The histone methyltransferase EZH2 primes the early differentiation of follicular helper T cells during acute viral infection

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Epigenetic modifications to histones dictate the differentiation of naïve CD4+ T cells into different subsets of effector T helper (T\textsubscript{eff}) cells. The histone methyltransferase enhancer of zeste homolog 2 (EZH2) has been implicated in the mechanism regulating the differentiation of T\textsubscript{eff}1, T\textsubscript{eff}2 and regulatory T (T\textsubscript{reg}) cells. However, whether and how EZH2 regulates follicular helper T (T\textsubscript{FH}) cell differentiation remain unknown. Using a mouse model of acute lymphocytic choriomeningitis virus (LCMV) infection, we observed abundant EZH2 expression and associated H3K27me3 modifications preferentially in the early committed virus-specific T\textsubscript{FH} cells compared to those in T\textsubscript{eff}1 cells. Ablation of EZH2 in LCMV-specific CD4+ T cells leads to a selective impairment of early T\textsubscript{FH} cell fate commitment, but not late T\textsubscript{FH} differentiation or memory T\textsubscript{FH} maintenance. Mechanistically, EZH2 specifically stabilizes the chromatin accessibility of a cluster of genes that are important for T\textsubscript{FH} fate commitment, particularly B cell lymphoma 6 (Bcl6), and thus directs T\textsubscript{FH} cell commitment. Therefore, we identified the chromatin-modifying enzyme EZH2 as a novel regulator of early T\textsubscript{FH} differentiation during acute viral infection.

INTRODUCTION

During pathogenic infections, follicular T helper (T\textsubscript{FH}) cells provide essential assistance to cognate pathogen-specific B cells that enable them to initiate and sustain germinal center (GC) reactions in B-cell follicles within secondary lymphoid tissues.1 GC reactions lead to both the rapid production of high-affinity antibodies that protect against the immediate infection and the subsequent generation of persistent humoral immune memory for long-term protection.2,12 In the early stage of GC reactions, intimate interactions between early differentiated T\textsubscript{FH} cells and newly activated cognate B cells in the interfollicular regions of secondary lymphoid tissues direct the migration of both cell types to B-cell follicles, in turn cooperating to prime early GC responses.3 During ongoing GC reactions, T\textsubscript{FH} cells promote the survival, proliferation, class switching and hypermutation of cognate B cells and eventually drive them to differentiate into long-lived memory B cells and antibody-secreting plasma cells by secreting important cytokines such as IL-21,4 IL-45 and IL-96 and engaging certain cell surface-bound receptors and their matching ligands, including CD40-CD40L,7 ICOS-ICOSL7 and PD-1-PD-L1.8 Unlike other lineages of CD4+ T cells, T\textsubscript{FH} cells are programmed to express the chemokine receptor CXCR5.9-11 In response to the chemottractant CXCL13,12,13 CXCR5+ T\textsubscript{FH} cells migrate to B-cell follicles, where they physically interact with cognate B cells.1

Given the critical role of T\textsubscript{FH} cells in B-cell-mediated humoral immunity, investigations of the early fate commitment of T\textsubscript{FH} cells are very important, as the differentiation of T\textsubscript{FH} cells occurs within 48 h after acute viral infections.14,15 The transcriptional repressor B cell lymphoma-6 (Bcl-6) functions as a "master regulator" to govern early T\textsubscript{FH} cell differentiation.16-18 A rapid induction of Bcl-6 expression in de novo activated virus-specific CD4+ T cells represents a key step toward the T\textsubscript{FH} fate commitment. A wide variety of transcription factors (TFs) have recently been shown to regulate Bcl-6 expression during T\textsubscript{FH} differentiation.1 STAT family TFs, including STAT1,19 STAT320 and STAT4,21 promote Bcl-6 expression in response to stimulation with the corresponding cytokines, such as IL-6, IL-12 and IL-21. Batf,22 IRF4,23 Notch1 and Notch224 have also been reported to induce Bcl-6 expression and promote T\textsubscript{FH} differentiation. In contrast, the TFs Blimp-116 and Foxo115 inhibit Bcl-6 expression and subsequently repress T\textsubscript{FH} differentiation. Despite the known positive and negative effects of these TFs on Bcl-6 expression, researchers have not conclusively determined whether these regulators exert their effects during the early stage of T\textsubscript{FH} commitment. Transcription factor-1 (TCF-1) initiates the T\textsubscript{FH} fate commitment by directly inducing Bcl-6...
expression and suppressing Blimp-1 expression during an acute viral infection. Although TCF-1 is expressed at high levels in naive CD4+ T cells, activated virus-specific CD4+ T cells must further increase TCF-1 expression levels for subsequent T FH conversion following an acute viral infection. Currently, additional factors that are responsible for the early induction of the fate commitment of T FH cells remain to be determined.

Epigenetic modifications of histones have been extensively implicated in the mechanisms regulating T cell differentiation. The epigenetic regulator enhancer of zeste homolog 2 (EZH2), which is the catalytic subunit of polycomb repressive complex 2 (PRC2), functions as a methyltransferase to induce the trimethylation of histone H3 at lysine 27. EZH2-mediated H3K27me3 plays a critical role in the differentiation and lineage stability of various types of CD4+ T cells, including T HEL, T FH, and regulatory T (Treg) cells. Additionally, EZH2 and H3K27me3 inhibit the transcription of both TFs and eventually suppress the expression of corresponding genes. EZH2-mediated H3K27me3 also promotes TH1 cell differentiation by increasing the stability of T-bet and inducing the production of T-bet cytokines. In Treg cells, CD28-induced EZH2 expression promotes H3K27me3 deposition at gene loci that are normally repressed in Treg cells and thus plays an important role in stabilizing the lineage specification of activated Treg cells. Despite the profound effects of EZH2 on T HEL, T FH, and Treg differentiation, whether and how EZH2 regulates the T FH fate commitment remains to be investigated.

