Epigenetic Control of Cdkn2a.Arf Protects Tumor-Infiltrating Lymphocytes from Metabolic Exhaustion

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Abstract

T-cell exhaustion in cancer is linked to poor clinical outcomes, where evidence suggests T-cell metabolic changes precede functional exhaustion. Direct competition between tumor-infiltrating lymphocytes (TIL) and cancer cells for metabolic resources often renders T cells dysfunctional. Environmental stress produces epigenome remodeling events within TIL resulting from loss of the histone methyltransferase EZH2. Here, we report an epigenetic mechanism contributing to the development of metabolic exhaustion in TIL. A multiomics approach revealed a Cdkn2a.Arf-mediated, p53-independent mechanism by which EZH2 inhibition leads to mitochondrial dysfunction and the resultant exhaustion. Reprogramming T cells to express a gain-of-function EZH2 mutant resulted in an enhanced ability of T cells to inhibit tumor growth in vitro and in vivo. Our data suggest that manipulation of T-cell EZH2 within the context of cellular therapies may yield lymphocytes that are able to withstand harsh tumor metabolic environments and collateral pharmacologic insults.

Significance: These findings demonstrate that manipulation of T-cell EZH2 in cellular therapies may yield cellular products able to withstand solid tumor metabolic-deficient environments.

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Introduction

The ability of the immune system to sustain T-cell function in a wide range of environments provides the immunosurveillance necessary to eliminate genetically damaged cells, protecting the host from developing overt malignancies. Cancer cells that escape the initial immune response can form solid tumors, which undergo further immunoediting, driving the generation of an immunosuppressive microenvironment, resulting in restricted T-cell infiltration and effector function (1). Features of the immune-hostile tumor environment include direct competition between tumor-infiltrating lymphocytes and cancer cells for metabolic resources often renders T cells dysfunctional. Environmental stress produces epigenome remodeling events within TIL resulting from loss of the histone methyltransferase EZH2. Here, we report an epigenetic mechanism contributing to the development of metabolic exhaustion in TIL. A multiomics approach revealed a Cdkn2a.Arf-mediated, p53-independent mechanism by which EZH2 inhibition leads to mitochondrial dysfunction and the resultant exhaustion. Reprogramming T cells to express a gain-of-function EZH2 mutant resulted in an enhanced ability of T cells to inhibit tumor growth in vitro and in vivo. Our data suggest that manipulation of T-cell EZH2 within the context of cellular therapies may yield lymphocytes that are able to withstand harsh tumor metabolic environments and collateral pharmacologic insults.

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by tumor-infiltrating lymphocytes (TIL) such as glucose deprivation and mitochondrial dysfunction (5, 6). Recent discoveries have highlighted various metabolic pathways as key intrinsic mechanisms through which T cells regulate their fate (7, 8). Nutrient availability is a primary driver of metabolic programming and is a necessary signal for maintenance of self-tolerance and protecting the host from tissue damage (9, 10). Loss of T-cell metabolic regulatory signals can be illustrated by the strong correlation between metabolic syndrome (obesity, hyperglycemia, dyslipidemia, and hypertension) and autoimmune diseases (11). T cells, like cancer cells, primarily utilize aerobic glycolysis for their energy needs. However, mitochondria are critical organelles for maintaining the integrity of effector T cells and the formation of memory T cells (12). TILs show a progressive loss of mitochondrial function and a reduction in glucose uptake (metabolic exhaustion), which is largely independent of checkpoint blockade or regulatory cell suppression (13).

Recent studies have begun to highlight the metabolic underpinnings of T-cell function and raised the possibility of metabolic manipulation aimed at vastly improving cancer immunotherapy (14, 15). The dynamic interplay between epigenetics and metabolic pathways has been revealed as primary mechanisms cells use to maintain the integrity of effector T cells and the formation of memory T cells (12). TILs show a progressive loss of mitochondrial function and a reduction in glucose uptake (metabolic exhaustion), which is largely independent of checkpoint blockade or regulatory cell suppression (13).

Tumor challenge
Mice were injected in the subcutaneous flank space with $1 \times 10^6$ tumor cells suspended in 100 μl of PBS. Tumor growth was monitored daily with caliper measurements of tumor length and width. Survival was plotted on Kaplan–Meier curves, the number of days after tumor engraftment where the tumor was less than 1,000 mm$^3$ (unless noted differently), with no ulceration. Tumor challenge studies were performed in all cases at least twice. For in vivo EZH2i, starting on day 5, mice were injected (orally) with vehicle (0.5% w/v methyl cellulose and 0.1% Tween-80) or 250 mg/kg tazemetostat (EPZ6438) twice daily for 5 consecutive days.

Primary cell culture
Naïve lymphocytes (CD8$^+$, CD4$^+$, or CD4$^+$CD25$^+$) were isolated from single-cell suspension of murine splenocytes using magnetic selection (Miltenyi). Purified lymphocytes were then activated using 5 μg/ml of plate-bound antiCD3e (Biolegend), 2 μg/ml soluble CD28 (Biolegend) and 50 U/ml IL2 (Peprotech) for indicated times. Lymphocytes were cultured in RPMI (Life Technologies) with 10% PBS, 55 μmol/L 2-mercaptoethanol, 2 mmol/L glutamine, penicillin, and streptomycin at 37°C and 5% CO$_2$.

