarching contract funds multiple immunotherapy research projects and clinical trials in an effort to determine the IO agents or combination treatments incorporating IO agents that provide the optimal benefit risk ratio for cancer populations and subpopulations.(when applicable)
Specific Aims: N/A
Role: PI
Overlap: None

Award ID: W81XWH-17-1-0627 (Elisseeff)
Title: Removal of trauma induced senescent cells as a new treatment for osteoarthritis
Effort: .24 calendar months
Supporting agency: US Department of Defense
Procuring Contracting/Grants Officer: TBD
Address of Grants Officer: Unknown
Performance period: 09/30/2017-09/29/2020
Level of funding: $382,573 annual direct costs
Project's Goal(s): The goal of this project is to answer the fundamental questions on how senescent cells induce OA after trauma (with implications also for age-related OA).
Specific Aims: 1.) Define the senescence and immunological profile of the articular joint after ACLT injury

2.) Determine the impact of senolytics and clearance of senescent cells on the immune profile and PTOA disease 3.) Develop and test controlled release senolytics in the PTOA murine model
Role: Co-Investigator
Overlap: None

Award ID: MFCR-MIC-001 (Pardoll)
Title: The MANAFEST Project
Effort: .6 calendar months
Supporting agency: The Mark Foundation for Cancer Research
Procuring Contracting/Grants Officer: Michele Cleary
Address of Grants Officer: 10 East 53rd St, Floor 13 New York, NY 10022
Performance period: 10/13/2017-10/12/2020
Level of funding: $1,000,000 annual direct costs
Project's Goal(s): The major goal of this project is to leverage and extend the existing Genomics Data Commons (GDC) towards the development of a Cancer Immunology Relational Database that integrates immunotherapy clinical and immunologic information in a fashion that can be efficiently mined. Specific Aims: 1.) Development of data standards for the newer immune related assays (such as neoantigen characterizations, immunophenotyping approaches. 2.) Map out a process for adapting the cBioPortal in which the features initially chosen have been shown to be important for modulating response to immunotherapies. 3.) Optimize, standardize and validate across institutions a new T cell assay platform – MANAFEST – together with informatics tools such as ImmunoMap, that will transform outputs into quantitative variables suitable for
populating the Cancer Immunology Relational Database.
Role: PI
Overlap: None

Award ID: N/A (Pardoll)
Title: Ervaxx-JHU Sponsored Research Agreement
Effort: .12 calendar months
Supporting agency: ERVAXX Limited
Procuring Contracting/Grants Officer: Timothy Edwards, Chief Operating Officer
Address of Grants Officer: 71, Kingsway, London WC2B 6ST, United Kingdom
Performance period: 12/20/2017-06/19/2020
Level of funding: $141,474 annual direct costs
Project's Goal(s): The major goals of this project are to address the following questions: **1.)** Are there detectable CD8 and CD4 responses against certain HERV-related peptide antigens in cancer patients, **2.)** Which HERV-related peptides generate the best and most consistent (ie among multiple patients) responses, in particular relative to normal controls, **3)** Can HERV-related peptide-specific T cell clones be found in the tumors? **4.)** How do HERV-related peptide-specific responses compare with MANA-specific responses.
Specific Aims: see major goal above
Role: Collaborator
Overlap: None

Award ID: (Anagnostou)
Title: Dynamics of neoantigen landscape during immunotherapy in lung cancer
Effort: .12 calendar months *effort, no salary support
Supporting agency: LUNGevity Foundation
Procuring Contracting/Grants Officer: Margery Jacobson
Address of Grants Officer: 228 S. Wabash Ave Suite 700, Chicago, IL 60604
Performance period: 11//01/2017-10/31/2020
Level of funding: $86,956 annual direct costs

Project's Goal(s): The goal of this project is to us the results of the proposed research including identification of putative neoantigens identified prior to and at the time of emergence of resistance and development of liquid molecular assays predictive of resistance to immune checkpoint blockade will be used to develop patient-specific immunotherapy approaches in a variety of cancer types and will launch investigator-initiated clinical trials at Hopkins and other institutions.
Specific Aims: N/A
Role: Collaborator
Overlap: None

AWARDED SINCE LAST SUBMISSION

Award ID: 18CHAL17 (Kachhap/Antonarakis)
Title: Concurrent Administration of Bipolar Androgen Therapy (BAT) and Nivolumab in Metastatic Castration-Resistant Prostate Cancer: The COMBAT-CRPC Trial
Effort: 0.2 calendar months
Supporting Agency: Prostate Cancer Foundation
Name of Procuring Contracting/Grants Officer: Howard Soule, Ph.D.
Address of Funding Agency: 1250 Fourth Street, Santa Monica, CA 90401
Performance Period: 02/28/2019-02/28/2021
Level of Funding: $500,000 annual direct costs
Project's Goal: The major goal of this project is to establish a new therapeutic paradigm for the treatment of both DNA repair-deficient and proficient advanced prostate cancer in the near-term, as well as to understanding the molecular, cellular, and immunological underpinnings predicting favorable responses to the BAT/nivolumab combination
Specific Aims: 1): Conduct a Biomarker-Rich Phase II Trial of BAT in Sequence with Nivolumab for mCRPC Patients. 2): Evaluate the Role of BAT-induced dsDNA Breaks and Assess its Contribution in Activating Innate and Adaptive Immune Responses. 3): Assessment of DNA Damage Markers, Immune Cell Infiltration, and Generation of Novel Mutation-Associated Neoantigens (MANAs) from Tumor Biopsy Specimens.
Role: Co-Investigator
Overlap: None

Award ID: MFCR Ref No: 19-028(Taube)
Title: Massively scalable, spatially-resolved analysis of the tumor immune microenvironment
Effort: 0.24 calendar months
Supporting Agency: The Mark Foundation for Cancer Research
Name of Procuring Contracting/Grants Officer: Rebecca Lui
Address of Funding Agency: 10 East 53rd St, Floor 13 New York, NY 10022
Performance Period: 07/01/2019-06/30/2021
Level of Funding: $1,131,253 annual direct costs
Project's Goal: Using approaches developed in planetary science, we are creating a platform for the next generation of pathologic analysis of the tumor microenvironment (TME) and using the resultant high-quality datasets for immuno-oncology biomarker development.
Specific Aims: 1.) Develop a unique facility to produce petabytes of tissue imaging data **2.)** Spatially-map specific cellular subsets, immune checkpoints, and immunoactive molecule expression in pre-treatment NSCLC and melanoma tumor specimens for the development of multiplex biomarkers. **3.)** Spatially-map specific cellular subsets, immune checkpoints, and immunoactive molecule expression in neoadjuvant and on-treatment specimens for an improved understanding of the therapeutic mechanisms and efficacy.
Role: Co-Investigator
Overlap: None

Award ID: OT123-397 TM239 PA32 (Pardoll)

Title: Affects of DGK1a/z inhibitor on the MANAFEST assays
Effort: 0.12 calendar months
Supporting Agency: Bristol Myers Squibb CO
Name of Procuring Contracting/Grants Officer: David Kacsue
Address of Funding Agency: 345 Park Avenue, New York, New York 10154
Performance Period: 07/01/2019-06/30/2020
Level of Funding: $126,744 annual direct costs
Project's Goal: The major goal of this project is to test whether adding DGKi to MANAFEST cultures will significantly enhance sensitivity of the assay.
Specific Aims: 1.) Test DGKi in CD8 MANAFEST assays. 2.) Test DGKi to enhance sensitivity of CD4 responses to MHIC II-restricted MANA.
Role: PD/PI
Overlap: None

Award ID: (Sears)
Title: ETBF Colon Tumorigenesis
Effort: 0.48 calendar months
Supporting Agency: Janssen Research and Development
Name of Procuring Contracting/Grants Officer: Joseph Erhardt
Address of Funding Agency: 920 U.S. Highway 202, Raritan, NJ 08869
Level of Funding: 435,927 annual direct costs
Performance Period: 07/10/2019-04/30/2021
Project's Goal: These studies will define whether co-colonization with a pair of microbes synergistically promote CRC development and test approaches to eliminate one or both from the colon as a means of CRC prevention.
Specific Aims: 1.) To determine the reproducibility of the enterotoxigenic Bacteroides fragilis (ETBF) and
pks+ Escherichia coli co-carcinogenic model given the new appreciation of azoxymethane(AOM) lot variability. **2.)** To determine the reproducibility of the enterotoxigenic Bacteroides fragilis (ETBF) and pks+ Escherichia coli co-carcinogenic model given the new appreciation of azoxymethane (AOM) lot variability**. 3.)** To develop purified BFT quantities required to accelerate translational discovery.
Role: Co-Investigator
Overlap: None

Award ID: P50CA098252 (Wu)
Title: SPORE in Cervical Cancer: Developmental Research Program (DRP)
Effort: 0.24 calendar months
Supporting Agency: NIH/NCI
Name of Procuring Contracting/Grants Officer: Jason Gill
Address of Funding Agency: 9609 Medical Center Drive, Rockville, MD 20850
Performance Period: 09/01/2019-08/31/2024
Level of Funding: $ 90,244 annual direct costs (DPR)
Project's Goal: The DRP provides means to respond to new opportunities, and it designed to encourage and facilitate new research efforts by providing funds for pilot projects with potential for development into full-fledged translational research avenues, collaborations, and new methodologies for integration into other Research Projects.
Specific Aims: 1) Develop a GLP freeze--dry protocol for a powder formulation of RG1--VLPs in alum and study its *in vitro* temperature stability, and in murine models test its immunogenicity and protective efficacy in comparison to Gardasil9. 2) To analyze the levels of protective antibodies in the serum of patients from the phase I study induced by RG1--VLP vaccination or Gardasil9
Role: Co-Leader, Developmental Research Program
Overlap: None

Award ID: N/A (Fakhry)
Title: The adjuvant therapy for high-risk HPV16 (Clinical Trial)
Effort: 0.12 calendar months
Supporting Agency: Astrazeneca LP
Name of Procuring Contracting/Grants Officer: Joanne Cornacchia, Sr. Contracts Manager, Oncology Business Unit
Address of Funding Agency: 1800 Concord Pike, Wilmington, DE 19850
Performance Period: 09/01/2019-08/31/2024
Level of Funding: $1,011,850 annual direct costs
Project's Goal: The major goal of this project is to determine whether anti-PD-L1 alone or together with an HPV vaccine will enhance HPV E6/E7-specific and/or mutation-associated neoantigen (MANA)-specific T cell responses and whether these responses correlate with enhanced clearance of HPV as measured by DNA in oral rinses and plasma.
Specific Aims: 1.) To determine whether combination immune checkpoint inhibitor and vaccine will result in clearance of HPV biomarkers for patients at risk of disease progression **2.)** To determine whether combination immune checkpoint inhibitor and vaccine will reduce risk of disease progression among HPV-OPC patients with evidence of HPV biomarkers after primary treatment. **3.)** To determine whether anti-PD-L1 alone or together with an HPV vaccine will enhance HPV E6/E7-specific and/or mutation-associated neoantigen (MANA)-specific T cell responses and whether these responses correlate with enhanced clearance of HPV as measured by DNA in oral rinses and plasma.
Role: Co-Investigator
Overlap: None

