Ezh2 Regulates Activation-Induced CD8+ T Cell Cycle Progression via Repressing Cdkn2a and Cdkn1c Expression

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Transition from resting to cell cycle in response to antigenic stimulation is an essential step for naïve CD8+ T cells to differentiate to effector and memory cells. Leaving the resting state requires dramatic changes of chromatin status in the key cell cycle inhibitors but the details of these concerted events are not fully elucidated. Here, we showed that Ezh2, an enzymatic component of polycomb repressive complex 2 (PRC2) catalyzing the trimethylation of lysine 27 on histone 3 (H3K27me3), regulates activation induced naïve CD8+ T cells proliferation and apoptosis. Upon deletion of Ezh2 during thymocyte development (Ezh2fl/flCd4Cre+ mice), naïve CD8+ T cells displayed impaired proliferation and increased apoptosis in response to antigen stimulation. However, naïve CD8+ T cells only had impaired proliferation but no increase in apoptosis when Ezh2 was deleted after activation (Ezh2fl/flGzmBCre+ mice), suggesting cell cycle and apoptosis are temporally separable events controlled by Ezh2. We then showed that deletion of Ezh2 resulted in the increase in expression of cyclin-dependent kinase inhibitors Cdkn2a (p16 and Arf) and Cdkn1c (p57) in activated naïve CD8+ T cells as the consequence of reduced levels of H3K27me3 at these two gene loci. Finally, with real time imaging, we observed prolonged cell division times of naïve CD8+ T cells in the absence of Ezh2 post in vitro stimulation. Together, these findings reveal that repression of Cdkn1c and Cdkn2a by Ezh2 plays a critical role in execution of activation-induced CD8+ T cell proliferation.

Keywords: EZH2, CD8+ T cells, cell cycle, Cdkn2a, Cdkn1c

CD8+ T cells deliver the main antigen-specific cytotoxic function in immune response. The typical sequential events of a CD8+ T cell response include proliferation of antigen-specific naïve CD8+ T cells post-antigenic challenge to generate sufficient number of effector cells, eliminate antigen-expressing target cells, and differentiate into long-lived memory CD8+ T cells (1, 2). At each stage of differentiation, CD8+ T cells gain different functions from cytokine production to cytotoxicity in effector cells and properties of rapid responsibility and longevity in memory cells (3–5). The foundation of differentiation is the ability of activation-induced cell cycle progression, which requires precise chromatin modifications in a timely fashion at specific gene loci through concerted efforts of multiple chromatin modifiers (6, 7). How these sequential events are coordinated at the
chromatin level in CD8+ T cells during activation have just begun to be understood.

The cell division cycle is directly controlled by both positive regulators (cyclins and cyclin-dependent kinase, Cdks) and negative regulators (cyclin-dependent kinase inhibitors, Cdkn). Two families of Cdkns are active in resting T cells. The inhibitors of CDK4 family, including Cdkn2a [p16 and alternative reading frames (Arf)], Cdkn2b (p15), Cdkn2c (p18), and Cdkn2d (p19), and the Cip/Kip family, including Cdkn1a (p21), Cdkn1b (p27), and Cdkn1c (p57), play an essential role in maintaining the G0/ G1 phase during T cell development, differentiation, and function (8, 9). Studies of individual Cdk inhibitor using genetically modified mice model reveal their distinct roles in T cell development and differentiation. Cdkn2a-deficient mice have thymic hyperplasia and increased peripheral T cell proliferation whereas increased expression of Cdkn2a leads to arrest of thymocyte development at DN3 stage (10, 11). However, deletion of the alternative reading frames (Arf) of Cdkn2a in mice does not affect cell proliferation but enhance apoptosis of dividing cells (12). Cdkn1c regulates cell division and is also involved in the p53-dependent apoptosis pathway (13, 14). Less is known about the upstream events of the epigenetic regulation of these cell cycle regulators during CD8+ T cell activation and proliferation.

Ezh2 is a methyltransferase that catalyzes trimethylation of Lys-27 on H3 (H3K27me3) and is a core component of the polycomb repressive complex-2 (15–17). Ezh2 plays critical roles in T cell differentiation and function (18, 19). Ezh2 was first reported to interact with Vav1 of TCR signaling via actin polymerization-dependent processes (20). Ezh2 is also capable of positively regulating cytokine expression during CD4+ T cell differentiation (21–23) and has been implicated in Treg cell differentiation through repressing corresponding transcription factors (24, 25). Another important phenotype of Ezh2-deficient T cells is enhanced T cells apoptosis during immune response (26, 27). More recently, it has been demonstrated that Ezh2 maintains the fate of terminal effector CD8+ T cells by repressing the pro-memory gene sets (28). Although it is reported that Ezh2 regulates Cdkn2a and Cdkn1c loci in tumor cell lines (29, 30), the involvement of Ezh2 in activation induced CD8+ T cell cycle progression and apoptosis has not been fully characterized.

Here, we focused on the cell cycle progression and apoptotic events during naive CD8+ T cell activation using two T cell specific Ezh2 knockout models (Cd4Cre as a stable deletion and GzmbCre as an activation-induced deletion) and direct monitoring of naive CD8+ T cell divisions using long-term live imaging. We found that stable deletion of Ezh2 (Ezh2fl/fl-Cd4Cre+) had both impaired proliferation and enhanced apoptosis whereas activation-induced deletion of Ezh2 (Ezh2fl/flGzmbCre+) was only impaired in proliferation but not apoptosis. At the gene level, Ezh2 repressed Cdkn2a (p16 and Arf) and Cdkn1c (p57), both of which are essential for naive CD8+ T cells entering cell cycle post-activation. Furthermore, in the absence of Ezh2, naive CD8+ T cells exhibited a substantial delay of cell cycle completion in response to antigen stimulation.