Cell lines
The B16F10 mouse cell line was purchased from the ATCC. The B16$^{C57}$ cell line was a kind gift from Thomas F. Gajewski (University of Chicago). Tumor cell lines were cultured in DMEM supplemented with 10% PBS, penicillin, and streptomycin at 37°C and 5% CO$_2$. All tumor cell lines were screened for contaminating pathogens (Ectromelia, EDIM, LCMV, MAV1, MAV2, MHV, MPV, MVM, Mycoplasma pulmonis, Polyoma, PVM, REO3, Sendai, TMEV) by IDEXX laboratories and do not contain any pathogens or Mycoplasma.

Generation of MC38SINFEKL
MC38 cells were engineered to express DsRed fused in frame with three repeated sequences encoding the model antigen SINFEKL followed by an AAY linker. The construct was generated by digesting a gBlock (IDT) encoding 3X-SINFEKL-AAY with flanking XhoI/BamHI cut sites. Note that 100 ng of the gBlock and 1 μg of pRetro-dsRed-monomer-N1 (Takara: 632465) were digested with XhoI and BamHI and gel purified (Qiagen). Purified digested gBlock and pRetro vectors were ligated together and transformed into Stbl3 (Fisher) cells. The insert region of the pRetro-SINFEKL-dsRed vector was sequenced to confirm that the insert was in-frame with dsRed without intervening stop codons. To generate the MC38.SINFEKL-dsRed cell line, Phoenix (ATCC) cells were transfected with the pRetro-SINFEKL-dsRed vector using Lipofectamine 3000 (Fisher) following the manufacturer’s protocol. Forty-eight hours after transfection, supernatant was collected, filtered through a 0.45 μm filter, and added to tumor cells with polybrene at 10 μg/ml. After expansion, cells were sorted based on dsRed expression. The cells were sorted 3 more times for the top 5% dsRed positive to ensure no dsRed cells remained.

IFNy ELISPOT assay
Mice were injected in the subcutaneous flank space with $1 \times 10^6$ tumor cells suspended in 100 μl of PBS. Spleens were harvested 7 days after injection for analysis. The enzyme-linked Immunospot assay (ELISPOT) was conducted with the BD mouse IFNy kit according to the manufacturer’s protocol. Splenocytes were plated at $10^6$ cells/well
and stimulated overnight with SIINFEKL peptide (160 nmol/L), or PMA (50 ng/mL) and ionomycin (0.5 μmol/L). IFNγ spots were detected using biotinylated antibody and avidin-peroxidase and developed using AEC substrate (Sigma-Aldrich).

**In vitro killing assay**

T-cell killing assays were carried out using preactivated CD8+ T cells from wt (control) or OT-1 (tumor-specific) mice. Activated T cells were washed and labeled with Cell Trace violet to enable their subsequent discrimination from target cells. Target cells (MC38SYNFEKL) were plated for 16 hours prior to culturing with T cells. The cocultures were conducted at different target:effector (T:E) ratios for 10 hours. Target cell viability was then determined by the percentage of Annexin V and propidium iodide (PI) cells.

**Adaptive transfers**

For in vivo survival experiments, 2 × 10^6 activated CD8+ T cells/mouse were injected i.v. into C57BL/6 mice. Cells were recovered 2 days later from the peripheral blood, spleen, and lymph nodes and then analyzed by flow cytometry. For adoptive cellular treatment experiments, 4 × 10^6 CD8+ OT-1+ or CD8+ OT-1- Lck-EZH2+ preactivated T cells/mouse were injected i.v. into tumor-bearing (MC38SYNFEKL) mice and measured for tumor volume growth.

**Western blotting and quantitative PCR**

For immunoblot analysis, harvested cells were lysed on ice for 30 minutes with RIPA (10 mmol/L Tris-Cl (pH 8.0), 1 mmol/L EDTA, 0.5 mmol/L EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mmol/L NaCl). Lysates were then cleared by centrifugation, and protein concentrations were determined by bicinchoninic acid (BCA) assay. For QPCR, RNA was extracted from activated lymphocytes using an RNeasy purification kit (Qiagen), and complementary DNA was synthesized (Bio-Rad). Target Primer sets below were used with SybrGreen (Bio-Rad) to measure relative transcript levels. Ubiquitin was used as a house keeping gene. See Supplementary Methods for the information of antibodies used.

For in vivo survival experiments, 2 × 10^6 T cells per well (≥28 wells per sample) were spun onto Cell-Tak (Corning)–coated seahorse 96-well plates and preincubated at 37°C for approximately 20 minutes in the absence of CO2. OCR and ECAR were measured in XF media (nonbuffered RPMI 1640 containing 10 mmol/L glucose, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate) under basal conditions and in response to 2 μmol/L oligomycin, 2 μmol/L fluoro-carbonyl cyanide phenylhydrazone (FCCP), 10 mmol/L 2-Deoxy-D-glucose, and 500 nmol/L rotenone + 500 nmol/L antimycin A.