Here, we first defined a strict lineage-specific mode of chromatin accessibility in virus-specific T FH cells compared to virus-specific T HEL cells in response to an acute infection. Bona fide differentiatied virus-specific T FH cells exhibited increased EZH2 expression and the associated H3K27me3 modification compared to naive T cells and T HEL cells in the early days after an acute lymphocytic choriomeningitis virus (LCMV) infection. Furthermore, EZH2 was required for governing the chromatin accessibility of a cluster of T FH lineage-associated genes, particularly Bcl6, which are essential for T FH fate commitment. Accordingly, inactivation of EZH2 in virus-specific CD4+ T cells led to a pronounced reduction in the early commitment but not late differentiation or maintenance of T FH cells.

**MATERIALS AND METHODS**

Mice, virus, bacteria and tamoxifen treatment

CD45.1 SMARTA mice were a gift from Dr. R. Ahmed (Atlanta, USA). The Ezh2fl/fl, Cd4-Cre transgenic, ERT2-Cre transgenic and C57BL/6J (CD45.1 and CD45.2) mice were obtained from Jackson Laboratories. The LCMV Armstrong strain was provided by Dr. R. Ahmed (Atlanta, USA), and 2 x 10^6 plaque-forming units (PFUs) of this strain were intraperitoneally injected into mice to establish an acute viral infection. Listeria monocytogenes expressing the LCMV glycoprotein-specific I-A^k/-restricted CD4+ T cell epitope Gp61–80 was created from a vector strain, and 1 x 10^7 colony-forming units (CFUs) of the recombinant bacteria were intravenously injected to establish a bacterial infection in mice. Six- to ten-week old mice of both sexes were infected without randomization or “blinding”. Bone marrow (BM) chimera mice were infected 2 months after reconstitution. Tamoxifen (T5648; Sigma-Aldrich; 10 mg/ml) in sunflower oil (S5007; Sigma-Aldrich) was intraperitoneally injected into mice at a daily dose of 1 mg/mouse for 4 days. Infected mice were housed in accordance with the institutional biosafety regulations of the Third Military Medical University. All mouse experiments were performed according to the guidelines of the Institutional Animal Care and Use Committees of the Third Military Medical University.

ATAC-Seq library preparation

The ATAC-Seq libraries were prepared as previously described. Briefly, 50,000 target cells were washed with PBS and then treated with lysis buffer, followed by labeling with the Nextera enzyme (15027865; Illumina). The labeled samples were immediately amplified by 9–10 cycles of polymerase chain reaction (PCR) with barcoded primers and sequenced with a HiSeq4000 instrument in a 150 bp/150 bp paired-end run or a NextSeq500 instrument in a 76 bp/76 bp paired-end run.

ATAC-Seq data preprocessing

Raw sequencing reads were first trimmed of adapters to improve the quality using Trim Galore! v0.4.4 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), which is a wrapper based on CutAdapt v1.14 (ref. 41) and FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Paired-end reads that passed quality control (QC) were then aligned to mm10 using Bowtie2 v2.2.9 (ref. 42). The resulting BAM files were then filtered again to remove unmapped reads, mate-unmapped reads, nonprimary aligned reads, reads that failed platform quality checks and PCR duplicate reads using SAMtools v1.4.1 (ref. 43) (F1 1804). In addition, reads mapped to ChrM were also removed and PCR duplicate reads were further identified and removed using Picard v2.16.0 MarkDuplicate (https://broadinstitute.github.io/picard/).

The insert size distributions were then calculated using Picard v2.16.0 CollectInsertSizeMetrics. Since Tn5 transposase binds as a dimer and inserts two adaptors separated by 9 bp, all aligned reads were shifted by + 4 bp on the positive strand and – 5 bp on the negative strand using deepTools v2.5.2 alignmentSieve. Afterward, peak calling was performed using MACS2 v2.1.1, with a q-value threshold of 0.01. Peaks that overlapped with the Encode black list regions were then removed using bedtools intersect v2.26.0 (ref. 44). We then merged peaks from all replicates and filtered peaks that are not reproducible, based on the Irreproducible Discovery Rate (IDR) (ref. 45) (IDR < 0.005), at least one pair of replicates in each sample group. Subsequently, depending on the comparisons required for different purposes, filtered peaks from multiple sample groups were merged using the bedtools v2.26.0 merge algorithm to create genome-wide atlas of accessible chromatin regions for further analysis.

Assignment of ATAC-Seq peaks to genes

Each ATAC-Seq peak was uniquely assigned to a gene, with the corresponding feature annotations (e.g., exon, intron and intergenic) in R v3.4.1 using ChipPeakAnno v3.10.2, 49 with TxDb. The histone methyltransferase EZH2 primes the early differentiation of...
factors) within each peak region of the corresponding atlas peak file using deepTools v.2.5.4 multiBamSummary in BED-mode. The resulting matrix was input to DESeq2 v1.16.1 (ref. 50) to calculate the differential accessibility of the peaks of the relevant pairs. Principal component analysis (PCA) plots were then generated using DESeq2 v1.16.1.