Award ID: P41EB028239 (Schneck)
Title: The Johns Hopkins Translational Immuno Engineering (JH-TIE) BTRC: TR&D2: Nanoimmunomaterials for Immune Engineering
Effort: 0.24 calendar months
Supporting Agency: NIH/NIBIB
Name of Procuring Contracting/Grants Officer: Katie Ellis
Address of Funding Agency: 6707 Democracy Blvd Bethesda MD 20892
Level of Funding: $194,028 annual direct costs
Performance Period: 09/15/2019-08/31/2024
Project's Goal: The major goal of TR&D2 is to bioengineer nanoimmunomaterial tools (NIMs) through the design and synthesis of new biomaterials and particles created for immunomodulation
Specific Aims: 1.) Engineer immune cell-specific polymeric nanoparticles for enhanced intracellular delivery of nucleic acids. **2.)** Engineer biomimetic nanoimmunomaterials as artificial APCs (aAPC) for immunostimulation or immunosuppression**. 3.)** Engineer nanoimmunomaterials for combined intracellular and extracellular signaling to target T cells.
Role: Co-Investigator
Overlap: None

Award ID: W81XWH1910724 (Kachhap)
Title: PC180630 - Innate Immune Signaling Induced by Androgens: Implications in tumor response to Bipolar Androgen Therapy
Effort: 0.36 calendar months
Supporting Agency: Department of Defense (DoD)
Name of Procuring Contracting/Grant Officer: TBD
Address of Funding Agency: 820 Chandler Street, Fort Detrick, MD 21702
Performance Period: 09/01/2019-08/31/2022
Level of Funding: $200,000

Project Goals: The overall objective of this proposal is to determine the role of nucleophagy and the molecular underpinnings that lead to immune signaling by suprahysiological testosterone in prostate cancer.
Specific Aims: (1) To determine the role of nucleophagy in clearing androgen-induced DNA damage; **(2)** To determine the mechanism by which SupT activate an innate immune response in PCa; **(3)** To evaluate the interferon signaling pathway as a tumor-intrinsic molecular determinant of response in tumor biopsies and serum samples of patients receiving BAT and BAT/nivolumab therapy.
Role: Co-Investigator
Projects Overlap or Parallel: No scientific or budgetary overlap

COMPLETED SINCE LAST SUBMISSION

Award ID: P50CA098252 (Wu)
Title: SPORE in Cervical Cancer
Effort: 0.24 calendar months
Supporting Agency: NIH/NCI
Name of Procuring Contracting/Grants Officer: Jason Gill
Address of Funding Agency: 9609 Medical Center Drive, Rockville, MD 20850
Performance Period: 09/01/04 – 08/31/19
Level of Funding: $1,702,596 annual direct costs
Project's Goal: The development research program role is to identify and select pilot projects with potential for development into full- fledged translational research avenues, collaborations, and new methodologies for integration into other research projects based on the described review criteria.
Specific Aims: 1) Provide initiating funds for novel explorations related to cervical cancer. 2) Integrate the awardee into the SPORE community by participation in monthly meetings, group communications, and opportunities for expanded funding and for collaborations. 3) Review progress and recommend avenues for continuation of successful projects
Role: Co- Director, Developmental Research Program
Overlap: None

Award ID: CA209-596 (Lim)
Title: ABTC: A Phase I Trial of Anti-LAG-3 or Anti-CD137 Alone and in Combination with Anti-PD-1 in Patients with Recurrent GBM (ABTC 1501)
Effort: .03 calendar months
Supporting agency: Bristol Myers Squibb
Procuring Contracting/Grants Officer: Fouad Namoun
Address of Grants Officer: 345 Park Avenue, New York, New York 10154
Performance period: 04/29/2016-05/01/2019
Level of funding: $488,373 annual direct costs
Project's Goal(s): The major goal of this project is assaying and analyzing the correlatives of CA209-596/ABTC1501 study.
Specific Aims: N/A
Role: Co-Investigator
Overlap: None

Award ID: N/A (Pardoll)
Title: Analysis of novel immunomodulatory ligands and receptors
Effort: .12 calendar months
Supporting Agency: Compugen Ltd.
Name of Procuring Contracting/Grants Officer: Anat Cohen-Dayag, Ph.D
Address of Funding Agency: 72 Pichas Rosen St., Tel Aviv 69512, Israel
Period of Performance: 01/01/2015-12/31/2019

Level of Funding: $895,033 annual direct costs
Project's Goal: The major goal of this project is to study the immunobiology and cancer immunotherapy relevance of multiple novel gene products identified as potentially immunomodulatory
Specific Aims: 1) Determine in-house phage display vs conventional hybridoma depending on level of conservation of molecule across species. 2) Expression studies in mice and humans-define target's expression tumor components of the TME, sorted cell populations, purified tumor infiltrates, myeloid and lymphocyte human-on selected targets. 3) In vitro testing of murine and human antibodies and Fc fusion molecules 4)Antibody/Recombinant Fc fusion experiments with emphasis on antibodies 5) Therapeutic synergy experiments
Role: PI
Overlap: None

Award ID: CA-209-358 (Topalian)
Title: Analysis of PD-1 Blockade in Virus-Associated Cancers on CA-209-358
Effort: .12 Calendar months
Supporting Agency: Bristol Myers Squibb Co
Name of Procuring Contracting/Grants Officer: Les Enterline
Address of Funding Agency: Route 206 and Providence Line Road, Princeton, NJ 08543
Period of Performance: 07/01/2016-12/31/2018
Level of Funding: $101,762 annual direct costs
Projects Goal: The goal of this project is to characterize changes in the tumor immune microenvironment in pre/post therapy biopsies from patients with advanced virus-associated cancers receiving anti-PD-1 therapy on clinical trial CA209-358, in order to understand response and resistance to therapy.
Specific Aims: N/A
Role: PI
Overlap: None

Individuals who have worked on the project

The Regents of the University of California

Name:	Dennis Slamon, M.D., Ph.D.
Project Role:	PI (Senior/Key Personnel)
Research Identifier:	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Slamon contributes clinical, translational, and genomic expertise to the project and is involved in the overall direction.
Funding Support:	See Other Support

Name:	Judy Dering, Ph.D.
Project Role:	Sr Public Analyst
Research Identifier:	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Dering is responsible for analyzing data from the microarray experiments.
Funding Support:	No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. See next pages for Dr. Slamon's Other Support.

OTHER SUPPORT

SLAMON, DENNIS

CURRENT

W81XWH-14-1-0385 (PI: Baylin)
Title: A New Paradigm for the treatment of Ovarian Cancer: The use of Epigenetic Therapy to Sensitize Patients to Immunotherapy and Chemotherapy.
Time Commitment: 0.5 Calendar Months
Supporting Agency: US Army Subaward with John Hopkins University
Procuring Contracting/Grants Officer: Melody Snow, M.H.S, Assistant Director, Outgoing Awards
Address of Grants Officer: John Hopkins University, School of Medicine, 1629 Thames Street, Suite 200
Baltimore, Maryland 21231
Performance Period: 9/30/2014-9/29/20
Level of Funding: $188,568
Project's Goal(s): To robustly prolong the survival of patients with serous ovarian cancer (OC) through introducing epigenetic therapy paradigms.
Specific Aims: 1) To uncover the mechanisms through which epigenetic therapy may, alone, achieve robust, durable responses in patients with advanced ovarian cancer (OC) 2) Study how epigenetic therapy may sensitize OC cells to subsequent chemotherapies 3) Study how epigenetic therapy may sensitize OC cells to subsequent immunotherapies which targets checkpoints which are driving immune tolerance 4) Develop new combinations of epigenetic drugs which may provide for the highest level of efficacy for management of OC
5) Bring all of the above studies to bear on leveraging clinical trials of epigenetic therapy on OC
Project Overlap or Parallel: No scientific or budgetary overlap.

P30 CA016042 (PI: Teitell)
Title: "Cancer Support Grant"
Time Commitment: 2.0 calendar
Supporting Agency: NIH/NCI
Procuring Contracting/Grants Officer: Amy Connolly, Grant Management Specialist
Address of Grants Officer: National Cancer Institute, Room 700, Mail Stop 8335
6116 Executive Blvd, Bethesda, MD 20852-8335
Performance Period: 4/23/2003-11/30/2018
Level of Funding: $103,442
Project's Goal(s): This Funding supports activities to increase scientific interaction among members of the Signal Transduction Program Area at Jonsson Cancer Center.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

CLEE0111F2301 (PI: Slamon)
Title: A randomized double-blind, placebo-controlled study of ribociclib in combination with fulvestrant for the treatment of postmenopausal women in hormone receptor positive, HER2-negative, advanced breast cancer who have received no or only one line of prior endocrine treatment.
Time Commitment: 0.12 calendar
Supporting Agency: Novartis
Procuring Contracting/Grants Officer: Karen Riccardello
Address of Grants Officer: One Health Plaza, East Hanover, NJ 07936-1080
Performance Period: 04/20/2015-05/08/2021
Level of Funding: $36,961

Project's Goal(s): To determine whether treatment with fulvestrant + ribociclib prolongs PFS compared to treatment with fulvestrant + ribociclib placebo in postmenopausal women with HR+, HER2- advanced breast cancer who received no or only 1 line of prior hormonal therapy for advanced breast cancer.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

CLEE0111H2301 (PI: Slamon)
Title: A phase III, multicenter, randomized, double-blind, placebo controlled study to evaluate efficacy and safety of ribociclib with endocrine therapy as an adjuvant treatment in patients with hormone receptor-positive, HER2-negative, intermediate risk early breast cancer.
Time Commitment: 0.12 calendar
Supporting Agency: Novartis
Procuring Contracting/Grants Officer: Karen Riccardello
Address of Grants Officer: One Health Plaza, East Hanover, NJ 07936-1080
Performance Period: 04/20/2015-05/08/2021
Level of Funding: $46,571
Project's Goal(s): To compare invasive disease-free survival (iDFS) for ribociclib + ET versus placebo + ET in patients with HR positive, HER2-negative, EBC with intermediate risk of recurrence.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