**Metabolic phenotyping**

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using the Seahorse XFe bioanalyzer. Note that 2 × 10^5 T cells per well (≥28 wells per sample) were spun onto Cell-Tak (Corning)–coated seahorse 96-well plates and preincubated at 37°C for approximately 20 minutes in the absence of CO2. OCR and ECAR were measured in XF media (nonbuffered RPMI 1640 containing 10 mmol/L glucose, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate) under basal conditions and in response to 2 μmol/L oligomycin, 2 μmol/L fluoro-carbonyl cyanide phenylhydrazone (FCCP), 10 mmol/L 2-Deoxy-D-glucose, and 500 nmol/L rotenone + 500 nmol/L antimycin A.

**ATP measurements**

Relative ATP levels were determined using Cell titer Glo (Promega) according to the manufacturer’s protocol. Note that 1 × 10^6 activated T cells were used per 96 well to determine relative luminescence unit for each condition.

**Cell death experiments**

Activated CD8+ lymphocytes were plated in fresh media at 0.5 × 10^6 cells/mL in 96-well plates. Cells were treated with glucose or glutamine withdrawal, H2O2, IFNγ, 2-DeoxyGlucose (Sigma), Staurosporine (Seleckchem), or vehicle control at indicated doses for 24 hours. Cell viability was determined by staining with Annexin V–FITC and DAPI staining. For withdrawal of glucose and glutamine, cells were cultured in dialyzed FBS.

**Histone mass spectrometry and post-translational modification identification**

Histone mass spectrometry was carried out as described previously (24). Histones were purified by acid extraction and resolved on a 4%–12% gradient SDS-PAGE gel. Histone bands were visualized by Coomassie staining. Histones were excised from the gel, destained, and treated with 20 μL/band of 30% D6-acetic anhydride in 50 mmol/L ammonium bicarbonate, which chemically adds isotopically heavy acetylation on unmodified and monomethylated lysines. Histones were then digested in-gel with 125 ng/band sequencing-grade trypsin at 37°C for overnight. Acidified tryptic peptides were separated using a 2.5 μm Waters XSelect CSH resin on a 150 mm X 0.075 mm column using a nanoAcquity UPLC system (Waters). Peptides were separated with a 60-minute chromatography gradient, with a 40-minute linear
separation gradient from 97% buffer A [0.1% formic acid in water (v/v)], 3% buffer B [0.1% formic acid (v/v), 99.9% acetonitrile (v/v)], to 80% of buffer A, 20% buffer B. Eluted peptides were ionized by electrospray (2,150 V) and analyzed on an Orbitrap Fusion Lumos mass spectrometer (Thermo) using data-dependent acquisition. Spectral count data were exported in tabular format and analyzed using R. Additional details regarding histone mass spectrometry and post-translational modification (PTM) identification are available in the Supplementary Materials.

**FASP bHPLC TMT mass spectrometry**

Purified proteins were reduced, alkylated, and digested using filter-aided sample preparation (25). Tryptic peptides were separated into 36 fractions on a 100 × 1.0 mm Acquity BEH C18 column (Waters) using an UltiMate 3000 UHPLC system (Thermo) with a 40-minute gradient from 99:1 to 60:40 buffer A:B ratio under basic pH conditions, and then consolidated into 12 superfractions. Each superfraction was further separated by reverse phase Jupiter Proteo resin (Phenomenex) on an in-line 200 × 0.075 mm column using a nanoAcquity UPLC system (Waters). Peptides were eluted using a 60-minute gradient from 97:3 to 67:33 buffer A:B ratio. Eluted peptides were ionized by electrospray (2.15 kV) followed by mass spectrometric analysis on an Orbitrap Fusion Tribrid mass spectrometer (Thermo) using multi-notch MS3 parameters. Scaffold Q+ (Proteome Software) was used to verify MS/MS-based peptide and protein identifications (protein identifications were accepted if they could be established with less than 1.0% false discovery and contained at least 2 identified peptides; protein probabilities were assigned by the Protein Prophet algorithm and to perform reporter ion-based statistical analysis; ref. 26). Additional details regarding TMT mass spectrometry are available in the Supplementary Materials.

**RNA sequencing**

RNA sequencing (RNA-seq) was carried out as before (27). For total RNA-seq, the Sequence library was prepared from 500 ng of total RNA using Illumina’s TrueSeq RNA Sample Preparation Kit v2 following the manufacturer’s protocol. CDNA Libraries were validated on the Arkansas Children’s Research Institute Genomics core Fragment Analyzer for fragment size peak of approximately 260 bp and were quantified by a Qubit fluorometer. Equal amounts of each library were pooled for sequencing on the NextSeq 500 platform using a high output flow cell to generate approximately 25 million 75-base reads per sample. cDNA libraries were constructed using Illumina’s TrueSeq stranded mRNA sample preparation kit according to the manufacturer’s protocol. All sequencing was conducted by the Center for Translational Pediatric Research Genomics Core Lab at Arkansas Children’s Research Institute (Little Rock, AR). For each comparison, edgeR’s tests relative to a threshold (glmTreat(), lfc=-1) method correcting for batch effects was used to identify differentially expressed genes between experimental groups. Genes with a fold change (FC) > 2 and multiple test corrected (FDR) P values < 0.05 were selected for further comparisons between treatments and analyzed by Ingenuity Pathway Analysis (IPA) for biological involvement. Additional details regarding RNA-seq analysis are available in the Supplementary Materials.