Coverage plots and chromatin accessibility heat map
For each sample group, shifted and RPM normalized bam files were first converted to bedgraph files by counting the normalized reads in 10 bp bins and removing reads in the Encode blacklisted regions. Then, reads obtained from replicates of the same sample group were pooled using bedtools v2.26.0 unionbedg, and the resulting merged bedgraph files were used to generate a single coverage plot for each sample group, which was visualized in IGV v2.4.4 (ref. 31) and used to generate the chromatin accessibility heat map. Using deepTools v2.5.4 computeMatrix, each atlas peak was extended to a ±1 kb region from the peak center, and the reads from the final coverage plot were then separated into 10 bp bins to be represented as a row in the heat map. Selected differential peaks were then stacked together to generate the overall heat map using deepTools v2.5.4 plotHeatmap. Bins with read counts greater than a threshold, which was defined as the 75th percentile +1.5 x inter quartile range (IQR), were capped at that threshold value to increase the visibility of low-signal regions. Capping was only performed to plot the heat map, and all other analyses were performed with uncapped values.

Flow cytometry and antibodies
Flow cytometry data were obtained with a FACSCan
to II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star). The major histocompatibility complex (MHC) class II (I-A^d) tetramer of LCMV epitope of GP66–77 was provided by Dr. Rafi Ahmed (Emory University). The antibodies and reagents used for flow cytometry are listed in Supplementary Table 3. CXCXR5 staining has been described previously.15 Surface staining was performed in PBS containing 2% fetal bovine serum (wt/vol) on ice. Intracellular staining of Foxp3, Ezh2, H3K27me3, TCF-1 and Bcl-6 was performed using the Foxp3/Transcription Factor Staining Buffer Set (00–5523; eBioscience). MHC II GP66–77 tetramer staining was performed by incubating cells with the tetramer for 1 h at 37 °C.

Immunofluorescence staining
Immunofluorescence staining was performed using previously described methods.52 Briefly, frozen sections of the spleen were fixed with cold acetone for 10 min at 4 °C and blocked with 5% normal goat serum for 30 min. Sections were then stained with biotin-conjugated PNA (RL-1072; Vector), Alexa Fluor 647-conjugated IgD (11–26c.2a; eBioscience) and PE-conjugated CD4 (RM4–5; BD Biosciences) antibodies, followed by Alexa Fluor 488-conjugated streptavidin (25–4317–B2; Invitrogen). Cover slips on which both types of cells were cultured were mounted on slides with the ProLong Antifade Kit (P-7481; Life Technologies) and examined under a Zeiss LSM 510 confocal fluorescence microscope. The images were processed with ImageJ software.

Retroviral constructs and transduction
The sequences encoding the codon-improved Cre (iCre) gene or Bcl6 were amplified and cloned into the vector MIGR1 (MSCV-ires-GFP) or MIGR2 (MSCV-ires-hCD2), respectively. Retroviruses were packaged by transfecting 293T cells with the retrovector plasmids along with the pCLeco plasmid. SMARTA cells were activated in vivo by injecting 200 μg of the GP61–77 peptide into SMARTA mice. Eighteen hours later, activated SMARTA cells were purified and "spin-infected" by centrifugation (800 g) with retrovirus supernatants, 20 ng/ml IL-2 (130–098–221; Miltenyi Biotec) and 8 μg/ml polybrene (H9268; Sigma-Aldrich) at 37 °C for 90 min.

SMARTA cells were then transferred into recipient mice, followed by the infection of the hosts with LCMV Armstrong.

Adaptive transfer
A total of 5 × 10^6 (for analysis on days 2, 3 or 5) or 1 × 10^7 (for analysis on day 8 or later) CD45.1<sup>+</sup> SMARTA cells (naive or retrovirus-transduced) were adoptively transferred into CD45.2<sup>+</sup> recipients. On the following day, the recipients were intraperitoneally injected with 1 × 10^6 PFUs of LCMV Armstrong (day 2 or 5) or 1 × 10^7 CFUs of LM-GP66 (day 3) or were intraperitoneally injected with 2 × 10^5 PFUs of LCMV Armstrong (day 8 or later). For the EPZ6438-treated SMARTA cell transfer experiment, naive CD45.1<sup>+</sup> SMARTA cells were treated with EPZ6438 (2 μM; E-7438, Active Biochem) or vehicle at 37 °C for 3 days, and then transferred into CD45.2<sup>+</sup> recipient mice, followed by infection with LCMV Armstrong.

BM chimeras
A total of 2 × 10^6 BM cells harvested from Ezh2<sup>Hfl<sub>M</sub>Ert2-Cre</sup> (CD45.2<sup>+</sup>) mice and C57BL/6J wild-type (WT) (CD45.1<sup>+</sup>) mice were mixed at a ratio of 4:6. Mixed BM cells were then intravenously injected into lethally irradiated (two doses of 550 rads each) CD45.1<sup>+</sup> WT recipients. After a 2-month reconstitution, the recipient mice were infected with LCMV Armstrong.

ELISA
The LCMV-specific serum IgG titers were measured using an ELISA, as previously described.53,54 Briefly, plates were coated with LCMV-infected cell lysates, and LCMV-specific antibodies were detected with HRP-conjugated goat anti-mouse IgG secondary antibodies (Southern Biotech).