TRIO-030 (PI: Slamon)
Title: A Presurgical Tissue-Acquisition Study to Evaluate Molecular Alterations in Human Breast Cancer Tissue Following Short-Term Exposure to the Androgen Receptor Antagonist ODM-201
Time Commitment: 0.12 calendar
Supporting Agency: TRIO
Procuring Contracting/Grants Officer: Launa Aspeslet
Address of Grants Officer: 9925 109th St NW, Suite 1100; Edmonton T5K2J8, Alberta, Canada.
Performance Period: 03/29/2017-03/29/2020
Level of Funding: $69,774
Project's Goal(s): To identify the molecular alterations that occur in hum BC tissue, following short-term exposure to ODM-201 in female subjects with EBC. To evaluate the safety and tolerability of short-term exposure to ODM-201 in female subjects with EBC.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

AWARDED SINCE LAST SUBMISSION

None

COMPLETED SINCE LAST SUBMISSION

R01CA182514-01A1 (PI: Curtis)
Title: Intergrated genomic analysis and multi-scale modeling of therapeutic resistance
Time Commitment: 0.24
Supporting Agency: NIH Subaward with Stanford University
Procuring Contracting/Grants Officer: Aida Vasquez, Vasquez@mail.nih.gov
240-276-6319
Performance Period: 09/12/14-8/31/19
Level of Funding: $74,773

Project's Goal(s): The major goals of this project are to i) perform an integrated genomic analysis of serial tissue specimens from HER2-positive patients enrolled in clinical trials to evaluate the efficacy of single or dual agent neoadjuvant lapatinib and or trastuzumab targeted therapy (NCT00769470/TRIO B07) in order to characterize mechanisms of resistance ii) delineate temporal patterns of clonal expansions under treatment selective pressure by analyzing longitudinal samples collected prior to, at run-in, and after therapy iii) to functionally characterize mechanisms of resistance to single and dual agent therapy in HER2-positive tumors and to phenotype resistant cell populations by analyzing patient-derived xenograft models and short-term primary cultures.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

CIRM DR3-07067 (PI: Slamon)
Title: "A Phase I dose escalation and expansion clinical trial of the novel first-in-class Polo-like Kinase 4 (PLK4) inhibitor, CFI-400945 in patients with advanced solid tumors"
Time Commitment: 3.60 calendar
Supporting Agency: California Institute for Regenerative Medicine
Procuring Contracting/Grants Officer: Doug Kearney, Grants Management Office
Address of Grants Officer: California Institute for Regenerative Medicine, 210 King Street
San Francisco, CA 94107
Performance Period: 05/01/2014-10/31/2018 (NCE)
Level of Funding: $8,469,697
Project's Goal(s): This proposal is aimed at a phase I clinical trial of CFI-400945, a first-in-class inhibitor of Polo-like Kinase 4 (PLK4). PLK4, a serine/threonine kinase functions at the intersection of mitosis, DNA repair, hypoxia and metabolism, and is expressed in a variety of solid tumors. Overexpression of PLK4 results in the excessive formation of centrioles and multinucleation in cells suggesting that the elevated expression of PLK4 in tumors could contribute to chromosomal instability (CIN) and aneuploidy. Of interest, PLK4 overexpression in neural stem cells drives centrosome amplification and is associated with tumor formation. Conversely, depletion of PLK4 in cancer cells by RNA interference prevents centriole duplication, causing mitotic defects and cell death. Notably, these effects are amplified in hypoxic conditions. Thus, PLK4 is an attractive target for the development of small-molecule therapeutics in cancer. The candidate molecule, CFI-400945 was developed as part of a collaborative effort funded by CIRM/CSCC (PIs: Dennis Slamon and Tak Mak) that supported a drug discovery effort, preclinical assessment, and IND enabling studies.
Specific Aims: This clinical trial described herein will be carried out in two parts. Part A will consist of the dose escalation phase of the first-in-human trial, where the primary objective will be to determine the maximum tolerated dose (MTD) of CFI-400945. In Part A, patients with any solid tumor refractory to conventional treatment will be enrolled in order to reach the MTD expeditiously. Part B will consist of the expansion phase, where the primary objectives are to further refine the MTD to assist in determination of the recommended phase II dose (RP2D), to further assess plasma pharmacokinetics and to evaluate preliminary evidence of antitumor activity patient populations dosed at the MTD. Up to 4 expansion cohorts of 6-12 patients each would be enrolled which may include: 1) cohorts restricted to a specific tumor histology and/or specific biomarker (predicated upon preclinical data) and a 2) a biomarker cohort to obtain tumor biopsy samples at pre-treatment, on-treatment, with the exploratory objective of evaluating pharmacodynamic effects and potential resistance mechanisms. We expect that the dose escalation will complete enrollment in approximately 1 year and an additional 12-18 months for completion of the expansion cohorts. We then expect an additional one year period will be required to collect data and complete a clinical study report (CSR). We believe that this Phase 1/1B trial will provide critical clinical and biomarker data that will demonstrate clinical proof of concept which will inform the Phase 2 development plan. Over the next 4 years, our Phase I trial will also advance a successfully completed CIRM funded-project for which an IND has already been filed
Project Overlap or Parallel: No scientific or budgetary overlap.

P30 CA016042 (PI: Teitell)
Title: "Cancer Support Grant"
Time Commitment: 2.0 calendar
Supporting Agency: NIH/NCI
Procuring Contracting/Grants Officer: Amy Connolly, Grant Management Specialist
Address of Grants Officer: National Cancer Institute, Room 700, Mail Stop 8335
6116 Executive Blvd, Bethesda, MD 20852-8335
Performance Period: 4/23/2003-11/30/2018
Level of Funding: $103,442
Project's Goal(s): This Funding supports activities to increase scientific interaction among members of the Signal Transduction Program Area at Jonsson Cancer Center.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

SNDX-275-0602 (PI: Slamon)
Title: A Randomized, Placebo-Controlled, Double-Blind, Multicenter Phase 2 Study of Atezolizumab With or Without Entinostat in Patients with Metastatic Triple Negative Breast Cancer, with a Phase Ib Lead in Phase (SYNDX-275-0602)
Time Commitment: 0.12 calendar
Supporting Agency: Syndax
Procuring Contracting/Grants Officer: Launa Aspeslet
Address of Grants Officer: 9925 109th St NW, Suite 1100; Edmonton T5K2J8, Alberta, Canada.
Performance Period: 4/28/2016-04/28/2019
Level of Funding: $231,440
Project's Goal(s): To determine the dose-limiting toxicities (DLT) and maximum tolerated dose (MTD) or recommended Phase 2 dose (RP20) or entinostate (SNDX-275) giving in combination with atezolizumab.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

C31003/TRIO-027 (PI: Slamon)
Title: An Open-Label Phase 2 Study of MLN0128 (A TORC1/2 Inhibitor) in Combination With Fulvestrant in Women with ER-Positive/HER2-Negative Advanced or Metastatic Breast Cancer That Has Progressed During or After Aromatase Inhibitor Therapy.
Time Commitment: 0.12 calendar
Supporting Agency: Millennium
Procuring Contracting/Grants Officer: Maria Alanlzas
Address of Grants Officer: 40 Landsdowne Street, Cambridge, MA 02139. USA
Performance Period: 10/26/2016-10/26/2019
Level of Funding: $255,829
Project's Goal(s): To compare the PFS of patients treated with the combination of fulvestrant+daily MLN0128 versus patients treated with single-agent fulvestratn. To compare the PFS of patients treated with the combination of fulvestrant+weekly MLN0128 versus patients treated with single-agent fulvestrant.
Specific Aims: N/A
Project Overlap or Parallel: No scientific or budgetary overlap.

Individuals who have worked on the project

Van Andel Research Institute

Name:	Peter Jones, Ph.D.
Project Role:	PI (Senior/Key Personnel)
Research Identifier:	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Jones serves as PI on this project.
Funding Support:	See Other Support

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. See next pages for Dr. Jones' Other Support.

OTHER SUPPORT

JONES, PETER A.

Ongoing Research Support:

R35CA209859 (Jones, P)	01/01/2017 – 12/31/2023	6.0 Cal Mths or 50% Effort
NCI		$7,579,702 Total Cost
Targeting DNA Methylation and the Cancer Epigenome The major goal of this project is to provide new approaches to treating cancers through the many epigenetic changes seen in the genomes of cancer cells.		
Role: PD/PI Overlap: None		
Agency Contact: Long Nguyen, nguyen1@mail.nih.gov Specific Aims: Future Question 1: Why are there so many mutations in chromatin modifiers and what are the effects of these mutations on the structure of the epigenome? Future Question 2: What are the functional consequences of activating the expression of cancer/testis genes by 5-Aza-CdR? Future Question 3: What double-stranded RNAs are activated by 5-Aza-CdR and how do these relate to cellular responses? Future Question 4: Can we design combinations of epigenetic drugs which might increase the effectiveness of 5-azanucleoside treatment? Future Question 5: Can cryo-EM help to visualize complexes relevant to chromatin structure and functions?		

SU2C-AACR-CT01-16 (Baylin, S; Hellmann, M)	04/01/2017 – 03/31/2020	0.3 Cal Mths or 2.5% Effort
AACR/SU2C-Merck Supported Catalyst Grant		$2,500,000 Total Cost
Combined Epigenetic Therapy and Pembrolizumab for Advanced NSCLC The primary objective of this project is to test a combined epigenetic therapy to determine whether it will result in a better immune response to cancer and improve the proportion of patients who respond. Note: Memorial Sloan Kettering is the lead collaborating clinical site where trial is being conducted.		
Role: Co-investigator Overlap: None		
Agency Contact: Mike Stewart, mike.stewart@aacr.org Specific Aims: N/A		

SU2C-AACR-CT08-17 (Jones, P; Plimack, E)	11/01/2017 – 10/31/2020	0.6 Cal Mths or 5% Effort
AACR/SU2C-Genentech Supported Catalyst Grant		$2,989,114 Total Cost
Overcoming Urothelial Cancer Atezolizumab Resistance by Epigenetic Therapy The major goal of this project is a clinical trial to evaluate the ability to resensitize relapsed/ refractory bladder cancer patients who have failed immunotherapy. Note: Fox Chase Cancer Center is the lead collaborating clinical site where trial is being conducted.		
Role: PD/PI Overlap: None		
Agency Contact: Mike Stewart, mike.stewart@aacr.org Specific Aims: N/A		

R37CA230748 (Shen, H)	07/01/2018 – 06/30/2023	0.6 Cal Mths or 5% Effort
NCI		$2,653,910 Total Cost

High-throughput Epigenomic Mapping of Regulatory Elements in Ovarian Cancer at Basepair Resolution The major goal of this project is to develop a cost-efficient way to profile the epigenetics of regulatory elements and understand ovarian cancer subtypes.
Role: Co-investigator Overlap: None
Agency Contact: Long Nguyen, nguyen1@mail.nih.gov Specific Aims: 1) will be to develop and optimize a cost-effective genome-wide assay pipeline and associated bioinformatics tools for base resolution profiling of regulatory elements. 2) will be to generate a genome-scale epigenetic regulatory landscape, along with transcriptomic sequencing for clear cell, endometrioid and serous ovarian tumors. 3) will be to characterize alterations in the regulatory network in different subtypes of ovarian cancer, and those associated with HNF1B activation and ARID1A mutation.