**ChIP and ChIP-seq**

Chromatin immunoprecipitation (ChIP)-qPCR and ChIP-sequencing (ChIP-seq) were carried out as before (28). For H3K27me3 and H3K36me3 ChIP-seq, 40 million of cells were cross-linked with 1% formaldehyde for 10 minutes, followed by addition of glycine to stop cross-linking. After washing, cell lysis, and sonication, the chromatin samples were incubated with antibody-conjugated Dynabeads (Invitrogen) at 4°C. Beads bound with chromatin were then subject to extensive washing and elution. Eluted chromatin was de–cross-linked overnight at 65°C, followed by protein digestion with proteinase K and DNA purification with Qiagen PCR purification kit. The obtained ChIP DNA samples were submitted to the UNC-Chapel Hill High-Throughput Sequencing Facility for preparation of multiplexed libraries and deep sequencing with an Illumina High-Seq platform according to the manufacturer’s instructions. Tag counts between DMSO controls and EZH2i-treated samples were then determined across the entire mouse genome using Bedtools multicov and log2 fold change and percent change (i.e., decrease or increase) was calculated (29). To compare DMSO controls with EZH2i-treated samples, Deeptools bamCoverage tool was used to calculate tag coverage for the following mouse genes: cdkn2a, Igf2bp3, Dab2ip, Gzma, Upp1, Tgm2, Fbxo2, and Kit (30). Additional details regarding ChIP sequencing analysis are available in the Supplementary Materials.

**Quantification and statistical analysis**

Please refer to the appropriate methods section for proteomics and sequencing data set statistical methods. Otherwise, comparisons for two groups were calculated using unpaired two-tailed Student t tests, and comparisons for more than two groups were calculated using one-way ANOVA followed by Bonferroni multiple comparison tests. Comparisons over time were calculated using two-way ANOVA followed by Bonferroni multiple comparison tests. For Kaplan–Meier plots of survival, log-rank test was used to determine P values. Data were analyzed using GraphPad Prism 7, R Studio, and Microsoft Excel.

**Results**

**EZH2 inhibition induces T-cell exhaustion and dysfunction**

To establish the repression of EZH2 (H3K27me3) during tumor infiltration, we purified TILs from an immune suppressive murine model of melanoma (B16F10; ref. 31). TILs from B16F10 tumors have been demonstrated to be exhausted, and here, we show infiltrating CD4+ and CD8+ T cells displayed a reduction in H3K27me3 compared with lymphocytes from tumor-draining lymph nodes and in vitro–activated T cells (Fig. 1A). To model acute inhibition of EZH2 in activated CD8+ T cells, we used highly specific and effective small-molecule inhibitors (EZH2i; Fig. 1B). We believe this approach, in contrast to genetic deletion, more accurately models the loss of T-cell EZH2 activity, which occurs within the tumor environment. Further, this approach also reveals potential unintended consequences affecting T-cell function that could occur with systemic EZH2i anticancer therapies. In vitro inhibition of EZH2 in primary CD8+ T cells leads to a minor effect on their in vitro proliferative capacity (Fig. 1C).

RNA-seq and proteomic analyses were performed on in vitro–activated cytotoxic T cells (CD8+) treated after activation (48 hours) with EZH2i for 48 hours as in Fig. 1B. RNA-seq and proteomic datasets revealed an exhausted phenotype induced by the in vitro inhibition of EZH2 (Fig. 1D). A significant increase in inhibitory receptors (Pdcd1, Lag3, Tigit) and loss of memory markers (Cxcr3, Tox, and Tox2) were also elevated in EZH2i-treated CD8+ T cells (Fig. 1E). In an in vitro setting, EZH2i had no effect on the ability of OT-1+ T cells to kill MC38 SINGFEK tumor cells. Target tumor cells were engineered to express the ovalbumin antigen SINGFEKL and were
Figure 1.
Systems approach uncovers drivers of T-cell exhaustion resulting from EZH2 inhibition. A, Western blot analysis of TDLN and B16F10 melanoma TIL populations. FACS was used to purify CD4+ and CD8+ lymphocyte populations and in vitro-activated (CD3ε/CD28) CD8+ T cells were used as a positive control. B, Western blot analysis of in vitro preactivated, primary CD8+ T cells treated with EZH2i (EPZ6438, 2.5 μmol/L) for 24 or 48 hours. In all experiments, CD8+ T cells were purified prior to activation. C, The proliferation of preactivated CD8+ T cells treated with EZH2i was determined using trypan blue exclusion (n = 5). Error bars, SEM. D, Subset of genes involved in T-cell effector function and exhaustion from RNA-seq and proteomic analysis of EZH2i-treated preactivated CD8+ T cells. The RNA-seq heatmap was generated using logCPM values (Z-score), and asterisks signify an adjusted P value < 0.05 (Holm–Sidak). The protein heatmap was generated from normalized tandem mass tag intensities (Z-score), and asterisks signify an adjusted P value < 0.05 (Holm–Sidak). E, Subset of exhaustion associated transcription factors from RNA-seq dataset. F, C57BL/6 (immune competent) or Rag1−/− (immune compromised) mice were injected subcutaneously with 1 × 10^6 B16SIY cells. Starting on day 5, mice were injected (orally) with vehicle (0.5% w/v methyl cellulose and 0.1% Tween-80) or 250 mg/kg EPZ6438 twice daily for 5 consecutive days. Tumor growth curves depict an average tumor volume in each group (n = 5–6). Error bars, SEM. Kaplan–Meier survival of recipient mice (tumor size > 500 mm^3). P value denotes statistical significance by log-rank test. G, Significantly altered gene lists from IPA of proteomic data. H, Venn diagram comparing significant genes identified in RNA-seq and proteomics data sets.
validated using Elispot (Supplementary Fig. S1A and S1B). This suggests that EZH2i does not simply lead to a decrease in the strength of activation.