Microarray
TFH cells were isolated using a previously described method.15 Briefly, total splenocytes obtained from WT and Ezh2<sup>Hfl<sub>M</sub></sup>Cd4-Cre<sup>+</sup> mice on day 8 after LCMV Armstrong infection were subjected to the depletion of lineage marker-positive cells (Lin<sup>+</sup> cells) using biotin-conjugated antibodies (anti-B220 (RA3–6B2), anti-CD8 (S3.6.7), anti-CD11c (N418), anti-TER-119 (TER-119) and anti-NK1.1 (PK136); all from BioLegend), followed by coupling to BeaverBeads Mag500 Streptavidin (22302; Beaver). The enriched Lin<sup>−</sup> cells were then stained with anti-CD4, anti-CD44, anti-GITR, anti-CD25 and anti-CXCR5 antibodies (all identified in Supplementary Table 3). The CD4<sup>+</sup> CD25<sup>−</sup> GITR<sup>−</sup> CD44<sup>+</sup> CXCR5<sup>+</sup> TFH<sup>+</sup> cells were sorted with a FACS Aria II cell sorter (BD Biosciences) and then immediately lysed with TRIzol LS reagent (10296; Life Technologies). Then, total RNA was extracted and submitted to CapitalBio for a microarray analysis.

Quantitative reverse-transcription PCR
Sorted TFH cells were sorted directly into TRIzol LS reagent (10296; Life Technologies). Total RNA was extracted and reverse transcribed with the RevertAid Minus First Strand cDNA Synthesis Kit (K1632; Thermo Scientific). The relative expression of various genes was determined using AceQ qPCR SYBR Green Master Mix (Q111; Vazyme) with a CFX96 Touch Real-Time System (Bio-Rad). The primers for the indicated genes are summarized in Supplementary Table 4.

ChIP
ChIP assays of sorted TFH<sup>+</sup> cells, TFH<sup>−</sup>1 cells and naive CD4<sup>+</sup> T cells were performed using a Simple Enzymatic Chromatin IP Kit (Magnetic Beads) (9003; Cell Signaling Technology). The resulting chromatin fragments were immunoprecipitated with an anti-H3K27me3 antibody (9733; Cell Signaling Technology), followed by binding to ChIP Grade Protein G Magnetic Beads (9006; Cell Signaling Technology) and purification with a PCR purification kit (28104; Qiagen). Sequence-indexed libraries were prepared from...
immunoprecipitated chromatin fragments using the Illumina TruSeq indexed pair-ended DNA library preparation protocol and ultimately sequenced using the NextSeq500 platform.

ChIP-Seq analysis and coverage plot
The raw sequencing reads obtained from the Chip-Seq analysis were first trimmed and filtered with Trim Galore! v0.4.4, and then aligned in single-end mode and searched against mm10 using Bowtie2 v2.2.9. The reads were then filtered using samtools to remove low-quality reads and unmapped reads. Duplicate reads were also filtered using Picard v2.16.0 MarkDuplicates. Peak calling was performed using MACS2 v2.1.1 with a q-value threshold of 0.05 and the −5SPMR flag. The resulting bedgraph files were used to build a coverage plot using MACS2 v2.1.1 bedgcmp with the logLR method and a P-value of 1e−5.

Statistical analysis
Statistical analyses were conducted with Prism 6.0 software (GraphPad). An unpaired two-tailed t-test with a 95% confidence interval was used to calculate P-values. For retroviral transduction, SMARTA cell cotransfer, spleen chimera and BM chimera experiments, and a paired two-tailed t-test with the 95% confidence interval were used to calculate P-values.

RESULTS
Chromatin states of virus-specific T\text{FH} and T\text{FH} cells in response to acute viral infection
In response to an acute viral infection, activated virus-specific CD4+ T cells differentiate into either T\text{FH} or T\text{FH} cells.\textsuperscript{15,55,56} We first adoptively transferred LCMV-specific naïve SMARTA cells (expressing a transgenic T cell receptor specific for the LCMV glycoprotein epitope I-A\textsuperscript{A}GP66–77) into WT C57BL/6J recipients and subsequently infected the chimERIC recipients with the LCMV Armstrong strain to investigate the potential regulatory regions involved in this bifurcated differentiation at the genome level. Then, we sorted virus-specific SMARTA T\text{FH} cells and T\text{FH} cells from the SMARTA chimera mice on days 2, 5 and 8 after the infection (Supplementary Figure S1a–c) and subsequently performed an ATAC-Seq assay to measure the transposable-accessible chromatin.\textsuperscript{56} We also sorted naïve SMARTA cells (CD4+CD25−CD62L−CD44+CD122+) as a control (Supplementary Figure S1d). QC of the ATAC-Seq libraries revealed the characteristic DNA fragment length distribution and the expected peak distribution across genomic features (Supplementary Figure S2a, b). With these high-quality libraries, we assessed the differences in chromatin-accessible regions (ChARs) and found that compared to naïve cells, dramatic changes in the numbers of ChARs emerged as early as day 2 postinfection in both virus-specific T\text{FH} and T\text{FH} cells (Supplementary Figure S2c). Furthermore, chromatin-accessible patterns were discerned in T\text{FH} and T\text{FH} cells, respectively, at different time points postinfection (Fig. 1a). The greatest differences in ChAR patterns were observed between T\text{FH} and T\text{FH} cells at day 8 postinfection (Fig. 1a), reflecting the multiscale differentiation of fully functional T\text{FH} and T\text{FH} cells.\textsuperscript{5} A ChAR-based PCA further revealed that T\text{FH} and T\text{FH} cells started to enter two distinct CD4+ T cell differentiation states on day 2 postinfection, and these differentiation trajectories continuously developed through the entire process (Fig. 1b).