F32CAGM129987 (Carpenter, B)	05/01/2019 – 04/30/2020	0.0 Cal Mths or 0% Effort
NCI		$61,610 Total Cost
Epigenetic Regulation of a non-coding RNA, nc886 The major goal of this project is to determine how genomic region containing a non-coding RNA, nc886, is epigenetically regulated and the functional consequences of this regulation.		
Role: Mentor Overlap: None		
Agency Contact: Lan Nguyen, nguyenla@mail.nih.gov Specific Aims: 1) Characterize how DNA methylation of the nc886 DMR alters genome-wide gene expression. 2) Define how variable SNP status and DNA methylation affects CTCF binding and chromatin architecture.		

R50CA24387 (Liu, M)	09/20/2019 – 08/31/2024	0.0 Cal Mths or 0% Effort
NCI		$435,605 Total Cost
Research Specialist Support-Targeting DNA Methylation and the Cancer Epigenome The award supports a Research Specialist in NCI-funded cancer research.		
Role: Unit Director / Other Significant Contributor Overlap: None		
Agency Contact: Long Nguyen, nguyen1@mail.nih.gov Specific Aims: N/A due to nature of award; same as NIH R35 listed above.		

Note: Changes in Other Support since last submission include the addition of the NIH R37, F32 and R50 awards, as well as the completion of an ACS postdoctoral fellowship that Dr. Jones' was co-mentor on, and this DoD subaward W81XWH14-1-0385 which ended at VARI on 09/29/2019. Dr. Jones has also been the Team Lead of an annual Michigan Economic Development Corporation institutional grant of $1,000,000 that supports new faculty recruitment and laboratories.

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 the progress report, 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, the work for which is a 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 two 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 the progress report, her work resulted in her paper now in press in PNAS 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 for a year now working on aspects of immunology related to OC.

Scientific training – Dr. Michael Topper: Dr. Topper completed a year of postdoctoral fellow with Dr. Baylin. 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 have just promoted him to faculty status in our Cancer Center as an Instructor in Oncology. He is now working with his oversight of trainees to extend the implications of his epigenetic therapy development for OC lung and other cancer types. As above, 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.

Additional training: As above.

Individual Development Plan (IDP) for Postdoctoral Fellows:
Johns Hopkins University School of Medicine requires postdoctoral fellows and their mentors to fill out an annual IDP. This allows the fellow and mentor to identify long-term and short-term goals for the postdoc's research progress as well as career development. All of our mentees have completed IDP's and this has been reviewed annually with Drs. Baylin and Zahnow.

Teal Innovator's Ovarian Cancer ambassadorship activities

As over the entire course of this TEAL award, Dr. Baylin has been requested to discuss, nationally and internationally the exciting results which are outlined in the progress 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. He will continue these interactions with this society with a 3 day lecture trip to Florida in March, 2020 visiting three different cities for talks.

9. APPENDICES

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.

The emerging role of epigenetic therapeutics in immuno-oncology

*Michael J. Topper[1,2], Michelle Vaz[1,2], Kristen A. Marrone[1], Julie R. Brahmer[1] and Stephen B. Baylin[1]**

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-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 pursued[16]. 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 instability[29]; 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 chemotherapy[33]. 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

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https://doi.org/10.1038/s41571-019-0266-5

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.

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 group[38]. 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. Coley[16]. 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 activation[55]. 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 development[55].

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 types[69]. 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 burden[70]. By contrast, findings in RCC samples demonstrated a positive correlation between TMB and a T cell inflamed transcriptional signature[71].

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.

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 site[224].

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 TME[227]. The expression of PD-L1 is a feedback response to IFNγ 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 response[227]. 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 cells[230].

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 machinery[238], ineffective TIL activation owing to a lack of co-stimulatory signals or T cell exhaustion[124]. These tumours can also contain immunosuppressive cells such as regulatory T cells and myeloid-derived suppressor cells[239]. 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 strategies[242]. 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 exhaustion[119] and to shift $CD8^+$ TILs to an effector and/or memory phenotype[144].

Epigenetic mechanisms and therapeutics

Basic principles of epigenetics

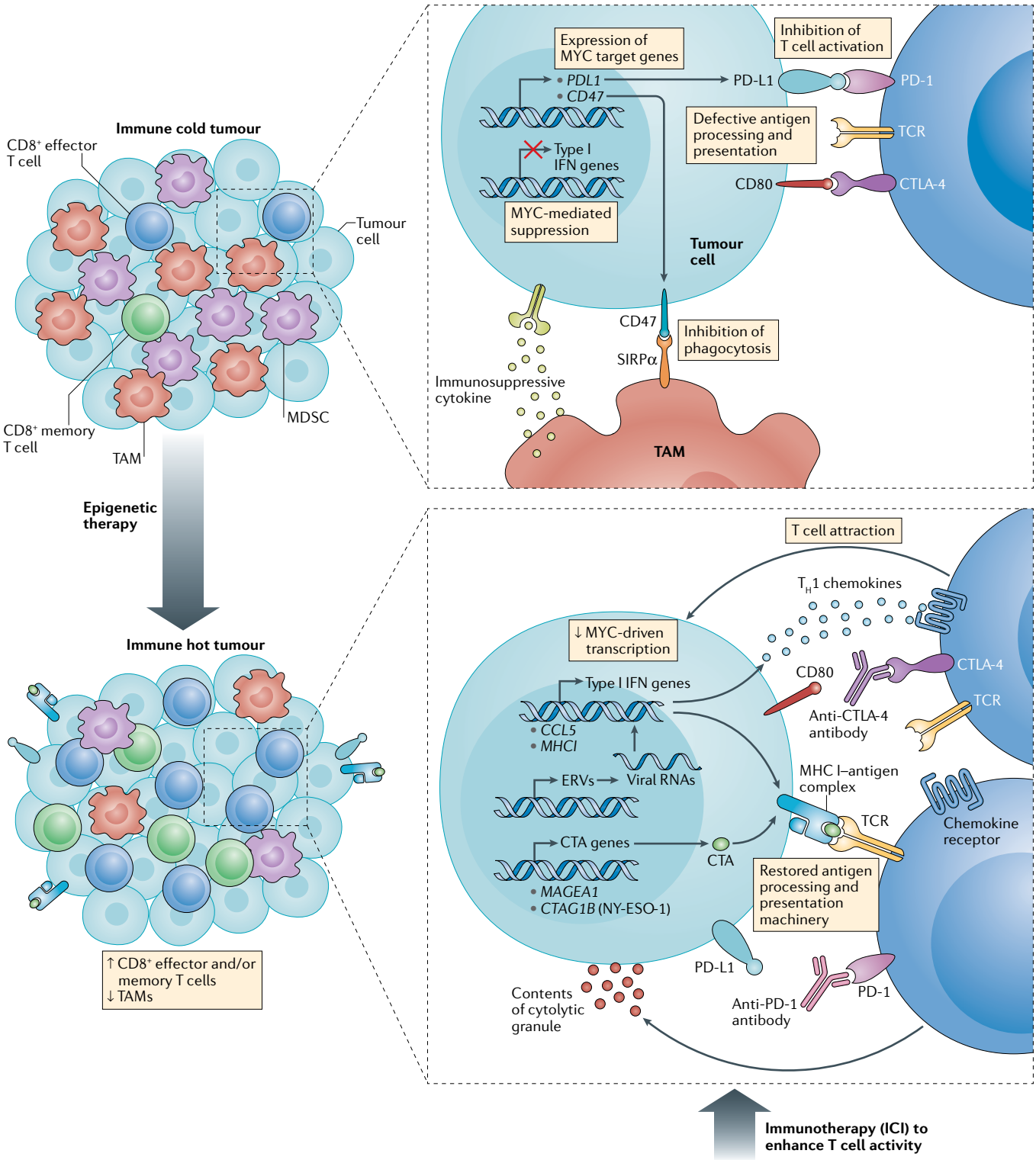
As extensively outlined in multiple reviews[72–77], epigenetics is the process by which changes mediating heritable patterns of gene expression are established without changing the sequence of DNA. Epigenetics can thus be viewed as a virtual 'software package' to control and utilize the information coded 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 expression[109]. 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

◀ 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 tumours[224], 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 microenvironment[224]. CTLA-4, cytotoxic T lymphocyte antigen 4; PD-1, programmed cell death 1; SIRPα, signal-regulatory protein α; TCR, T cell receptor; T_H1 cell, type 1 T helper cell.

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 cells[114] (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 evolve[116]. 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 functions[123].

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 IFNγ[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 cells[125]. In the context of immunotherapy, PD-1 is an important target, the expression of which is regulated by both DNA methylation[126] and alterations of chromatin accessibility[127]. 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 model[119]. 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

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 macrophages[244]. 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 presentation[245]. This interaction triggers mitogen-activated protein kinase and phospholipase C signalling downstream of the TCR, inducing nuclear factor-κB and activator protein 1 activation, and culminating in transcription and expression of IL-2 (REF.[246]). 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 populations[247]. 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* mRNA[250]. 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 survival[251]. Signal 3 results from cytokines and/or chemokines present in the microenvironment, usually derived directly from APCs[251]. This signal has a potent effect on T cell differentiation, with factors such as IL-12 and IFNα, IFNβ and IFNγ 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 phase[244]. 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].

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 macrophages[136].

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, guadecitabine[140]. 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 impeded[120]. 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 programme[119] (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 antibodies[119].