MATERIALS AND METHODS

Animals and Cells

Ezh2 (Ezh2fl/fl) mice were generated as described (20) and obtained from MMRRRC repository. Cd4Cre was obtained from Taconic, and GzmbCre, OT-I, ROSA-26Sor1m39(CAG-hsp/EYFP), and B6.SJL-Ptpraj mice from Jackson Laboratory. Ezh2fl/fl mice were crossed with Cd4Cre to generate the Ezh2fl/fl-Cd4Cre+ (Ezh2-c-KO) strain. Ezh2fl/fl-Cd4Cre mice were further crossed with OT-I to generate Ezh2fl/fl-Cd4Cre-OT-I (Ezh2-c-KO OT-I) strain. Ezh2fl/fl was also crossed with GzmbCre and Gt(Rosa)26Sor1m39(CAG-hsp/EYFP)Hze to create Ezh2fl/flGzmbCre-YFP (Ezh2-g-KO) mice. All mice were maintained under specific pathogen free conditions at the animal facility of National Institute on Aging, and animal care was conducted in accordance with the guidelines of NIH.

CD8+ T cells were isolated from splenocytes or blood obtained from different murine strains. Naïve CD8+ T cells were defined as CD44+CD62L+ and purified using StemCell CD8+ Naïve T cell isolation kit with a final purity of more than 95%. Memory precursor and central memory CD8+ T cells were defined as CD127+KLRG1- and CD44+CD62L+, respectively. Cells were cultured in RPMI-1640 with 10% FBS, 10 mM HEPES, 0.11 nM beta-metacaptoethanol, and 1× Pen/Strep/Glu from Thermo-Fisher. CD8+ T cell stimulations performed using plate coated anti-CD3 (2C11, 5 μg/ml) and soluble aCD28 (37.51, 1 μg/ml) (Biolegend).

Listeria Infection

Listeria monocytogenes (10403S) with engineered OVA was a gift from Dr. Hao Shen of University of Pennsylvania and cultured in the Brain Heart Infusion (BHI) media with 10 μg/ml erythromycin. Mice were immunized via tail vein injections of 5 × 104 cfu of Listeria monocytogenes as infection model. All infection experiments were conducted under BSL-2 condition with approved protocol.

Adoptive Transfer

Naïve CD8+ T cells were isolated and purified from the spleens of CD45.2+ Ezh2-c-KO or Ezh2-c-KO-OT1 mice and adoptive transferred into CD45.1+ B6.SJL-Ptpraj animals. The mice were immunized with Listeria monocytogenes on the next day and sacrificed at the indicated time for different experiments or bled at the indicated time for flow cytometric analysis.

Flow Cytometry Analysis and Cell Sorting

The antibodies used for detection of surface and intracellular molecules such as CD4, CD8, CD45.1, CD45.2, CD127, KLRG1, CD69, CD25, CD44, CD62L, IFNγ, TNFα, IL2, Gzmb, BrdU, and annexin V conjugated to various fluorescent dyes were purchased from Biolegend and OVA-dextramer from Immudex. For activation-induced expression of activation markers, CD8+ naïve T cells were stimulated by anti-CD3/CD28 antibodies for 72 h and harvested for surface expression of CD69, CD25, and CD44. For activation-induced cytokine expression, naïve CD8+ T cells were stimulated by anti-CD3/CD28 antibodies for 68 h, and PMA (20 ng/ml), Ionomycin (1 μg/ml), and Golgi plug (1 μl/ml) were
added and incubated 4 additional hours before proceeding for cytokines and granzyme B analysis. Cells were permeabilized with Fix/Perm buffer (BD bioscience) and blocked with 7% normal rat serum (Stemcell technology) to reduce nonspecific antibody staining. Permeabilized cells were stained in Perm buffer (contains saponin) with cytokine specific or representative isotype control antibodies. Flow cytometry data were acquired on a BD FACSCantoII or Accuri C6 flow cytometer, and the results were analyzed with FlowJo (10.3) (Tree Star).

Microarray Data Collection and Analysis
Total RNA was extracted from Ezh2-c-KO and WT naïve CD8+ T cells after stimulation (0, 16, or 72 h) with RNAeasy Kit on QIAcube (Qiagen) in three biological replications. RNA was labeled using Quick Amp Labeling two-color kit (Agilent) with Cy3 dye used for sample RNA and Cy5 dye for home-made T cell RNA control for all array and hybridized to Agilent-028005 SurePrint G3 Mouse Gene Expression v2 8 x 60K Microarray or Agilent-026655 Whole Mouse Genome v2 4 x 44K Microarray according to the manufacturer’s protocols. The hybridized chips were scanned with SureScan Microarray Scanner D (Agilent), and the expression level was determined with Agilent Feature Extraction software 11.5.1.1. Microarray data are submitted to GEO/NCBI database, accession number GSE106426. Data were log-transformed and then normalized based on Cy5 signal intensity. Data from 60K and 44K microarray platforms were combined using batch normalization by equalizing median log-transformed gene expression values for seven samples that were hybridized to both array platforms. To avoid redundancy, these seven samples were further analyzed using only 60K data. The microarray data were further analyzed with ExAtlas (NIA, NIH) and BRB Array Tools (NCI, NIH).