K-means clustering of the differential peaks revealed four distinct areas, and the third and fourth clusters (gene lists are provided in Supplementary Tables S1 and S2) revealed opened chromatin-accessible regions (ChARs) and found that compared to naïve cells. Notably, when focusing on cluster 4, we found an array of T\text{FH} lineage-associated gene loci that were enriched in this region, including Bcl6, Tcf7, Id3,\textsuperscript{51} Asc2,\textsuperscript{58} Cxcr5 and Il21 (Fig. 1d). The T\text{FH}1-associated genes Tbx21, Pردm1, HAvcr2, Ccl3, Ccl4 and Ccl5 were observed in cluster 3 (Fig. 1e). Further analysis of the ChARs for each individual gene locus revealed the stringent lineage-specific mode of chromatin accessibility; i.e., the chromatin accessibility of T\text{FH}1-associated genes was more prominent in T\text{FH}1 cells than in T\text{FH}1 cells, and vice versa (Fig. 1d and e). Based on these results, chromatin remodeling is tightly associated with the T\text{FH}1 but not T\text{FH}1 lineage commitment and differentiation in response to an acute viral infection.

Dynamic EZH2 expression and H3K27me3 modification in virus-specific T\text{FH} cells
The EZH2-mediated H3K27me3 modification plays a critical role in chromatin remodeling.\textsuperscript{59} Next, we sought to investigate the relationship between EZH2 expression and H3K27me3 modification during T\text{FH} cell differentiation in response to an acute viral infection. We first adoptively transferred naïve SMARTA cells into WT C57BL/6J recipients, followed by an infection with the LCMV Armstrong strain. On day 2 after infection, we compared EZH2 expression and H3K27me3 modification between naïve (CD44loCD25−), T\text{FH}1 (CD25+CXCR5+) and T\text{FH}1 (CD25+CXCR5+) SMARTA cells. We observed the highest levels of EZH2 expression in T\text{FH}1 cells compared to those in T\text{FH}1 and naïve cells, and T\text{FH}1 cells expressed a relatively higher level of EZH2 than the naïve cells (Fig. 2a). This phenotypic pattern was consistent with the levels of the EZH2-mediated H3K27me3 modification in T\text{FH}1 and naïve SMARTA cells (Fig. 2b). Next, we analyzed the kinetics of EZH2 expression in T\text{FH} SMARTA cells at different time points after infection and found that virus-specific T\text{FH} cells rapidly increased EZH2 expression upon infection and reached a peak on day 2 postinfection (Fig. 2c). EZH2 expression then decreased to a comparable level to naïve cells on day 8 postinfection (Fig. 2c). Consistent with the EZH2 expression kinetics, the direct target of EZH2, H3K27me3, exhibited similar kinetics during T\text{FH} cell differentiation (Fig. 2d).

The fate commitment of virus-specific T\text{FH} cells occurs within the first 48 h after an acute viral infection. Therefore, the high expression of EZH2 in early differentiated T\text{FH} cells on day 2 postinfection prompted us to examine whether the level of EZH2 expression correlated with T\text{FH} cell commitment. For this purpose, on day 2 after infection, we divided SMARTA cells into three subsets, EZH2hi, EZH2inter and EZH2lo, according to their EZH2 expression levels and observed that the EZH2hi subset was much more poised to adopt the TFH fate than the EZH2inter and EZH2lo subsets (Fig. 2e, f), while the opposite phenotype was observed during T\text{FH}1 differentiation (Fig. 2e, g). Compared to the EZH2inter and EZH2lo subsets, the EZH2hi subset expressed a significantly higher level of Bcl-6 (Fig. 2h), suggesting the enhanced propensity of this subset to become T\text{FH} cells. Consistent with these findings, the extent of the H3K27me3 modification was also positively correlated with EZH2 expression and an early T\text{FH} fate choice in SMARTA cells on day 2 after an acute viral infection (Fig. 2i–k).

Collectively, high EZH2 expression and the associated H3K27me3 modification in activated virus-specific CD4+ T cells preferentially drove them to differentiate into T\text{FH} cells during the early response to acute viral infection, while the cells displaying low EZH2 and H3K27me3 levels were more prone to differentiate into T\text{FH} cells.