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

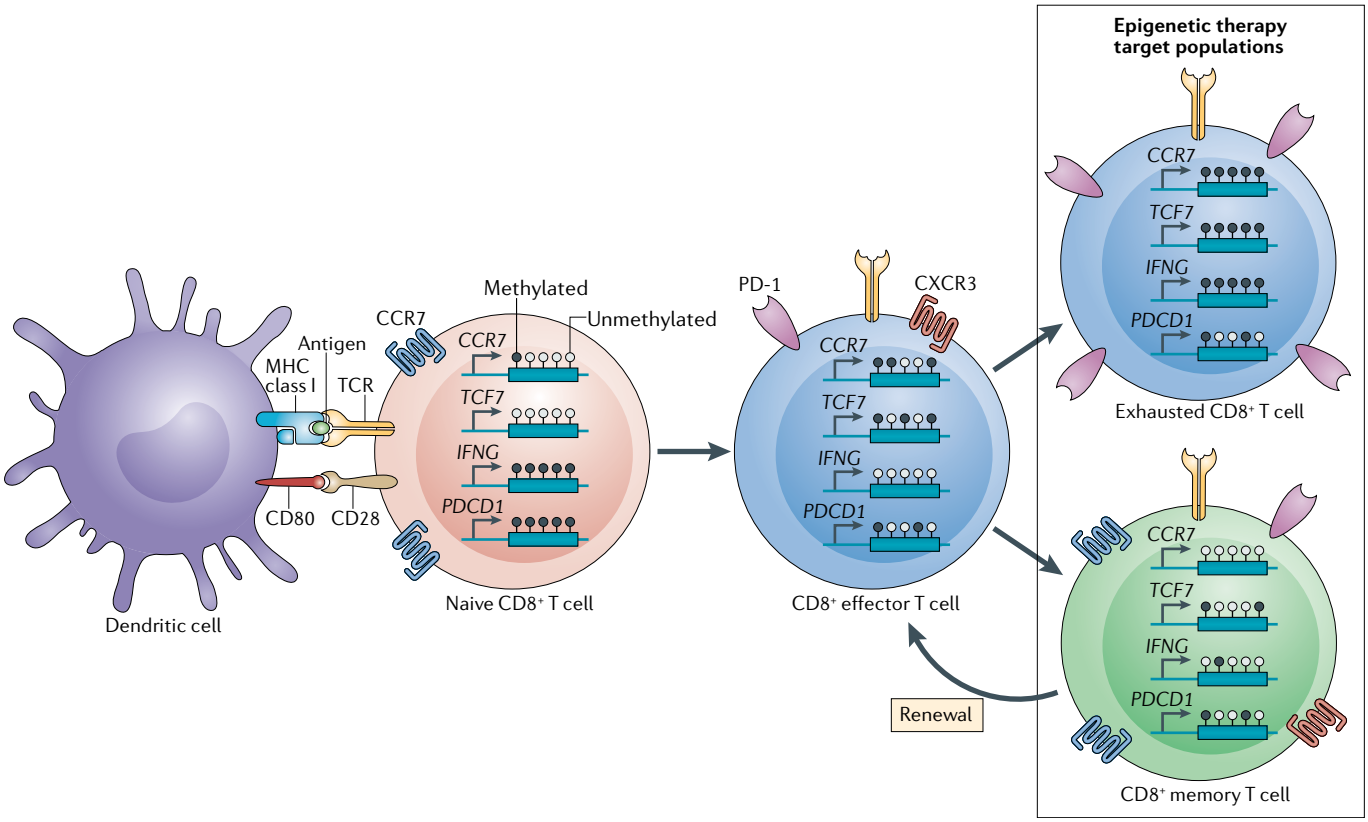


Fig. 2 | **Implications of DNA methylation-associated programmes on T cell differentiation.** T cell activation from the naive to an effector state is induced by interaction between the T cell receptor (TCR) and corresponding MHC class II–peptide complex on professional antigen-presenting cells or MHC 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 stimuli[255]. 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 process[245]. 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 CXC-chemokine receptor 3 (CXCR3) and CC-chemokine receptor 7 (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 CXC-chemokine ligand 9 (CXCL9), CXCL10 and CXCL11, which tend to emanate from sites of inflammation[256]. 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 signalling[258]. 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 antigen[259]. 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 IFNγ[119]. The post-effector fate of $CD8^+$ T cell generally involves the acquisition of either of two major phenotypes, namely exhausted or memory[260]. The exhausted state is characterized by whole-genome gains in DNA methylation, including sites in *TCF7*, *IFNG* and *CCR7* (REF.[119]). 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].

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].

Table 1 | **Summary of therapeutics targeting epigenetic modifiers**

Epigenetic agent	Stage of clinical development	Combination therapies
DNMT inhibitors[a]		
Azacitidine	FDA approved: MDS and AML	In combination with the BCL-2 antagonist venetoclax in AML
	Phase I/II: various solid carcinomas, lymphomas and/or other haematological malignancies; Phase III: haematological malignancies	HDAC inhibitors, immune-checkpoint inhibitors and/or chemotherapeutic agents
Decitabine or THU-DAC	FDA approved: MDS	NA
	Phase I/II: various solid carcinomas, lymphomas and/or other haematological malignancies; Phase III: haematological malignancies and primary neoplasia of ovary	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
HDAC inhibitors[a]		
Entinostat	FDA breakthrough drug designation: advanced-stage breast cancer	Exemestane
	Phase I/II: various solid carcinomas, lymphomas and/or other haematological malignancies; Phase III: hormone receptor-positive breast cancer	Immunotherapies
Vorinostat	FDA approved: CTCL	NA
	Phase I/II: various solid carcinomas, lymphomas and/or other haematological malignancies; Phase III: CTCL, multiple myeloma and ALL	Immunotherapies, PARP inhibitors or chemotherapeutic agents
Romidepsin	FDA approved: CTCL	NA
	Phase I/II: Hodgkin lymphoma, PTCL, multiple myeloma, NHL and/or solid carcinomas; Phase III: T cell lymphomas	Immunotherapies or chemotherapeutic agents
Panobinostat	FDA approved: multiple myeloma	Bortezomib (proteasome inhibitor) plus dexamethasone
	Phase I–III: various solid carcinomas, lymphomas and/or other haematological malignancies	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	NA
	Phase I/II: T cell leukaemia or lymphoma, MDS or AML, glioblastoma or various other solid carcinomas and haematological malignancies	Zidovudine (± IFNα-2b), pevonedistat (NEDD8-activating enzyme inhibitor) or temozolomide (chemotherapy) plus radiotherapy
HMT inhibitors		
CPI-1205 (EZH2 inhibitor)	• Phase Ib/II: mCRPC • Phase I/II: various advanced-stage solid tumours previously treated with a PD-1 or PD-L1 inhibitor • Phase I: B cell lymphoma	• Enzalutamide or abiraterone (anti-androgens) and prednisone • Ipilimumab (anti-CTLA-4 antibody) • NA
Tazemetostat[a] (EZH2 inhibitor)	• Phase II: various advanced-stage NHLs and solid tumours, including *INI1*-negative tumours, tumours with *EZH2*, *SMARCB1* or *SMARCA4* mutation, or mesothelioma with *BAP1* 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: *KMT2A*-rearranged AML	Standard chemotherapies
LSD1 inhibitors		
IMG-7289	Phase I: myelofibrosis	NA
Seclidemstat (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)

Table 1 (cont.) | **Summary of therapeutics targeting epigenetic modifiers**

Epigenetic agent	Stage of clinical development	Combination therapies
BET inhibitors		
RO6870810	• Phase I: multiple myeloma • Phase I: DLBCL or high-grade B cell lymphoma with *MYC*, *BCL2* and/or *BCL6* 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 • 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	• Fulvestrant (selective oestrogen receptor degrader) • 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.
[a]Phase 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.

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 genome[165], 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 entinostat[170] 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 NSCLC[170], 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 model[171]. 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 antibodies[172]. 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 responses[119]. 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 model[174]. In this study[174], 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 observation[174]. 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 IFNγ production by tumour-specific $CD8^+$ T cells as well as decreased numbers of splenic regulatory T (T_{reg}) cells[175]. 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 activity[177]. Furthermore, evidence suggested that HDAC inhibition might promote the differentiation of this cell population[177].

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 therapy[117]. 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 mechanisms[144] 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 outcomes[183]. Additionally, in animal models of NSCLC, the application of epigenetic therapy prevents the aforementioned exhausted phenotype in tumour-associated $CD8^+$ T cells[119], with acquisition of effector and/or memory phenotypes noted[144].

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 (REF.[144]). 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 adenocarcinoma[184]. 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 cells[184]. 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 models[185]. Notably, CD47, termed the 'do not eat me' antigen, facilitates antagonization of macrophage-dependent immune surveillance through interaction with signal-regulatory protein α (SIRPα) on macrophages. Targeting of the CD47–SIRPα axis is an emerging paradigm in immunotherapy[186].

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 above[144], 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-naive 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 NSCLC[188]. 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 interruptions[188]. 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), respectively[189]. 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 antibodies[198]. 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 models[200]. Several EZH2 inhibitors have entered clinical testing (TABLE 1). Together, these studies provide initial indications of synergy between EZH2 inhibition and immune-checkpoint inhibtion 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 marks[201]. LSD1 can also demethylate a number of nonhistone substrates, including DNMT1, and the loss of LSD1 expression is correlated with a decrease in DNMT1 levels owing to increased methylation and destabilization of this protein[202]; 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-renewal[212]. 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 lines[213]. 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 sites[214]. 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 histones[215], 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 identified[219]. 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* deletion[220]. 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 alone[220]. 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].

Published online 23 September 2019

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Acknowledgements
The work of the authors is supported by grants from The Dr Miriam and Sheldon G. Adelson Medical Research Foundation and the Defense Health Program through the Department of Defense Ovarian Cancer Research Program (Teal Innovator Award No. OC130454/W81XWH-14-1-0385). Opinions, interpretations, conclusions and recommendations presented in this manuscript are those of the author and are not necessarily endorsed by the Department of Defense. The authors also receive funding from The Hodson Trust (S.B.B.), the Commonwealth Foundation (S.B.B. and J.R.B.), the Emerson Cancer Research Award (S.B.B.), the Rising Tide Foundation for Clinical Research (S.B.B. and J.R.B.), the Stand Up To Cancer Jim Toth Sr Breakthrough Prize in Lung Cancer (S.B.B. and J.R.B.), the Van Andel Research Institute through the Van Andel Research Institute–Stand Up To Cancer Epigenetics Dream Team (to S.B.B.; Stand Up To Cancer is a program of the Entertainment Industry Foundation that is administered by AACR), and the NIH National Cancer Institute award number P30CA006973 (SKCCC Core Grant to S.B.B.). The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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. recieves grant research funding from AstraZeneca/MedImmune, BMS and Merck. K.A.M. is a consultant for AstraZeneca. All other authors declare no competing interests.

Peer review information
Nature Reviews Clinical Oncology thanks M. Maio and the other, anonymous, reviewers for their contribution to the peer review of this work.

Supplementary information
Supplementary information is available for this paper at https://doi.org/10.1038/s41571-019-0266-5.

Published OnlineFirst May 14, 2019; DOI: 10.1158/0008-5472.CAN-18-4018

Tumor Biology and Immunology

Cancer Research

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 (5AZA-C) activates type I interferon signaling to increase IFNγ^+ T cells and natural killer (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*, 5AZA-C and DFMO, either alone or in combination, significantly increased survival, decreased tumor burden, and caused recruitment of activated (IFNγ^+) CD4$^+$ T cells, CD8$^+$ T cells, and NK cells. The combination therapy had a striking increase in survival when compared with 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 5AZA-C + 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.