Quantitative Reverse Transcription Polymerase Chain Reaction
Total RNA was extracted with RNAeasy Kit on QIAcube (Qiagen) from naïve CD8+ T cells. RNA was reverse transcribed to cDNA using Super Script III (Invitrogen). Quantitative PCR was performed with SYBR green (Qiagen) by standard protocol. The probes and primers used for PCR are listed in Table S3 in Supplementary Material.

Proliferation Assay
Proliferation assays were carried out with CFSE and BrdU incorporation assay. For CFSE incorporation assay, naïve CD8+ T cells were labeled with 1 μM CFSE or CellTrace Violet (Invitrogen) according to the manufacturer’s instructions and then stimulated with anti-CD3/CD28. The labeled cells were collected, and flow cytometric analysis was performed at different time points, with or without counting beads (Spherotech). For BrdU assay, naïve CD8+ T cells were first stimulated with anti-CD3/CD28. One hour before the measurement, the activated cells were labeled with 10 μM BrdU (Sigma) and stained with surface markers, intracellular αBrdU antibodies and 7-AAD. Data were acquired on BD FACSCantoII and analyzed with FlowJo (Tree Star).

Apoptosis Assay
Apoptosis assays were performed with annexin V staining, caspase 3/7 staining, and TUNEL assay. For annexin V staining, prepared cells were first stained with cell surface antibodies and then with fluorophore-conjugated annexin V and 7-AAD in the annexin V staining buffer. The cells were acquired immediately on a flow cytometer. Caspase 3/7 staining was performed with Vybrant FAM Caspase-3 and -7 Assay Kit (Thermo-Fisher) according to the manufacturer’s protocols. Briefly, cells were incubated with FLICA working solution 1 h before measurement and stained with other fluorescent conjugated cell surface antibodies and 7-AAD. The stained cells were acquired immediately on a flow cytometer. The TUNEL assay was performed with APO-BrdU TUNEL Assay Kit (Thermo-Fisher) according to the manufacturer’s protocol. Briefly, cells were fixed with 1% (w/v) paraformaldehyde in PBS on ice for 15 min, and then resuspended in ice-cold 70% ethanol and further incubated on ice for a minimum of 30 min. The fixed cells were labeled with BrdU with TdT enzyme for 60 min, and then stained with fluorescent-conjugated anti-BrdU antibody. The cells were acquired on a flow cytometer and analyzed as mentioned earlier.

Chromatin ImmunoPrecipitation
Naïve CD8+ T cells were isolated freshly and stimulated with anti-CD3/CD28 in vitro for 72 h. The cells were digested by 0.2U MNaše per million cells for 10 min and sonicated (Diagenode) with a power setting of 5 for 4 repeats of 30 s followed by 30 s break between each repeat in ice-cold water to obtain the average size between 100 and 300 bps. The sonicated products were dialyzed with 10000 MWCO Cassette G2 (Thermo-Fisher) in 400 ml RIPA buffer for 2 h at 4°C and then incubated overnight with 2 μg anti-H3K27me3 antibody (Millipore, 07-449) or IgG-conjugated Dynabeads Protein G (Thermo-Fisher). The beads were washed with RIPA, LiCl, and TE buffer sequentially and recovered overnight at 65°C with TE supplemented with 10% SDS and proteinase K (MBiotech). The ChIP DNA was purified with phenol/chloroform (Invitrogen) and precipitated for quantitative PCR. The primers used for ChIP are listed in Table S3 in Supplementary Material. The specific H3K27me3 ChIP was calculated by subtracting the value of IgG (% of H3K27me3 in input – % of IgG in input). We also used the primer covering non-promoter region of these gene loci (P0) as additional control. The P0 regions were selected based on published H3K27me3 ChIP-seq data from other cell lines.

Microscopy and Image Analysis
Naïve CD8+ T cells were isolated and labeled with CellTrace Far Red in Phenol Red-free RPMI-1640 media. 35-mm dishes were coated with anti-CD3 and a 4-well silicone micro-insert (ibidi) placed in the same dish to hold the cells. Cells to be imaged were mixed with soluble anti-CD28 and pipetted into the micro-inserts. Cells were also plated at a density of 1 × 10⁷ in the surrounding area of the insert. The insert was fully submerged by adding additional Phenol Red-free RPMI-1640 media. Cells were imaged on a Zeiss LSM880 platform. Image acquisition was performed using the Tile scan (6 × 4 or 5 × 5) feature of the Zen 2.3 (black)
Ezh2 Genotyping at Single Cell Level
Naïve CD8+ T cells from WT, Ezh2-c-KO, or Ezh2-g-KO mice were isolated, labeled with CFSE, and stimulated with anti-CD3/CD28 for 72 h in vitro. The undivided and high divided (>3 division) stimulated cells were sorted into a 96-well plate containing 1× PCR reagents. The sorted plates were immediately spin down at 1,400 rpm for 5 min and heated at 95°C for 5 min, following with Ezh2 genotyping PCR and checked with gel electrophoresis. The primers used for Ezh2 genotyping were listed in Table S3 in Supplementary Material.

Statistical Analysis
Unless specifically indicated, p values were calculated using Student’s t-test. ANOVA test was used for comparison of multiple samples. *, **, and *** represent p < 0.05, 0.01, and 0.001, respectively.

Accession Numbers
Microarray data were available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under GSE106426 (GSE106424 and GSE106425).