In vivo, EZH2 inhibition blocks immune control of the highly immunogenic murine model of melanoma, B16F10SY (Fig. 1F). B16F10SY cells are engineered to express the model antigen, SIY (SIYRYYGL), and have a well-documented growth delay in immune-competent recipient mice due to strong immune detection (32). B16F10 cells are resistant to EZH2 inhibition and provide an opportunity to assess the effects of EZH2 inhibitors (EZH2i) on T-cell antitumor activity (33). Treatment with EZH2i increased the growth rate of B16F10SY tumors in vivo and decreased survival in WT mice. This was not observed in the immune-compromised, RAG1−/− mice, suggesting a role in the repression of TILs. However, this does not inform us on the extent or the capacity in which T cells require EZH2 for their function.

**Multomics approach uncovers mitochondrial dysfunction as a driver of EZH2 inhibition-induced T-cell exhaustion**

In addition to the functional markers above, we quantified the relative abundance of 11,006 protein coding transcripts and 9,301 proteins. EZH2i treated treatment resulted in 62 transcripts differentially expressed (Log2FC > 2.5, P value < 0.05; Supplementary Fig. S2A) and 34 protein levels differentially expressed (Log2FC > 1.25, P value < 0.05; Supplementary Fig. S2B). As expected, inhibition of the repressive PRC2 complex generated mostly increases in transcript and protein levels, visualized through volcano plots (Supplementary Fig. S2C and S2D). To prioritize upstream candidate genes and pathways contributing to the exhaustion phenotype, we used two approaches. First, IPA of differentially expressed proteins predicted pathways most significant cell pathways in EZH2i-treated T cells largely correspond to mitochondrial dysfunction (Fig. 1G). The genes that define this gene set include: Gzma, Mapk12, Tgms1, mt-Atp6, Cdkn2a, and Cox6d1. Interestingly, elevation of Tec kinase signaling was also identified in the analysis and is known for its role in TCR signaling leading to IL2 induction, which can be a sign of overstimulation and exhaustion (34). Our second approach to prioritize candidates was based on the understanding that EZH2i primarily regulates genes at the transcript level, and therefore most upstream candidates are likely different at both the transcript and protein levels. We regarded the genes found in the union of this multomics approach as possible drivers of exhaustion in EZH2i-treated T cells (Fig. 1H). Taken together, these data suggest EZH2i-driven T-cell exhaustion is, at least in part, due to mitochondrial dysfunction.

**Loss of EZH2 function leads to metabolic exhaustion in CD8+ T cells**

T-cell activation leads to rapid and robust metabolic changes essential to support proliferation and function (35). To further understand possible metabolic changes in response to EZH2 inhibition, we measured OCR and ECAR. Metabolic flux analysis of CD8+ T cells treated with EZH2i revealed a defect in basal OCR and substantial loss of spare respiratory capacity (OCRbasal/OCRtotal), which are key measurements of mitochondrial respiration and oxidative phosphorylation (OxPhos; Fig. 2A). Naive T cells were used for comparison and to illustrate the drastic loss in oxygen consumption. EZH2 inhibition resulted in an increased dependence on glycolytic metabolism, indicated by the ratio of basal ECAR/OCR (Glycolysis/OxPhos; Fig. 2B). Parallel analysis of cytotoxic (CD8+), helper (CD4+, CD25−), and regulatory (CD4+, CD25+) T cells reveals a similar loss in OCR (Fig. 2C; Supplementary Fig. S3A). Consistent with mitochondrial dysfunction, cytotoxic, helper, and regulatory T cells all display a loss of mitochondrial membrane potential (Fig. 2D) and an increase in mitochondrial-derived superoxide species (Fig. 2E) when treated with EZH2i. The metabolic phenotype induced by EZH2i inhibition was replicated in CD8+ T cells with an alternative EZH2i, GSK503 (Supplementary Fig. S3C and S3D). Surprisingly, cellular ATP levels were not affected in EZH2i-treated T cells (Supplementary Fig. S3E). This suggests glycolytic metabolism compensates for the loss of mitochondrial respiration and provides the necessary ATP in an *in vitro* glucose rich environment. Transmission electron microscopy revealed an abnormal mitochondrial morphology in CD8+ T cells treated with an EZH2i (Fig. 2F; Supplementary Fig. S3F). We also observed a loss of mitochondrial mass using Mitotracker FM (Fig. 2G).