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.

Introduction

Although ovarian cancer has a low incidence rate of 1.3% 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 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 intratumoral CD8$^+$ T cells and high immunosuppressive cell populations such as myeloid-derived suppressor cells (MDSC) 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 patients with ovarian cancer.

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 antitumorigenic, producing proinflammatory 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 (DNMTI) and histone deacetylase inhibitors (HDACI; refs. 13–19). 5-Azacytidine (5AZA-C) is a demethylating agent, which incorporates into nucleic acids as a cytidine analog that cannot be methylated by DNA methyl transferases (DNMT). 5AZA-C is FDA approved for myelodysplastic syndrome (MDS), and low nanomolar doses lead to decreased DNA promoter methylation and restored expression of

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Cancer Res 2019;79:3445–54

doi: 10.1158/0008-5472.CAN-18-4018

Published OnlineFirst May 14, 2019; DOI: 10.1158/0008-5472.CAN-18-4018

hypermethylated genes in cancer (20). Additionally, 5AZA-C treatment induces the reexpression of hypermethylated, silenced endogenous retroviruses (ERV) *in vitro*, which can elicit an antiviral, interferon immune response that leads to T-cell activation *in vivo* (15, 18). Furthermore, 5AZA-C treatment of an ovarian cancer mouse model leads to increased immune cells in the tumor microenvironment, and combination 5AZA-C and HDACi sensitized tumors to α-PD-1 therapy (18). Although 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.

Although 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 antiviral, interferon response, whereas PBT attenuates immunosuppressive cells such as MDSCs.

We have therefore tested the hypothesis that the combination of 5AZA-C 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 5AZA-C 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. Because 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, Charleston, SC). 5AZA-C was purchased from Sigma-Aldrich (Catalog No. 320-67-2). 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- to 8-week-old) were purchased from Envigo International Holdings, Inc. 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

A total of 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 GFP. Although 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 WT. 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 5AZA-C/saline, Monday to Friday, every other week and continuous 2% DFMO in drinking water. Two hundred micrograms of α-PD-1 or IgG was injected intraperitoneally four times total on days 17, 20, 24, and 27 after intraperitoneal injection of VDID8 cells. Two hundred micrograms of α-CSF1R or IgG was injected intraperitoneally twice weekly beginning 2 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 4 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; 00-4975-93) to cause aggregation of secreted proteins inside the cell.

Flow cytometry

Cells were washed and blocked with FcR Blocking Reagent (Miltenyi Biotec; 130-092-575) and stained for cell-surface markers including Live/Dead (eBioscience; 65-0865-14), 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

Published OnlineFirst May 14, 2019; DOI: 10.1158/0008-5472.CAN-18-4018

(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 IFNγ (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; 130-092-575) and stained for cell-surface markers including Live/Dead (eBioscience; 65-0865-14), 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 FACSAria 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). Two hundred nanograms of RNA were used for cDNA synthesis using qScript cDNA SuperMix (Quanta Biosciences), followed by SYBR green-mediated real-time PCR (Universal SYBR Green Supermix; Bio-Rad) 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 (1,000 rpm) for 15 minutes, and 1,000 μL of supernatant was collected and stored at −80°C. Circulating CSF1 levels in mice treated with IgG versus 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 2 groups using the one-way ANOVA or Kruskal–Wallis test dependent upon normality results from the Shapiro–Wilk test. If only 2 sets of data were compared, either the Mann–Whitney (nonparametric) or Student 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 criterion (37). Significances are shown as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Results

Combination 5AZA-C 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, VDID8 cells were treated *in vitro* and *in vivo* and polyamine levels were determined (Supplementary Fig. S1A and S1B). *In vitro* treatment of VDID8 tumor cells led to a significant decrease in putrescine and spermidine with DFMO alone and when combined with 5AZA-C. However, 5AZA-C alone appeared to have a stimulatory effect on putrescine and spermidine synthesis (Supplementary Fig. S1A). In bulk ascites cells from treated animals, combination treatment led to a decrease in all 3 polyamines, including spermine (Supplementary Fig. S1B). No significant changes to the polyamine pools were observed with 5AZA-C treatment alone, but putrescine and spermidine were decreased (although not significantly) by DFMO treatment (Supplementary Fig. S1B).

To test the hypothesis that addition of DFMO to therapy using the DNMTi 5AZA-C would reduce tumor burden and improve overall survival in a mouse model of ovarian cancer, immunocompetent C57BL/6 mice were injected intraperitoneally with 250,000 VDID8 syngeneic MOSE cells. Mice were treated intraperitoneally with 5AZA-C (0.5 mg/kg) or saline vehicle, DFMO (2% in water), or combination 5AZA-C and DFMO beginning 3 days posttumor injection (Fig. 1A). Hemorrhagic ascites fluid consistently develops at approximately 4 to 5 weeks after VDID8 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 posttumor injection), mice treated with single-agent 5AZA-C 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 with that of single-agent 5AZA-C or DFMO of approximately 44 days (Fig. 1C). Although total numbers of lymphocytes are significantly increased by single-agent 5AZA-C or DFMO compared with vehicle, these numbers are not further enhanced with combination 5AZA-C + DFMO treatment (Fig. 1D and E).

5AZA-C and DFMO combination treatment significantly increases $IFN\gamma^+$ natural killer cells

To pursue further whether changes in lymphocyte populations might account for the dramatic increase in survival observed with 5AZA-C + DFMO combination therapy, the numbers and activity of specific lymphocyte subpopulations in hemorrhagic ascites fluid at week 5 posttumor injection were analyzed. Single-agent 5AZA-C or DFMO led to significant increases in T-cell, natural killer (NK)-cell, and $IFN\gamma^+$ lymphocyte populations examined in

Published OnlineFirst May 14, 2019; DOI: 10.1158/0008-5472.CAN-18-4018

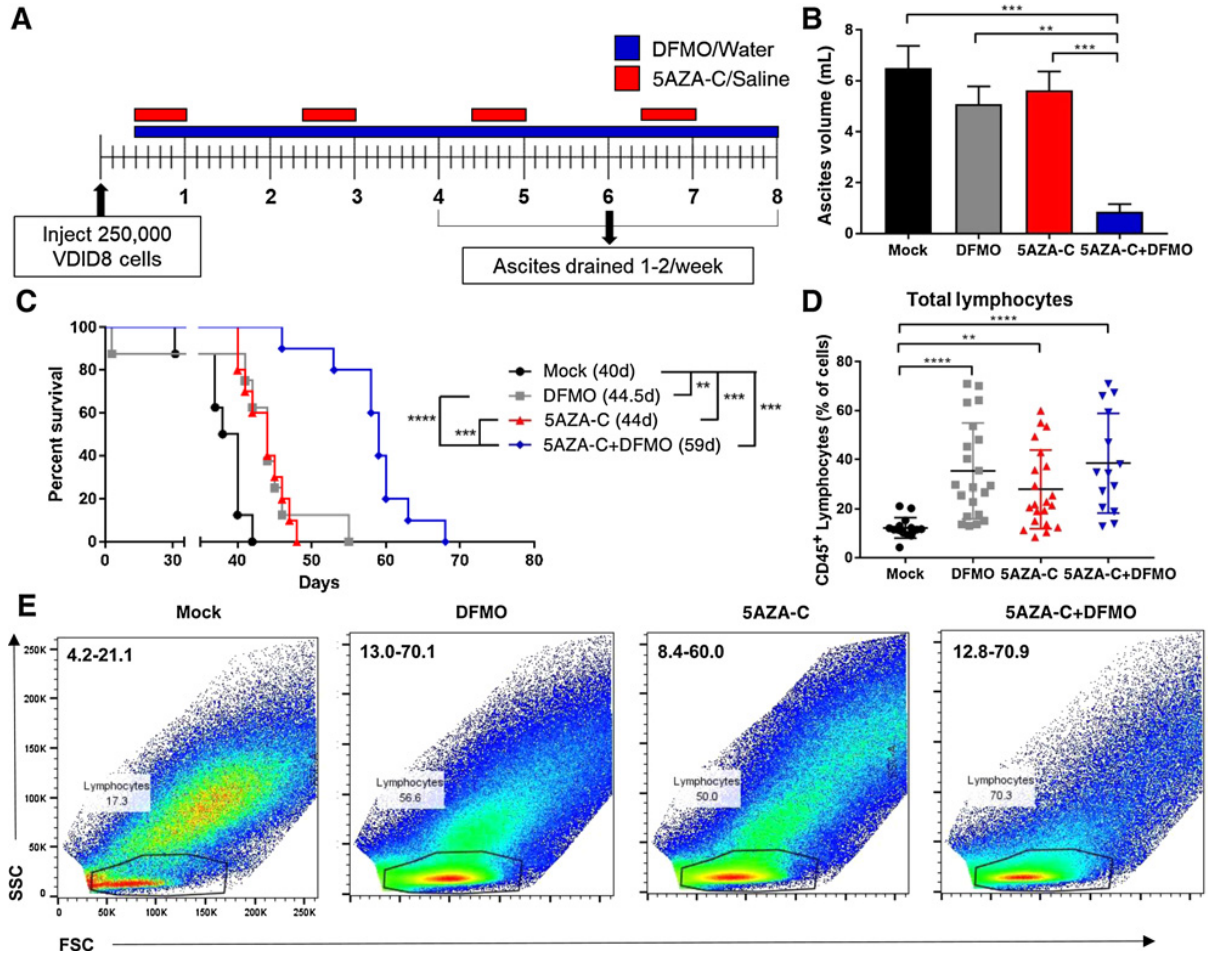

Figure 1.