RESULTS
Ezh2-Deficient Naïve CD8+ T Cells Have Diminished Activation-Induced Expansion and Differentiation During Listeria Infection
To determine the roles of Ezh2 in naïve CD8+ T cell function, we used Ezh2 conditional knockout mice (Ezh2fl/fl-Cd4Cre- referred as Ezh2-c-KO, and both Ezh2fl/fl-Cd4Cre and Ezh2WT-Cd4Cre as WT) (20) and challenged with Listeria-OVA (LM-OVA) (31). Although both total CD8+ and OVA-specific CD8+ T cells from WT mice increased significantly in response to Listeria infection, little to no expansion of OVA(OVA257-264)-specific CD8+ T cells was observed in Ezh2-c-KO mice (Figures 1A,B). The deletion of Ezh2 in CD8+ T cells from Ezh2-c-KO mice was completed (Figure S1 in Supplementary Material). To rule out potential differences in the number of OVA-specific CD8+ T cells between WT and Ezh2-c-KO mice, we adaptively transferred the same number of isolated CD8+ T cells (1 × 10⁵) from OT-I or Ezh2-c-KO OT-I mice into the congenic CD45.1 mice followed by LM-OVA infection. Again, no detectable expansion of OVA-specific CD8+ T cells was observed in Ezh2-c-KO mice throughout 10-day post infection period, while OVA specific CD8+ T cells from OT-I mice had...
significant expansion at day 5, peaked at day 7, and reduced at day 10 (Figures 1C,D). In addition to lack of expansion, CD8+ T cells from Ezh2-c-KO mice were all effector cells (CD127–KLRG1+) with little memory precursor cells (CD127+KLRG1−) at day 12 post LM-OVA infection (Figures 1E,F). These results demonstrate a role of Ezh2 in activation-induced expansion and differentiation of CD8+ T cells.

**Ezh2-Deficient CD8+ T Cells Are Activated but Undergo Increased Apoptosis**

To further investigate the defects in the absence of Ezh2, CD8+ naïve T cells from Ezh2-c-KO and WT mice were isolated and activated in vitro with anti-CD3 and anti-CD28 antibodies (anti-CD3/CD28). We examined the expression of three activation markers (CD69, CD25, and CD44), three cytokines (Ifn-γ, Il-2, and Tnf-α), and one effector molecule (Granzyme B) after 72 h of stimulation. We found no significant differences in expression of three activation markers between Ezh2-c-KO and WT mice (Figure 2A). In agreement with increase effector cells (CD127+KLRG1+), CD8+ naïve T cells from Ezh2-c-KO mice had significantly higher MFI of Ifn-γ, Il-2, and Granzyme B than that from WT mice (Figure 2B). These findings show that Ezh2-deficient CD8+ T cells have no defects in in vitro activation-induced expression of activation markers but enhanced expression of two cytokines (Ifn-γ and Il-2) and one effector molecule (Gzmb).

We further observed significantly reduced number of live cells after stimulation as a consequence of increased death of Ezh2-deficient CD8+ T cells. First, there was a significant increase in apoptotic cells defined by annexin V+ and 7AAD+ 16 h after stimulation (Figures 2C–E). Increased apoptosis in activated Ezh2-deficient CD8+ T cells was also observed with the increase in DNA fragmentation by TUNEL assay (Figure S2 in Supplementary Material). Consequently, there were significantly fewer numbers of CD8+ T cells from Ezh2-deficient mice than from WT mice (WT and Ezh2WT-Cd4cre+) after in vitro stimulation (Figure 2E). To determine whether increased apoptosis occurred under in vivo conditions, we used adoptive transfer with LM-OVA infection model and found that Ezh2-deficient CD8+ T cells had more apoptosis (Figures 2F,G) and higher percentage of cells expressing key apoptotic activators, caspase 3/7, than WT CD8+ T cells (Figures 2H,I). Together, these findings demonstrate that Ezh2-deficient CD8+ T cells have normal response to activation-induced expression of key functional molecules but significantly increased apoptosis.

**Ezh2-Deficient Naïve CD8+ T Cells Have Impaired Activation-Induced Proliferation**

To further determine if the reduction in the number of Ezh2-deficient naïve CD8+ T cells post-activation was also influenced by a reduction in cell proliferation, we used the CellTrace fluorescent dye incorporation assay and found that Ezh2-deficient CD8+ T cells had more undivided cells as shown by a decrease of Division Index (compared divided cells over undivided cells) after stimulation in vitro compared to WT naïve CD8+ T cells (Figures 3A,B). We then analyzed the cell cycle progression after activation using the BrdU incorporation assay and found that Ezh2-deficient CD8+ T cells were fewer in S-phase but more in G0/G1 phase compared to WT cells (Figures 3C,D). These results indicate that Ezh2-deficient CD8+ T cells have impaired cell cycle progression from G0/G1 to S phase in response to stimulation.