As the loss of mitochondrial function was clear, we next examined the sensitivity of EZH2-inhibited T cells to metabolic-stress induced cell death. EZH2 inhibition rendered T cells more sensitive to increasing doses of 2-deoxy-D-glucose (glycolysis inhibitor; Fig. 2H) and to the withdrawal of glucose (Fig. 2I). Conversely, EZH2 inhibition reduced the sensitivity to the withdrawal of glutamine, a mitochondrial substrate (Fig. 2J). EZH2i inhibition also led to a decrease in the sensitivity of T cells to oxidative stress (H2O2; Supplementary Fig. S3G) and pan-kinase inhibition (Staurosporine; Supplementary Fig. S3H). Thus, EZH2i inhibition causes a drastic shift in T-cell dependency on glycolytic metabolism, rendering them metabolically exhausted and sensitive to further insult including glucose withdrawal.

**EZH2 inhibition leads to histone epigenetic reprogramming at the Cdkn2a locus**

The connection between EZH2 inhibition and mitochondrial dysfunction has not been previously explored. To further prioritize our target genes driving the mitochondrial phenotype, we sought to connect the expression analysis directly to histone epigenetic control. First, to determine the histone PTM landscape after EZH2i inhibition in T cells, we performed a high-resolution mass spectrometric analysis of acid-extracted histones from T cells after EZH2 inhibition (Supplementary Fig. S4A). We quantified the relative abundance of approximately 30 unique histone modifications (monomethylation, dimethylation, trimethylation, phosphorylation, and acetylation) and combinatorial status where possible (Fig. 3A and B; Supplementary Fig. S4B–S4D). Relative spectral counting of PTMs on the functionally distinct histone H3.1 (DNA synthesis) and H3.3 (DNA synthesis independent) revealed a global reduction H3K27me2/3 as expected. We did not detect a large-scale remodeling of the histone landscape, and most of the PTMs detected were not altered between the groups. However, aside from K27me2/me3 changes, we detected significant changes in s28ph, k36me3, and k37me/me2 on H3.1. Interestingly, we observed an elevation of H3K36me3 on both H3.1 and H3.3, a histone PTM thought to be involved in gene elongation and alternative splicing (36). Time course analysis revealed that T cells have near undetectable levels of H3K36me3, and this changed as H3K27me2/3 is lost due to EZH2i inhibition (Fig. 3C). The presence of H3K36me3 was confirmed using parallel reaction monitoring (PRM) and targeted mass spectrometry (Fig. 3D; ref. 37). These data indicate that a loss of the repressive mark, H3K27me3, coincides with a certain degree of accumulation of H3K36me3 in EZH2i-treated cells.

In accordance with the histone proteomics, ChIP-sequencing revealed a genome-wide reduction in H3K27me3 level in the EZH2i-treated cells. For instance, in a window of ±5 kb from the transcription start site (TSS), we demonstrated a significant decrease in average read count (ARC) of H3K27me3 (ARC: 0.2), compared with control (0.4; Fig. 3E). Although the EZH2i inhibition does not...
Figure 2.
Loss of EZH2 leads to metabolic exhaustion in CD8⁺ T cells. A, Representative trace of OCR in preactivated CD8⁺ T cells treated for 48 hours with EZH2i. Arrows, injection time of respective inhibitors, oligomycin (ATP synthase inhibitor), FCCP (uncoupler), and rotenone + antimycin (complex I and III). Naïve T cells were used for comparison. B, Glycolytic dependency determined by the ratio of ECAR and OCR. Data represent the mean, and error bars represent the SEM. P value was determined by unpaired t test. C–E, Parallel analysis of activated cytotoxic (CD8⁺), helper (CD4⁺ CD25⁻), and regulatory (CD4⁺ CD25⁺) T cells with EZH2i. OCR (C), mitochondrial membrane potential (TMRM; D), and mitochondrial superoxide species (MitoSOX; E). Histogram median fluorescent intensity (MFI) is indicated. F, Representative transmission electron microscopy of activated CD8⁺ T cells with EZH2i. G, Relative mitochondrial mass FACS analysis (MitoTracker FM) from T cells with EZH2i. Data represent the mean (n = 3), and error bars represent the SEM. P value was determined by unpaired t test. H and I, Viability was determined by Annexin-V/DAPI staining in CD8⁺ T-cell cultures treated with increasing doses of 2-DG (H), glucose withdrawal (I), and glutamine withdrawal (J) for 24 hours. Data represent the mean (n = 3), and error bars represent SEM. Sum-of-squares F test was used for statistical comparison of IC₅₀.
necessarily affect the occupancy or enrichment of H3K36me3 at the ±5 kb of global TSS site (Supplementary Fig. S5A), we observed enrichment of H3K36me3 at the body of several differentially overexpressed (Log₂FC > 2.5 genes, N = 18), including Tgm2, Gzma, Dab2ip, or Kit of the most significant candidates from the RNA-seq and proteomic data sets (Fig. 3F). Interestingly, EZH2i induced an elevation of H3K36me3 at the Cdkn2a promoter (Fig. 3G). The Cdkn2a promoter was previously reported to contain bivalent chromatin marks, overlapped at the hypomethylated CpG Island. Induced demethylation resulted in complete loss of H3K27me3 at the gene promoter, leading to a gain in H3K27ac (38). Herein, we observed that induced removal of H3K27me3 is concomitant with accumulation of H3K27ac at the promoter. Increment in gene expression (Log₂FC = 4.36) hence can be reasonably correlated to the switch in occupancy from H3K27me3 to the activating H3K27ac, etc. marks at the Cdkn2a promoter in the EZH2-inhibited cells. The shift in epigenetic regulation at the Ink4a-Arf (Cdkn2a) locus and release of this gene had significant downstream effects, as
Arf induces metabolic exhaustion independent of p53

The effect on the intrinsic apoptotic machinery (BCL-2 family). For example, p53 is well known to induce the expression of the proapoptotic PUMA, which we did not observe (Supplementary Fig. S6D and S6E). Also, there was no detectable cleavage of the caspase target, PARP (Supplementary Fig. S6E). Interestingly, the transcript levels of p53 dropped in response to EZH2 inhibition (Supplementary Fig. S6F). These data suggest Arf acts independently of p53 in these cells.