Role of EZH2 in early T\text{FH} commitment during acute viral infection
Considering the positive correlation between the EZH2 expression level and T\text{FH} commitment, we next investigated whether EZH2 is indeed essential for early T\text{FH} differentiation upon acute viral infection. We crossed mice harboring IoxP-flanked \text{Ezh2} alleles (\text{Ezh2}\text{fl/fl}) with TCR-transgenic SMARTA mice that expressed the conjugic marker CD45.1 to generate \text{Ezh2}\text{fl/fl} SMARTA mice (hereafter designated \text{Ezh2}\text{fl/fl} SM). Subsequently, we used a retroviral transduction system to overexpress codon-improved Cre recombinase (iCre) for the conditional deletion of EZH2 in virus-specific \text{Ezh2}\text{fl/fl} SM cells. Then, we transferred...
Fig. 1 Chromatin states of the virus-specific T_{FH} and T_{H1} cells in response to an acute viral infection. a Numbers of chromatin peaks with differential accessibility (FDR < 0.05; FC > 4) between SMARTA T_{FH} cells and SMARTA T_{H1} cells at the indicated time points after LCMV Armstrong infection. b PCA plot of the peak accessibilities in naïve SMARTA CD4^{+} T cells, SMARTA T_{FH} cells (days 2, 5 and 8 postinfection) and SMARTA T_{H1} cells (days 2, 5 and 8 postinfection). Each dot represents a replicate of the indicated group. c Chromatin accessibility heat map of differential peaks from a. Each row represents one of the 15,600 differential peaks that was center-aligned and extended upstream and downstream by 1 kb from the center. The peaks are K-means clustered. d ATAC-Seq signal profiles of T_{FH} lineage-associated gene loci. e ATAC-Seq signal profiles of T_{H1} lineage-associated gene loci. Differential peaks are highlighted in gray (d and e). The data were obtained from one experiment with three biological replicates (pooled from at least five mice per group) (a–e)
The histone methyltransferase EZH2 primes the early differentiation of...
retrovirus-transduced and nontransduced Ezh2/fl mice or WT SM cells (Ezh2/+ SM) into naïve WT recipients that expressed the congeneric marker CD45.2 and subsequently infected animals with the LCMV Armstrong strain (Fig. 3a). On day 2 after infection, we visualized the efficient deletion of EZH2 expression in Ezh2/fl SM cells overexpressing iCre (Supplementary Figure S3a). Notably, only 4.7% of iCre-expressing Ezh2/fl SM cells differentiated into CD25lo CXCR5+ Foxp3+ cells, whereas ~20% of virus-specific Ezh2/+ SM cells (with and without iCre recombinase) and Ezh2/fl SM cells (without iCre recombinase) were committed to the Tfh cell fate (Fig. 3b, c), highlighting the importance of EZH2 in Tfh fate determination.

We bred Ezh2/fl mice with SMARTA mice that transgenically expressed Cre recombinase downstream of theCd4 enhancer, promoter and silencer sequences (hereafter called Ezh2/fl SM-Cre) to further confirm the role of EZH2 in the early differentiation of virus-specific Tfh cells. We next mixed equal numbers of Ezh2/+ SM-Cre cells with Ezh2/fl SM-Cre cells and cotransferred these cells into WT recipients, which were subsequently infected with the LCMV Armstrong strain (Fig. 3d). On day 2 after infection, we were able to visualize the efficiency of Ezh2 deletion in Ezh2/fl SM-Cre cells (Supplementary Figure S3b) and observed a remarkable reduction in the Tfh cell commitment of the Ezh2/fl SM-Cre cells compared to Ezh2/fl SM-Cre cells (Fig. 3e, f). Furthermore, we compared the expression levels of molecules that are closely associated with Tfh fate commitment, including TCF-1, Bcl-6, ICOS and CXCR5, between Tfh cells that differentiated from Ezh2/fl SM-Cre cells and Ezh2/+ SM-Cre cells (cells within the red circle in Fig. 3e). The levels of all examined molecules were substantially reduced in Ezh2/fl SM-Cre cell-derived Tfh cells compared to those in Tfh cells that differentiated from Ezh2/+ SM-Cre cells (Fig. 3g). Additionally, these phenotypes characterizing the early Tfh differentiation of Ezh2/fl SM-Cre cells in response to LCMV infection were largely confirmed to be the same cells upon an acute intracellular bacterial infection with a recombinant L. monocytogenes strain that expressed the LCMV glycoprotein epitope I-A/GP61–80 (Supplementary Figure S3e–i).

Next, we investigated whether the inhibition of the EZH2-mediated H3K27me3 modification also led to defects in the Tfh cell commitment of virus-specific CD4+ T cells in response to a viral infection. For this purpose, we treated SMARTA cells with vehicle or the small molecule EPZ6438, which is a specific inhibitor of the H3K27me3 modification, in vitro for 3 days and subsequently transferred these cells into WT recipients that were then infected with the LCMV Armstrong strain (Fig. 3h). Treatment with EPZ6438 efficiently reduced the levels of the H3K27me3 modification in SMARTA cells without affecting EZH2 expression (Supplementary Figure S3c and d). Notably, the inhibition of the H3K27me3 modification by the EPZ6438 treatment resulted in a substantial reduction in Tfh cell differentiation on day 2 postinfection compared to the Tfh cell commitment of vehicle-treated SMARTA cells (Fig. 3i, j). Furthermore, similar to results obtained using Ezh2/fl SM-Cre cell-derived Tfh cells (Fig. 3g), Tfh cells that differentiated from EPZ6438-treated SMARTA cells also exhibited lower expression of Tfh lineage-associated molecules, including TCF-1, Bcl-6, ICOS and CXCR5, compared to that of Tfh cells derived from vehicle-treated SMARTA cells (Fig. 3k). Taken together, these data validated the hypothesis that EZH2 expression and the subsequent H3K27me3 modification in virus-specific CD4+ T cells were important for early commitment to the Tfh cell fate in response to an acute viral infection.