**Ezh2-Deficient CD8+ T Cells Have Altered Expression of Apoptosis and Cell Cycle Related Genes**

To further investigate the mechanism of the observed defects in Ezh2-deficient CD8+ T cells, we analyzed the transcriptome by microarray of naïve CD8+ T cells from Ezh2-c-KO and WT mice at three different time points: freshly isolated and in vitro stimulated by anti-CD3/CD28 for 16 and 72 h, respectively. We observed 1,626 and 1,404 genes with increased and decreased expression (>2-fold change and FDR < 0.05) in freshly isolated Ezh2-c-KO naïve CD8+ T cells compared to WT cells, respectively. After stimulation, there were 2,192 and 1,015 genes with increased expression and 2,121 and 842 genes with decreased expression (Ezh2-c-KO vs. WT) at 16 and 72 h, respectively (Figure 4A). Further functional analysis suggested that freshly isolated naïve CD8+ T cells from Ezh2-c-KO mice had altered expression in RNA related, DNA related, apoptosis, immune response, inflammatory response, actin and migration related biological processes. After stimulation, additional alteration in proliferation, cytokines and chemokines, histone-related biological processes was observed (Figure 4B). This indicates that Ezh2 deficiency affects a wide range of molecular and cellular function of naïve CD8+ T cells in a temporal manner (Tables S1 and S2 in Supplementary Material).

We then focused on apoptosis and cell cycle related genes which may explain the cellular defects we observed in the Ezh2-c-KO mice. Among 335 increased apoptosis related genes found in Ezh2-c-KO mice, 99 were detected in freshly isolated naïve CD8+ T cells (Figures 4B,C). As Ezh2 deletion occurs during T cell development in thymus in the Ezh2-c-KO mice, mature naïve CD8+ T cells in periphery had already carried these defects and activation further exacerbated the apoptosis process. In contrast, the expression of most cell cycle checkpoint genes were not significantly different between Ezh2-deficient and WT naïve CD8+ T cells but several cycle checkpoint genes including Cdkn2a and Cdkn2b were significantly increased after 72 h post-activation in Ezh2-deficient CD8+ T cells compared to WT CD8+ T cells (Figures 4B,C). Together, the transcriptome analysis provides evidence of altered gene expressions that is responsible for activation-induced increased apoptosis and cell cycle defects in the absence of Ezh2.

**Ezh2 Regulates H3K27me3 of Cdkn1c and Cdkn2a Locus**

To understand how early the changes in expression of cell cycle related-genes happens in the absence of Ezh2, we included four additional early time points (1, 2, 4, and 8 h after stimulation) during the course of 72 h after anti-CD3/CD28 stimulation. Ezh2 mRNA levels were increased at 8 h, peaked around 48–72 h after stimulation (Figure 5A). We then compared expressions of 12
cell-cycle regulators and found that *Cdkke1c* (p57) was dramatically increased at 2 h and peaked at 4 h (>2-folds higher in Ezh2-deficient CD8+ T cells than in WT CD8+ T cells) after stimulation (Figure 5A). For *Cdkk2* family, expression of *Cdkk2aV1* (Arf) and *Cdkk2aV2* (p16), key regulators for apoptosis and cell cycle, respectively, were increased in Ezh2-deficient naïve CD8+ T cells compared to WT naïve CD8+ T cells from 8 to 72 h after stimulation (Figure 5A). *Cdkk2b* (p15) was also expressed higher in
Ezh2 deficient than in WT naïve CD8⁺ T cells from 8 to 16 h (Figure S3 in Supplementary Material). However, we did not find significant differences in gene expressions of either cell cycle activators such as Ccn2, Ccn3, Cdk2, Cdk4, Cdk6, E2F1, c-Myc, and Gadd45α or cell cycle inhibitors Cdkn1a (p21) and Cdkn1b (p27) between Ezh2-deficient and WT CD8⁺ T cells of Figure S3 in Supplementary Material. Together, these findings show that Ezh2 may regulate expression of Cdkn1c, Cdkn2aV1 (Arf), and Cdkn2aV2 (p16) in naïve CD8⁺ T cells.

To further determine whether the enhanced expressions of Cdkn1c, Cdkn2aV1 (Arf) and Cdkn2aV2 (p16) were the consequence of loss of Ezh2, we analyzed H3K27me3 in these genomic loci using ChIP assay. We found that H3K27me3 was present in the promoter region of Cdkn1c locus (Figures 5B,C). The H3K27me3 occupancy was higher at the promoter region of exon 1a, but not exon 1b (Figures 5D,E) which indicates that naïve CD8⁺ T cells activation induce more repression of Cdkn2aV1 rather than repressing Cdkn2aV2. These data suggest that Ezh2-mediated H3K27me3 regulates Cdkn1c, Cdkn2aV1 (Arf), and Cdkn2aV2 (p16) expressions during naïve CD8⁺ T cell activation.