Combination 5AZA-C + DFMO reduces tumor burden and increases survival in an ovarian cancer mouse model. **A,** Tumor cell injection and treatment schematic. Mice were injected intraperitoneally with 250,000 VEGF-DEFB ID8 MOSE cells (VDID8). 0.5 mg/kg of 5AZA-C was given intraperitoneally 5 days a week, every other week. Two percent of DFMO was provided in water bottles. Mice were treated throughout the duration of the experiment. Upon 25% to 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 posttumor injection. Data are from the second ascites drain procedure; the first was 4 weeks posttumor injection. Representative data (mean ± SEM shown, $n = 10$; 4 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 criterion. **C,** Representative survival curve (median survival in days; $n = 10$; 4 biological replicates). Significance determined using log-rank Mantel–Cox test. **D,** Total lymphocyte populations in bulk ascites fluid of mice at week 5 bulk; $n = 14$–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 versus FSC, demonstrating an increase in lymphocyte populations in ascites fluid at week 5 posttumor injection with 5AZA-C, DFMO, and 5AZA-C+DFMO treatment. Range of total lymphocyte population percentages are included in the top left-hand corner for each plot. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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 IFNγ$^+$ NK cells observed in combination-treated mice versus 5AZA-C or DFMO alone (Fig. 2G). It was hypothesized that the observed increase in IFNγ$^+$ 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 5AZA-C treatment did not further decrease tumor burden in the mice, nor did it increase survival (Supplementary Fig. S2A–S2F). 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 (Supplementary Fig. S2G and S2H). 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 5AZA-C and DFMO treatment led to elevated IFNγ$^+$ NK cells and modest increases in T cells, it does not appear that the differences between

Published OnlineFirst May 14, 2019; DOI: 10.1158/0008-5472.CAN-18-4018

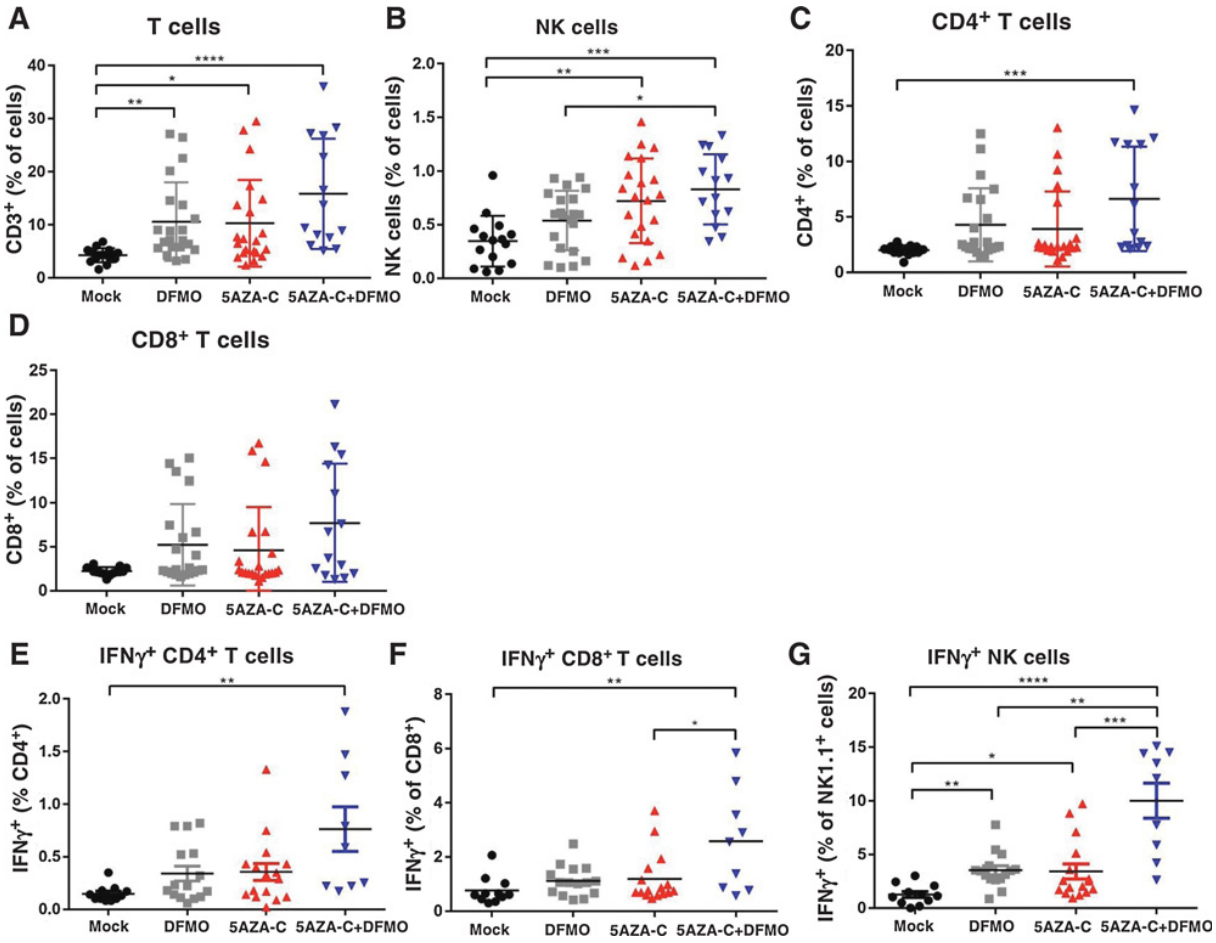

Figure 2.

Combination 5AZA-C + DFMO elevates lymphocyte populations and IFNγ$^+$ 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 demonstrates that combination treatment of 0.5 mg/kg 5AZA-C 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** and **F**) and NK cells (**G**) showed an increase in IFNγ$^+$ cells with combination treatment. IFNγ$^+$ NK cells were significantly increased with combination 5AZA-C + DFMO compared with both single agent 5AZA-C or DFMO alone. Each data point represents cells harvested from one 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**, and **E**–**G**) or Kruskal–Wallis test (**C** and **D**), dependent upon normality results from Shapiro–Wilk test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

combination treatment and single agents 5AZA-C or DFMO were significant enough to explain the dramatic increase in survival seen with combination treatment (Fig. 1C).

Combination 5AZA-C + 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 nonlymphocyte or MDSC populations was observed after treatment with 5AZA-C and DFMO (Fig. 3A and B). Instead, total macrophage populations in the tumor microenvironment were consistently decreased with 5AZA-C 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 5AZA-C, DFMO, and 5AZA-C + DFMO treatment compared with vehicle (Fig. 3D and E; Supplementary Fig. S3A and S3B). Importantly, MHC II expressing cells were increased significantly with combination treatment compared with single-agent 5AZA-C, suggesting a possible explanation for the dramatic increase in survival (Figs. 1C and 3D). 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.

Published OnlineFirst May 14, 2019; DOI: 10.1158/0008-5472.CAN-18-4018

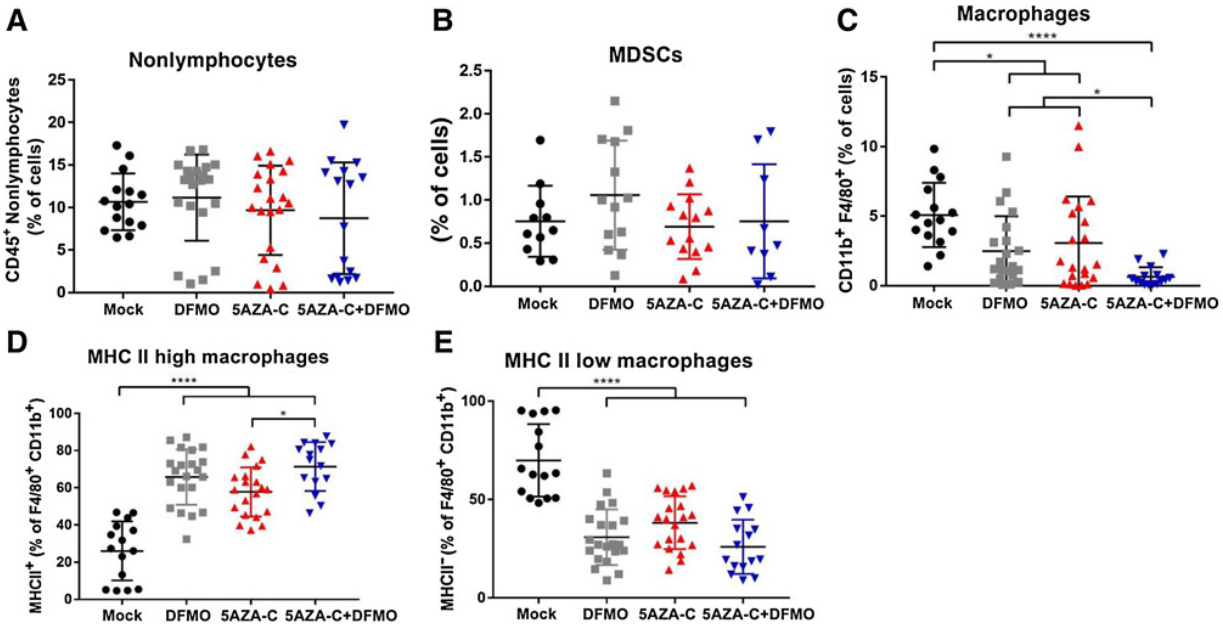

Figure 3.

5AZA-C + 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 nonlymphocytes (**A**) or MDSCs (**B**). A significant decrease in total macrophages was observed in combination-treated mice compared with vehicle, as well as a significant decrease compared with 5AZA-C alone (**C**). Further analysis of macrophage populations revealed that the MHCII low (M2-like) population was decreased in all treatment arms (**D**), whereas 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. *, $P < 0.05$; ****, $P < 0.0001$.

Combination 5AZA-C + 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; Supplementary Fig. 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 with CD206$^-$ macrophages (Fig. 4C and D), and MHC II$^+$ macrophages had increased expression of iNOS2 compared with 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 5AZA-C + 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 (Supplementary Fig. S5A and S5B). Macrophages in vehicle-treated mice were therefore assessed at 3 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 5AZA-C + DFMO response in the ovarian cancer mouse model

To test whether the increase in M1 macrophages was important in the response to 5AZA-C and DFMO treatment, macrophages were blocked in the ovarian cancer mouse model using an antibody to CSF1R (Fig. 5A; ref. 45). 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 5AZA-C + 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 5AZA-C + DFMO mice receiving α-CSF1R (Fig. 5D and E). This decrease in macrophages also led to a decrease in overall survival, compared with 5AZA-C + DFMO mice that received IgG control (Fig. 5F). Analysis via flow cytometry of M1 and M2 surface markers showed that with IgG control, 5AZA-C + DFMO mice had increased M1 macrophages and decreased M2 macrophages compared with vehicle, as was previously seen (Figs. 5G and H and 4A