Requirement for EZH2 expression in endogenous virus-specific Tfh cell differentiation
We bred Ezh2/fl mice with C4d-Cre transgenic mice to further analyze the role of EZH2 in non-Tcr transgenic, endogenous virus-specific Tfh cell differentiation. The resulting Ezh2/fl C4d-Cre mice conditionally lost EZH2 expression in their CD4+ T cell population (Supplementary Figure S4a). On day 8 after infection with the LCMV Armstrong strain, Ezh2/fl C4d-Cre mice exhibited a remarkable reduction in the virus-specific GP66–77 tetramer-positive CXCR5+ICOS+ Tfh cell population in the spleen compared to that in Ezh2/fl control mice (Fig. 4a, b). Furthermore, the tetramer-positive Tfh cells from Ezh2/fl C4d-Cre mice exhibited decreased levels of Tfh differentiation-associated molecules, such as Bcl-6, TCF-1, PD-1 and CXCR5, compared to Tfh cells from Ezh2/+ control mice (Fig. 4c). These data revealed the effect of EZH2 deficiency on the Tfh differentiation of endogenous virus-specific CD4+ T cells, which were highly consistent with our observations in the Ezh2-null TCR-transgenic SMARTA cells shown in Fig. 3. Additionally, we compared the Tfh differentiation of bulk virus-activated Foxp3+ C4d/+ T cells from Ezh2/fl C4d-Cre mice and Ezh2/fl control mice after LCMV infection. Similar to the impaired Tfh differentiation of GP66–77 tetramer-positive CD4+ T cells in Ezh2/fl C4d-Cre mice upon infection, the bulk-activated Foxp3+ C4d/+ C4d+ T cells from these mice also exhibited compromised C4d+CXCR5+ Tfh differentiation, as evidenced by the decreases in the proportion of Tfh cells, the Tfh to Tfh number and the absolute Tfh number compared to that in Ezh2/fl control mice (Supplementary Figure S4b–d). As expected, Tfh cells lacking EZH2 expression displayed reduced levels of an array of Tfh lineage-associated proteins, including TCF-1, Bcl-6, PD-1 and ICOS (Supplementary Figure S4e).

Consistent with the defective Tfh differentiation caused by Ezh2 deficiency in CD4+ T cells, Ezh2/fl C4d-Cre mice exhibited remarkably reduced frequencies and absolute numbers of PNA+FAS+GC B cells in the spleen on day 8 postinfection (Supplementary Figure S4f, g). The typical GC structure was also rarely observed in tissue sections of the spleens of Ezh2/fl C4d-Cre mice compared to sections from Ezh2/+ control mice, as revealed by a confocal microscopy analysis (Supplementary Figure S4h). Consistently, we noted less differentiation of B220+CD138+ plasma cells in the spleens of Ezh2/fl C4d-Cre mice than that in the spleens of Ezh2/fl control mice (Supplementary Figure S4i and j). Given the defective Tfh differentiation and consequent scarcity of the GC B cell population in response to LCMV infection, Ezh2/fl C4d-Cre mice exhibited substantially reduced LCMV-specific IgG titers on days 8 and 90 postinfection compared with those of Ezh2/+ controls (Supplementary Figure S4k).

We generated BM chimeras by mixing congenitally marked BM cells from Ezh2/fl ERT2-Cre mice (expressing a fusion protein with a Cre recombinase sensitive to tamoxifen and Cre; Cd45.2, 40%) with BM cells from WT mice (Cd45.1, 60%) and subsequently injecting these mixtures into irradiated WT recipients to more precisely evaluate the role of cell-autonomous EZH2 in the mechanism regulating endogenous Tfh differentiation (Fig. 4d). After reconstitution, we first administered tamoxifen to efficiently delete EZH2 (Supplementary Figure S5a) and then infected these chimeric mice with the LCMV Armstrong strain. On day 8 after infection, a decreased percentage of Bcl-6+CXCR5+ Tfh cells was observed among GP66–77 tetramer-positive CD4+ T cells originating from Ezh2/+ ERT2-Cre mice compared with that of cells of WT origin (Fig. 4e, f). Significantly lower levels of TCF-1, Bcl-6, PD-1 and CXCR5 were also detected in Ezh2/-deficient Tfh cells (Ezh2/fl ERT2-Cre) than those in control Tfh cells (WT) (Fig. 4g). We observed similar phenotypes in bulk Tfh cells from Ezh2/fl ERT2-Cre and WT mice (Supplementary Figure S5b–e). Altogether, these data highlighted the importance of cell-intrinsic EZH2 in the mechanism regulating endogenous Tfh differentiation in response to an acute viral infection.

EZH2 is not required for the late differentiation and maintenance of virus-specific Tfh cells during an acute viral infection
We next determined the role of EZH2 in the late differentiation of Tfh cells during an acute infection. BM chimeras were generated by mixing BM cells from Ezh2/fl ERT2-Cre mice (Cd45.2, 40%) with...
The histone methyltransferase EZH2 primes the early differentiation of... X Chen et al.

**Fig. 3** Role of EZH2 in early TFH commitment during an acute viral infection. 

**a** Experimental setup. A retrovirus overexpressing iCre was introduced into CD45.1\(^+\)Ezh2\(^{+/+}\) SMARTA (SM) cells and CD45.1\(^+\)Ezh2\(^{+/+}\) SM cells, which were transferred into CD45.2\(^+\) WT recipients. Then, the recipients were infected with the LCMV Armstrong strain and analyzed on day 2 postinfection. **b** Flow cytometry analysis of Ezh2\(^{+/+}\) and Ezh2\(^{+/+}\) SM cells transduced with a retrovirus expressing iCre. The numbers adjacent to the outlined areas indicate the percentages of CD25\(^{lo}\)CXCR5\(^+\) TFH cells, which are summarized in **c**.