Epigenetic Control of T-cell Metabolic Exhaustion

Our data suggest that EZH2 acts as a critical mediator for mitochondrial dysfunction. Arf has been reported to interact with the mitochondrial protein p32/C1QBP, which was shown to lead to mitochondrial damage (43). In addition, there is an N-terminally truncated Arf protein (smArf) that lacks the residues required for p53 activation and has been reported to localize to mitochondria and trigger mitophagy (44, 45). To directly assess the contribution of Arf expression to T mitochondrial dysfunction, we utilized an Arf knock out (Arf-KO) mouse model, which only disrupts Arf expression at the Cdkn2a locus (Fig. 4C, ref. 39). Arf deficiency was able to partially restore the loss of OCR induced by EZH2 inhibition in T cells (Fig. 4D). Arf plays a role in driving T-cell metabolic dysfunction in the tumor environment, though this datum suggests that there are additional or compensatory mechanisms at play.

Our data suggest that EZH2 acts as a critical mediator for mitochondrial sufficiency in part through regulation of the Arf locus. To demonstrate the involvement of Arf signaling in T-cell dysfunction, we challenged Arf-deficient (Arf-KO) mice with a murine model colon
adenocarcinoma (MC38; Fig. 4E). Constitutive deletion of Arf leads to an enhanced ability to control tumor growth and significantly increased median survival. However, this did not amount to complete protection and is consistent with the partial rescue of the metabolic phenotype.

Exogenous expression of EZH2<sup>Y641F</sup> improves tumor control

Approaches to enhance the survivability and metabolic sufficiency of tumor-specific T cells will be important for advancing this approach (46). We assessed whether reprogramming T cells with the gain-of-function EZH2<sup>Y641F</sup> mutant would enhance their ability to control tumor growth in an ACI mouse model. The EZH2<sup>Y641F</sup> mutation causes a gain of function of EZH2 by altering its substrate specificity. This mutation increases its activity toward the dimethylated substrate and in concert with a WT allele, causing an increase in the trimethylation of H3K27 (Fig. 5A; ref. 47). Tumor-specific T cells were generated from OT-1 and OT-1-Lck-EZH2<sup>Y641F</sup> mice. Activated CD8<sup>+</sup> T cells expressing the EZH2<sup>Y641F</sup> mutant had slightly elevated rates of oxygen consumption (Fig. 5B) and were superior at killing of MC38<sup>SINFEKL</sup> tumor cells <sup>in vitro</sup> (Fig. 5C). In a model ACI, tumor (MC38<sup>SINFEKL</sup>) bearing mice that received EZH2<sup>Y641F</sup>-positive OT-1 T cells were able to control tumor growth significantly better than mice that received either tumor-specific (OT-1) and nonspecific (wt) control cells (Fig. 5D). To determine if EZH2<sup>Y641F</sup> expression simply enhanced the survival of peripheral T cells <sup>in vivo</sup>, we adoptively transferred control and EZH2<sup>Y641F</sup>-positive T cells into congenic, CD45.1<sup>+</sup> recipient mice and tracked donor (CD45.2<sup>+</sup>) survival (Supplementary Fig. S7A). We found no significant difference in EZH2<sup>Y641F</sup> T cells within the spleen, lymph nodes, or peripheral blood 2 days after transfer. These findings are consistent with the idea that EZH2 plays a role in the protection of TILs from metabolic stress–induced dysfunction and that manipulation of EZH2 in ACI therapies would be beneficial.

Discussion

Understanding the mechanisms T cells use for protecting metabolic circuits may lead to new strategies and improve the efficacy of cancer...
immunotherapies. Engineering adoptive T cells, unconstrained by tolerance-based metabolic circuits, may allow for further advancement of cellular therapies against solid tumors. There have been some successes in overcoming inhibitory tumor metabolism, largely through enforcing mitochondrial respiration (7, 13). For example, the exogenous expression of the transcription factor Pgc1a, which directly controls mitochondrial biogenesis and effectively changes the metabolic circuit. However, recent discoveries have highlighted dynamic metabolic processes as drivers of T-cell function. This suggests that genetically or pharmacologically influencing T-cell metabolism will negate their ability to dynamically adapt. The goal therefore is to generate T cells that can withstand metabolic stresses, allowing for metabolic plasticity.