Published OnlineFirst May 14, 2019; DOI: 10.1158/0008-5472.CAN-18-4018

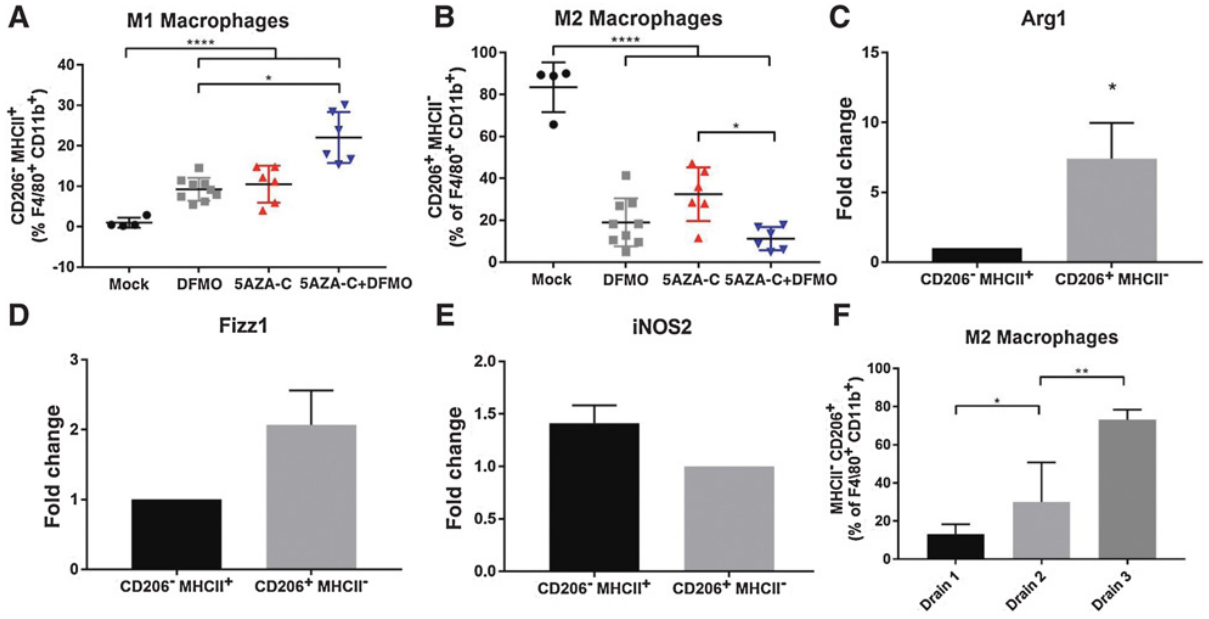

Figure 4.

5AZA-C + DFMO treatment reduces M2 polarization and increases M1 polarized macrophages in the tumor microenvironment. **A,** Percentage of M1 macrophages (MHC II$^+$ CD206$^-$) were increased with DFMO and 5AZA-C treatment, and further increased with combination 5AZA-C + DFMO treatment. **B,** Percentage of M2 macrophages (MHC II$^-$ CD206$^+$) was reduced in all treatment arms, with the greatest reduction observed in combination 5AZA-C + DFMO treatment. Macrophages were sorted from bulk ascites fluid collected from mice at week 5 posttumor 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 confirm 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 posttumor 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** and **B**) or *t* test (**C–E**). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

and B). Interestingly, although 5AZA-C + 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 with 5AZA-C + DFMO mice receiving IgG control (Fig. 5G and H). These results indicate that the presence of M1 macrophages is important for the mechanism of action of this combination drug therapy, as 5AZA-C + DFMO-treated mice receiving α-CSF1R had decreased survival and increased tumor burden compared with 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 5AZA-C alone led to an increase in IFNγ$^+$ NK cells, CD4$^+$ T cells, and CD8$^+$ T cells, as has been demonstrated before (14, 18, 19). Signaling of IFNγ via its receptor IFNGR1 on tumor cells can lead to increased expression of PD-L1 on tumor cells, thereby making this increase in IFNγ 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 5AZA-C and DFMO. These results are in contrast to previous studies using 5AZA-C and HDACi where the addition of α-PD-1 produced a significant therapeutic response (18). Histone acetylation is essential for transcription of IFNγ, therefore the use of an HDACi may explain the sensitization to α-PD-1 therapy previously seen, as increasing histone acetylation even further increased IFNγ levels in lymphocytes (47).

Analysis of the tumor microenvironment after the combination treatment with 5AZA-C and DFMO indicated that the impacts on macrophage polarization are critically important in this model. 5AZA-C treatment has been shown to decrease macrophages in the tumor microenvironment, although 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 IFNγ and IL12, 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). In addition, 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

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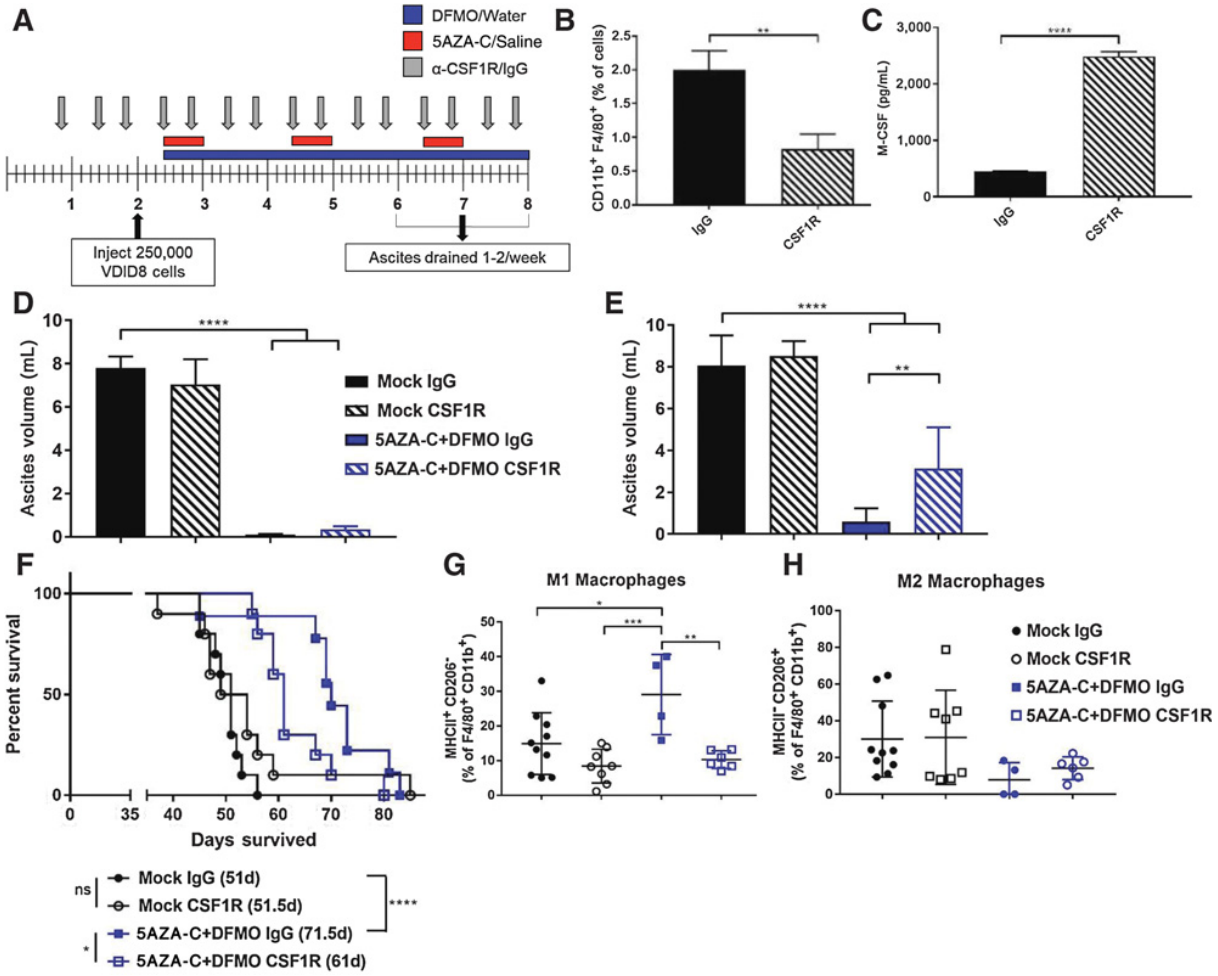

Figure 5.

Increased M1 macrophages are essential to the efficacy of combined 5AZA-C + DFMO treatment in an ovarian cancer mouse model. **A,** Treatment schematic for dosing with macrophage block antibody α-CSF1R. All mice were 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 M-CSF levels demonstrating an increase in circulating M-CSF in the presence of receptor block CSF1R. **D,** Tumor burden represented by ascites volume in mice treated with 5AZA-C + 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 5AZA-C + DFMO mice receiving CSF1R. **F,** Survival curve of 5AZA-C + DFMO–treated mice receiving CSF1R antibody. Mice with decreased macrophages due to the antibody demonstrated a decrease in survival compared with 5AZA-C + DFMO mice receiving IgG. **G,** M1 macrophages (MHC II$^+$ CD206$^-$) analyzed via flow cytometry. 5AZA-C + DFMO–treated mice receiving CSF1R showed no increase in M1 macrophages. **H,** M2 macrophages (MHC II$^-$ CD206$^+$) analyzed via flow cytometry. M2 macrophages were reduced in both 5AZA-C + DFMO treatment arms, compared with 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** and **C**) or one-way ANOVA (**D, E, G,** and **H**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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, whereas 5AZA-C may help increase M1 macrophages via its interferon response and production of IFNγ, a cytokine that drives M1 polarization (15, 18, 19, 49).

Depletion of macrophages in the tumor microenvironment using a CSF1R antibody significantly diminished the efficacy of combination 5AZA-C 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 5AZA-C 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.

Published OnlineFirst May 14, 2019; DOI: 10.1158/0008-5472.CAN-18-4018

Disclosure of Potential Conflicts of Interest

K.E. Bachman has ownership interest (including stock, patents, etc.) in Johnson & Johnson. C.A. Zahnow reports receiving Commercial Research Grant from Janssen Pharmaceuticals Inc. No conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Travers, S.M. Brown, M. Dunworth, C.E. Holbert, K.R. Wiehagen, J.R. Foley, M.L. Stone
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Travers, S.M. Brown, M. Dunworth, K.R. Wiehagen, R.A. Casero, Jr., C.A. Zahnow
Writing, review, and/or revision of the manuscript: M. Travers, C.E. Holbert, K.E. Bachman, S.B. Baylin, R.A. Casero, Jr., C.A. Zahnow
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Travers, S.M. Brown, K.R. Wiehagen
Study supervision: K.E. Bachman, R.A. Casero, Jr., C.A. Zahnow

Acknowledgments

This work was supported by the NCI under award number R01CA204345 (to R.A. Casero, Jr.) and P30CA006973 (SKCCC Core Grant). The content is solely the responsibility of the authors and does not represent official views of the NIH. This work was also supported in part by Janssen, the SWCRF Collaboration for a Cure Grant, the Irving A. Hansen Memorial Foundation, and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation.

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Received December 20, 2018; revised March 25, 2019; accepted May 7, 2019; published first May 14, 2019.

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DFMO and 5-Azacytidine Increase M1 Macrophages in the Tumor Microenvironment of Murine Ovarian Cancer

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Cancer Res 2019;79:3445-3454. Published OnlineFirst May 14, 2019.