### Immediate Deletion of Ezh2 Affects Activation-Induced Cell Cycle but Not Apoptosis in CD8⁺ T Cell

The deletion of Ezh2 in Ezh2 floxedCd4Cre+ mice happened at double positive thymocyte stage and naïve CD8⁺ T cells of spleen had enhanced expression of apoptosis-related genes. To investigate the immediate effects of losing Ezh2, we crossed Ezh2floxed with GzmBCre and Gr(ROSA)26Sor tm39(CAG-hop/EYFP)Hze.
The deletion of Ezh2 took place only after activation of T cells which turned on GzmbcCre hence leading to Ezh2 deletion and EYFP expression (Ezh2<sup>fl/fl</sup>GzmbcCre<sup>+</sup> as Ezh2-g-KO and Ezh2<sup>fl/fl</sup>GzmbcCre<sup>-</sup> as WT) (Figure S4 in Supplementary Material). We then used Ezh2-g-KO mice to determine the immediate effects of deleting Ezh2 in naïve CD8<sup>+</sup> T cell differentiation in vivo.
Ezh2-g-KO and WT (Ezh2<sup>fl/fl</sup> GzmbCre<sup>−/−</sup>) mice were infected with LM-OVA, and OVA-specific CD8<sup>+</sup> T cells were measured at day 7 after immunization. Comparable to the Ezh2-c-KO mice, Ezh2-g-KO mice also showed a marked defect in CD8<sup>+</sup> T cell differentiation after LM-OVA infection in vivo (Figures 6A,B). However, unlike Ezh2-c-KO mice, CD8<sup>+</sup> T cells of Ezh2-g-KO mice had comparable percentages of live, apoptosis, and dead cells with CD8<sup>+</sup> T cells of WT mice after in vitro stimulation (Figures 6C,D) and total live cells (Figure 6E). In contrast, we observed significantly decreased percentage of cells in S-phase and more in G0/G1 phase in naïve CD8<sup>+</sup> T cells of Ezh2-g-KO mice compared to that of WT (Figures 6F,G). Together, these findings indicate that regulation of cell cycle and apoptosis by Ezh2 are temporally separated events and loss of Ezh2 first affects cell cycle progression in naïve CD8<sup>+</sup> T cells.

To determine whether altered transcriptional changes of cell cycle regulators and apoptosis related genes in naïve CD8<sup>+</sup> T cells observed from Ezh2-c-KO mice were also found in the Ezh2-g-KO mice, we sorted activated naïve CD8<sup>+</sup> T cells from WT or Ezh2-g-KO mice into undivided and highly divided (more than three divisions) populations after 72 h of stimulation (anti-CD3/CD28) in vitro to allow GzmbCre expression (Figure 7A). We found that both undivided and high divided naïve CD8<sup>+</sup> T cells showed significantly decreased Ezh2 levels from Ezh2-g-KO compared to the WT mice (Figure 7B). Furthermore, expression of Cdkn2aV1 and Cdkn2aV2 were significantly increased, but no change in expression of Cdkn1c and apoptotic related genes could be observed in naïve CD8<sup>+</sup> T cells from Ezh2-g-KO compared to that from WT mice (Figures 7C,D).

**Naïve CD8<sup>+</sup> T Cells Take Longer to Complete Cell Division in Response to Activation After Ezh2 Deletion**

The increased G0/G1 and reduced S phase of naïve CD8<sup>+</sup> T cells from Ezh2-g-KO mice suggested that immediate deletion of
Ezh2 altered activation-induced cell cycle. To directly test this, we developed a method to track naïve CD8+ T cell division after anti-CD3/CD28 stimulation in real-time using live cell imaging (Videos S1–S3 in Supplementary Material). We used 2.0 mm × 1.5 mm silicone inserts to culture activated naïve CD8+ T cells (Videos S1–S3 in Supplementary Material). We used 2.0 mm silicone inserts to culture activated naïve CD8+ T cells (Videos S1–S3 in Supplementary Material).

Because we detected lower Ezh2 mRNA (Figure 7B) and the division time of some CD8+ T cells from Ezh2-c-KO mice had similar time as that of CD8+ T cells from WT mice, we suspected that the complete deletion of Ezh2 in Ezh2-g-KO mice may not have occurred. We further performed single cell genotyping of Ezh2 in naïve CD8+ T cells of these mice after in vitro stimulation and found that Ezh2 was completely deleted in 28.3% cells, deletion of one allele happened in 17% of cells, and the remaining 54.7% of cells had no deletion (Figure 8D). Thus, the delay of cell cycle completion would have been more severe had the deletion taken place in those 54.7% cells of the Ezh2-g-KO mice. Taken together, we tracked highly motile CD8+ T cells through their division events in real time after activation and demonstrated the precise time delay of cell cycle in Ezh2 deleted naïve CD8+ T cells.

**DISCUSSION**

Ezh2 catalyzes H3K27 trimethylation to repress genes involving multiple critical biological processes in development and differentiation of T cells. In this study, we demonstrated that the role of Ezh2 in regulation of cell proliferation and apoptosis are two temporally separable events during naïve CD8+ T cell activation. Naïve CD8+ T cells from spleen of Ezh2-c-KO mice had altered expression of cell cycle and apoptosis related genes prior to activation (Figure 4) and display cell cycle defects and increased apoptosis after in vitro and in vivo activation. However, if the deletion of Ezh2 in naïve CD8+ T cells occurred only after activation in the Ezh2-g-KO mice, only cell cycle defect but no increase apoptosis could be observed in these activated naïve CD8+ T cells. This shows that cell cycle regulators (Cdkns) are...
more sensitive to the loss of Ezh2 than apoptosis related genes, and Ezh2 plays a critical role in repressing the expression of Cdkn genes during naïve CD8+ T cell activation-induced proliferation.