The ability of T cells to generate diverse phenotypes is in part due to histone modifications during the extensive chromatin remodeling occurring upon activation (48). Genetic deletion studies have recently implicated EZH2 (H3K27me3) in the control T-cell effector function and memory precursor formation (49, 50). However, this approach does not simulate an acute loss of EZH2, which TILs undergo during infiltration. In addition, genetic deletion of EZH2 can be compensated for by EZH1 (51). Perhaps more importantly, EZH2 thymic T cells are actively being considered for treatment of a variety of advanced solid tumors (e.g., NCT02601950, NCT03213665) and in some cases in combination with immune checkpoint inhibition (NCT03525795). These studies are motivated by a recently realized toxicity to T regulatory cells and the ability of H3K27me3 to mediate Th1-type chemokine expression in tumor cells (20, 52). However, our work sheds light on the deleterious effects of EZH2 inhibition on other T-cell subsets and suggests the use of these drugs in combination with immunotherapy may be challenging.

In the present study, we report the identification of EZH2 (H3K27me3) as a protector of T-cell metabolic sufficiency. Through a systematic evaluation of H3K27me3 loss, which occurs during tumor infiltration, we have discovered an epigenetic mechanism that contributes to the development of tumor-induced metabolic exhaustion in T cells. Loss of PRC2-repressive activity, through acute inhibition of EZH2, leads to a metabolic insufficiency in T cells. This has been characterized by the loss of mitochondrial respiration, loss of mitochondrial membrane potential, and increased sensitivity to glucose withdrawal. Our data emphasize the coordination of histone epigenetics and T-cell metabolic exhaustion. These findings are consistent with recent work defining a role for EZH2 in metabolic reprogramming of cancer cells (53, 54).

We identified the EZH2 inhibition–induced mitochondrial dysfunction phenotype using cutting-edge proteomics and gene set enrichment analysis. Using metabolic flux analysis to measure OCR (mitochondrial respiration) and acidification rates (glycolysis), we validated the loss of mitochondrial sufficiency in both CD4+ and CD8+ T cells using multiple specific inhibitors of EZH2. The elevated glycolytic rates induced by EZH2 inhibition are consistent with mitochondrial damage and indicate a strong glycolytic dependence. This metabolic phenotype is consistent with metabolic exhaustion in TILs, which is thought to be a driving force of functional exhaustion (55). To mimic the glucose withdrawal present in solid tumors, we treated with 2-Deoxy-D-Glucose (glycolysis inhibitor) or altered the concentration of glucose. EZH2 inhibition sensitized T cells to these metabolic stresses, a process that we believe occurs during tumor infiltration.

The ability of EZH2 to protect mitochondrial integrity has not been previously explored. Our comparison of proteomics, RNA-seq, and ChIP-sequencing approaches greatly narrowed down the H3K27me3-controlled candidate genes involved in driving mitochondrial dysfunction. Surprisingly, we did not find that EZH2 was directly regulating any conical metabolic pathways (fatty acid metabolism, glucose metabolism, electron transport, etc.). However, Cdkn2aAF/- was a top candidate in all these approaches and known to be repressed by EZH2 (H3K27me3) in cancer cells (56). The regulation of the Cdkn2a locus has been previously described in EZH2-KO T cells and was associated with prolonged division times, leading to a minor reduction in proliferation (57). This is likely due to the fact that in activated T cells, the expression of Arf does not lead to the canonical stabilization of p53, which is necessary for downstream cell-cycle arrest or cell death due to a strong repression of the Trp53 through engagement of TCR signaling (58). This observation leads us to propose alternative p53-independent mechanisms. We show that genetic deletion of Arf can rescue the metabolic defect induced during EZH2 inhibition. Thus, we propose that Arf induces mitochondrial dysfunction and therefore the downstream metabolic exhaustion. This p53-independent mechanism has been proposed and is thought to work through an interaction with the mitochondrial protein p32/CtIBP (43). p32 is predominantly localized to the mitochondria where it has a functional role in maintaining oxidative phosphorylation (59, 60). Furthermore, we show that Arf-/- animals have better control of colon adenocarcinoma (MC38) growth.

Therapeutically, this study suggests that enhancing/protecting EZH2 in cytotoxic T cells would be beneficial. One of the most common mutations of EZH2 in cancer cells is Y641F, a gain-of-function mutation that elevates H3K27me3 (61). We engineered tumor-specific T cells to express the EZH2Y641F mutation, and it did, in fact, enhance the ability to control tumor growth in vitro and in adoptive T-cell transplant experiments. Additional studies will be necessary to fully explore the potential of utilizing EZH2 gain-of-function mutants in cellular transplant studies.

**Limitations**

As outlined above, our results demonstrate that EZH2 through the placement of H3K27me3 contributes to the protection of mitochondrial sufficiency within T cells. However, we did not explore potential nonenzymatic roles for EZH2, which have been proposed to be an important aspect of EZH2 biology (62). Understanding the full scope of EZH2 in T-cell biology is challenging, especially when paired with conflicting roles of EZH2 within the context of tumor immunology and anticancer therapies. There are many important facets to T cells, and EZH2 exerts broad regulatory control through modifying the epigenome. Although Arf was clearly a top candidate gene in our studies, we did identify other potentially important candidates including Gema, which has also been implicated in mitochondrial dysfunction (63). Nevertheless, our findings provide an innovative perspective on the control of metabolic exhaustion of T cells during tumor infiltration and provide rationale for the clinical development of gain-of-function EZH2 T-cell therapies.

**Disclosure of Potential Conflicts of Interest**

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Epigenetic Control of *Cdkn2a.Arf* Protects Tumor-Infiltrating Lymphocytes from Metabolic Exhaustion


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