Expression of cell cycle inhibitors such as Cdkns is tightly regulated in T cells (9). Here we show that loss of Ezh2 resulted in altered expression of several key cyclin-dependent kinase inhibitors including Cdkn2a (p16 and Arf) and Cdkn1c (p57) in naïve CD8+ T cells after activation, which explained the cell cycle impairment of Ezh2-deficient naïve CD8+ T cells in response to antigenic stimulation. However, loss of Ezh2 had limited impact on other key Cdkns including Cdkn2c (p18), Cdkn2d (p19), Cdkn1a (p21), Cdkn2b (p15), and Cdkn1b (p27) (Figure S2 in Supplementary Material). In addition, a dramatic increase (70-fold) in expression of Cdkn1c (p57) in Ezh2 deficient naïve CD8+ T cells after 4 h stimulation also contributed to p53 mediated apoptosis pathway (8, 9) and increased apoptosis along with alteration of apoptosis-related genes. These findings provide evidence of the regulatory role of Ezh2 in cell cycle inhibitors and apoptosis related gene expression via modification of H3K27me3 levels at these loci. Loss of Ezh2 during thymocyte development resulted in apparent

**Figure 7** | Increased expression of Cdkns but not apoptosis-related in stimulated naïve CD8+ T cells from Ezh2-g-KO mice. Naïve CD8+ T cells were isolated from WT or Ezh2-g-KO mice and labeled with CellTrace Violet and stimulated with anti-CD3/CD28 for 72 h. The undivided (Division 0) and high divided (≥Division 3) cells were sorted, and the expression of selected Cdkns and apoptosis genes were measured by real time RT-PCR. (A) Cell sort scheme. (B–D) Relative amount of mRNA is presented after normalization to RPL32 including Ezh2, Cdkn1c, Cdkn2a, and apoptosis related genes (Bad, Bax, Trp53). The experiments were repeated twice, with three mice of each repeat.
alteration of gene expression observed in freshly isolated and 16 h stimulated naïve CD8+ T cells. But this transcriptional difference became less obvious at 72 h stimulation probably reflecting the selection of those less damaged dividing cells. However, in addition to the direct effects of Ezh2, the impairment of cell cycle progression of naïve CD8+ T cells over the course (several days) of analysis after stimulation in the absence of Ezh2 could also be secondary effects resulting from the defects of gene expression regulators controlled by Ezh2. Thus, it was necessary to dissect the direct and indirect effects of Ezh2 in regulation of cell cycle and apoptosis during naïve CD8+ T cell activation.

Applying GzmbCre-mediated Ezh2 deletion allowed us to examine the early effects of losing Ezh2 in naïve CD8+ T cells and differentiated cell cycle defects from apoptosis. Several reasons could explain the less severe defects observed in naïve CD8+ T cells from Ezh2-g-KO mice compared to naïve CD8+ T cells from Ezh2-c-KO mice. First, it takes time for the effect of Cre-mediated Ezh2 deletion to be observed phenotypically. Ezh2 deletion occurred in thymocyte development in Ezh2-c-KO mice whereas after activation of naïve CD8+ T cells of Ezh2-g-KO mice. The different degree of impairments reflects the degree of cumulated defects in the absence of Ezh2. Second, deletion of
Ezh2 was completed in CD8+ T cells of Ezh2-c KO, whereas we observed various degree of deletion of Ezh2 in stimulated naïve CD8+ T cells from Ezh2-g-KO mice by single cell genotyping. Less than half (45%) of activated naïve CD8+ T cells had complete and one allele deletion of Ezh2, indicating that the impact of activation-induced immediate deletion of Ezh2 in naïve CD8+ T cells of Ezh2-g-KO mice could have been more severe if it were feasible to remove cells containing the undeleted Ezh2 loci from the analysis. Together, these findings show the sensitiveness of Ezh2 in processes involved inactivation-induced naïve CD8+ T cell proliferation.

The importance of cell division for T cell function during an infection is apparent yet the precise time required for the resting naïve CD8+ T cells completing first and subsequent cell cycle has not been directly measured. Previous methods calculates indirectly estimated the average cell division time based on the dilution of cell division tracking dye (32–34). In this study, we used live imaging to record cell divisions of naïve CD8+ T cells from WT, Ezh2-c-KO, and Ezh2-g-KO mice in response to in vitro stimulation with anti-CD3/CD28. To increase our efficiency of tracking motile T cells and their subsequent divisions over a long period of time, we cultured stimulated T cells in silicone inserts and imaged the entire chamber. The seeding number of cells was also optimized to accommodate their growth over the course of 72 h. With these optimizations in place, we directly observed several cells that underwent first and second round of divisions in response to stimulation. The average time for naïve CD8+ T cells to complete the first division was approximately 28 h. The deletion of Ezh2 in naïve CD8+ T cells resulted in significant delay of the first cell cycle completion. Although the subsequent second cell division took approximately one-third of time (8.5 h) in WT mice, the time of second cell division was further delayed in both Ezh2 KO mice. These observations reflect the cumulative defects in the absence of Ezh2. The live imaging setup can also track the localization and dynamics of specific fluorophore-tagged proteins and can serve as a crucial tool for understanding role of key regulators in cell division. In conclusion, our findings reveal a critical role of Ezh2 in regulation of cell cycle during naïve CD8+ T cell activation by establishing a repressing state of Cdkn2a and Cdkn1c.

ETHICS STATEMENT

This study protocol was carried out in accordance with the guidelines of the National Institute on Aging and approved by the Institutional Review Board. Study subjects were participants of the Baltimore Longitudinal Study of Aging (BLSA) and gave written informed consent.

AUTHOR CONTRIBUTIONS

GC and N-pW designed experiments. GC, KS, SC, JL, and JK performed experiments. GC, SC, JL, and JK performed data analysis. AS helped array data analysis, XM and RW helped perform experiments. M-HS and N-pW supervised the research projects. GC and N-pW supervised the research projects. GC and N-pW wrote the manuscript with approval from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00549/full#supplementary-material.

FIGURE S1 | Deletion of Ezh2 in Ezh2-c-KO CD8 T cells before and after in vitro stimulation: Naïve CD8+ T cells were isolated from spleen of WT or Ezh2-c-KO mice and stimulated with anti-CD3/CD28 for 72 h. With these optimizations in place, we directly observed several cells that underwent first and second round of divisions in response to stimulation. The average time for naïve CD8+ T cells to complete the first division was approximately 28 h. The deletion of Ezh2 in naïve CD8+ T cells resulted in significant delay of the first cell cycle completion. Although the subsequent second cell division took approximately one-third of time (8.5 h) in WT mice, the time of second cell division was further delayed in both Ezh2 KO mice. These observations reflect the cumulative defects in the absence of Ezh2. The live imaging setup can also track the localization and dynamics of specific fluorophore-tagged proteins and can serve as a crucial tool for understanding role of key regulators in cell division. In conclusion, our findings reveal a critical role of Ezh2 in regulation of cell cycle during naïve CD8+ T cell activation by establishing a repressing state of Cdkn2a and Cdkn1c.

FIGURE S2 | TUNNEL assay in Ezh2 deficient CD8+ T cells. WT or Ezh2-c-KO CD8+ naïve T cells were isolated and stimulated with anti-CD3 and anti-CD28 for 72 h in vitro. The cells were fixed with 1% (W/V) paraformaldehyde in PBS on ice for 15 min, and then resuspended in ice cold 70% ethanol, and incubated on ice for a minimum of 30 min. The fixed cells were labeled with BrdU with TdT enzyme for 60 min, and then stained with fluorescent conjugated anti-BrdU antibody. The cells were acquired on Flow Cytometer (A) and the percentages normalized in (B). This figure is supplementary to Figure 2.

FIGURE S3 | Gene expression profiles in Ezh2-deficient CD8+ T cells. Wild type or Ezh2-c-KO CD8+ naïve T cells were stimulated with anti-CD3 and anti-CD28 in vitro, and collected at different time points post-stimulation for qPCR analysis. The gene expression level was normalized to the level of Rp18, and further normalized to the unstimulated wild type samples. This figure is supplementary to Figure 5.

FIGURE S4 | Validation of Ezh2 deletion in naïve CD8+ T cells from Ezh2-g-KO mice. (A,B) WT or Ezh2-g-KO CD8+ naïve T cells were isolated, labeled with CellTrace Violet and stimulated with anti-CD3 and anti-CD28 in vitro for 4 days. (A) YFP expression was detected in the divided Ezh2-g-KO CD8+ T cells. (B) The YFP+ divided cells in Ezh2-g-KO and YFP- divided cells in WT were sorted with Flow Cytometry and used for Ezh2 mRNA expression by qPCR. The experiments were repeated twice. (C-E) Ezh2-g-KO genotyping. (C) Ezh2-g-KO or WT activated CD8+ T cells were sorted into undivided and >3 times divided population, and genomic DNA was isolated for Ezh2 genotyping by PCR. Lane L: DNA ladder. Lanes 1–6 were as following 1. WT, 2. Ezh2-c-KO, 3. equal mix of
WT and Ezh2−/−, 4. undivided, and 5. >3 divided cells from Ezh2−/− and 6. water control. The up and low bands represented Ezh2+/+ and deleted Ezh2 PCR products, respectively. (D) Single Ezh2−/− or WT activated CD8+ T cells were sorted into 96 wells plate and genotyped for Ezh2 deletion by PCR. The representative gel image from one of the three repeats is shown. Lane L: DNA ladder. Lanes 1–16 were Ezh2−/− single cells, respectively. The up and low bands represented Ezh2+/+ and deleted Ezh2 PCR products, respectively. This figure is supplementary to Figures 6–8.

**FIGURE S5** | Tile scan profile of live cell imaging. WT, Ezh2−/− or Ezh2−/− CD8+ naïve T cells were labeled with CellTrace Far Red and stimulated with plate coated anti-CD3/CD28 (see Materials and Methods for details). The representative tile scan profile shows the complete imaging area of the chamber of interest in the silicone micro-insert. The edge effects as seen on the scan profile had no effect on data quantification. This figure is supplementary to Figure 8.

**VIDEO S1** | Live Imaging of naïve CD8+ T cells of WT mice. Time lapse movie (2 fps) of overlaid DIC and Far Red (CellTrace) channels of naïve CD8+ T cells obtained from WT mice. A representative cell that was tracked over time is indicated with a white arrowhead. The cell undergoing first and second division is marked with cyan and yellow arrowhead, respectively. Duration in hh:mm at the top-left corner indicates the time of acquisition post-stimulation of the cells.

**VIDEO S2** | Live Imaging of naïve CD8+ T cells of Ezh2−/− mice. Time lapse movie (2 fps) of overlaid DIC and Far Red (CellTrace) channels of naïve CD8+ T cells obtained from Ezh2−/− mice. A representative cell that was tracked over time is indicated with a white arrowhead. The cell undergoing first and second division is marked with cyan and yellow arrowhead, respectively. Duration in hh:mm at the top-left corner indicates the time of acquisition post-stimulation of the cells.

**VIDEO S3** | Live Imaging of naïve CD8+ T cells of Ezh2−/− mice. Time lapse movie (2 fps) of overlaid DIC and Far Red (CellTrace) channels of naïve CD8+ T cells obtained from Ezh2−/− mice. A representative cell that was tracked over time is indicated with a white arrowhead. The cell undergoing first and second division is marked with cyan and yellow arrowhead, respectively. Duration in hh:mm at the top-left corner indicates the time of acquisition post-stimulation of the cells.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.