**d** Experimental setup. Ezh2\(^{+/+}\) Cd4\(^{-}\)-Cre SMARTA cells (Ezh2\(^{+/+}\) SM-Cre; CD45.1\(^+\)CD45.2\(^-\)) and Ezh2\(^{+/+}\) Cd4\(^{-}\)-Cre SMARTA cells (Ezh2\(^{+/+}\) SM-Cre; CD45.1\(^+\) CD45.2\(^-\)) were cotransferred into WT recipients (CD45.1\(^-\)CD45.2\(^+\)), which were infected with the LCMV Armstrong strain and assessed on day 2 postinfection. **e** Flow cytometry analysis of Ezh2\(^{+/+}\) SM-Cre and Ezh2\(^{+/+}\) SM-Cre SMARTA cells. The numbers adjacent to the outlined areas indicate the percentages of CD25\(^{lo}\)CXCR5\(^+\) TFH cells, which are summarized in **f**.

**g** Quantification of TCF-1, Bcl-6, ICOS and CXCR5 levels in the CD25\(^{lo}\)CXCR5\(^+\) TFH cells shown in **e**. **h** Experimental setup. After treatment with either EPZ6438 or vehicle for 3 days, CD45.1\(^+\) SMARTA cells were transferred into WT CD45.2\(^+\) recipients that were subsequently infected with the LCMV Armstrong strain. The adoptively transferred SMARTA cells were analyzed on day 2 postinfection. **i** Flow cytometry analysis of EPZ6438-treated and vehicle-treated SMARTA cells. The numbers adjacent to the outlined areas indicate the proportions of CD25\(^{lo}\)CXCR5\(^+\) TFH cells, which are summarized in **j**. Total numbers of CD25\(^{lo}\)CXCR5\(^+\) TFH cells in **e** are presented in **j** (right panel). **k** Quantification of TCF-1, Bcl-6, ICOS and CXCR5 levels in the CD25\(^{lo}\)CXCR5\(^+\) TFH cells shown in **j**. NS not significant; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (paired two-tailed t-test **c, f and g** or unpaired two-tailed t-test **j and k**). The data are representative of two independent experiments with at least three mice (**c, f, g, j and k**) per group (error bars in **j and k** indicate the s.d.).
BM cells from WT mice (CD45.1, 60%) and subsequently injecting the mixtures into irradiated WT recipients (Fig. 5a). After reconstitution, these chimeric mice were initially infected with the LCMV Armstrong strain and then administered tamoxifen from days 4 to 7 after infection to induce the deletion of EZH2 (Fig. 5b). Notably, we observed comparable virus-specific TFH cell proportions between cells from Ezh2fl/flERT2-Cre and WT mice (Fig. 5c, d). Consistent with the intact late TFH cell differentiation, levels of molecules related to TFH cell differentiation, including Bcl-6, PD-1 and ICOS, were also similar between cells from Ezh2fl/flERT2-Cre and WT mice (Supplementary Figure S6a, b) generated from WT TFH cells, Ezh2-KO TFH cells and naive CD4+ T cells. Compared to naive CD4+ cells, both WT TFH and KO TFH cells showed significant changes in opening and closing peaks in the genome, suggesting the presence of different chromatin states (Fig. 6a). This finding was clearly revealed in the PCA plot (Fig. 6b); the KO TFH cells showed a chromatin state that differed from WT TFH cells and naive T cells. The EZH2 deficiency resulted in the remodeling of a cluster of specific gene loci that are closely associated with TFH differentiation, which were almost identical to the loci identified in Fig. 1d, including Bcl6, Tcf7, Ascl2, Ccnc5, Icos, Il21 and Sh2d1a (Fig. 6c). Notably, these loci were less open in Ezh2 KO TFH cells than those in WT TFH cells (Fig. 6c). Because the EZH2-directed H3K27me3 modification is generally associated with a closed state of chromatin accessibility, we further probed whether these loci were marked by the H2K27me3 modification. Therefore, we sorted virus-specific TFH cells and T cells from the SMARTA chimeric mice on day 2 after the LCMV Armstrong infection and naive CD4+ T cells (CD4+CD25-CD62L-CD44+) from naive mice and subsequently performed H3K27me3 ChIP-Seq experiments. Strikingly, Tfh-associated genes (e.g., Bcl6, Tcf7, Ascl2 and Ccnc5) were selectively marked by the EZH2-associated H3K27me3 modification in Tfh cells, but not in T cells, during the bifurcated CD4+ T cell differentiation (Fig. 6d) Thus, although EZH2 functions as a chromatin repressor, it appeared to rewind the chromatin states of certain Tfh-associated genes to favor TFH differentiation.

EZH2 remodels TFH lineage-associated chromatin accessibility during viral infection

We investigated the impact of the EZH2 deficiency on chromatin state changes in virus-specific TFH cells to obtain an understanding of the epigenetic modifications mediated by EZH2 to regulate TFH differentiation. Therefore, we analyzed ATAC-Seq libraries (Supplementary Figure S6a, b) generated from WT TFH cells, Ezh2-KO TFH cells and naive CD4+ T cells. Compared to naive CD4+ cells, both WT TFH and KO TFH cells showed significant changes in opening and closing peaks in the genome, suggesting the presence of different chromatin states (Fig. 6a). This finding was clearly revealed in the PCA plot (Fig. 6b): the KO TFH cells showed a chromatin state that differed from WT TFH cells and naive T cells. The EZH2 deficiency resulted in the remodeling of a cluster of specific gene loci that are closely associated with TFH differentiation, which were almost identical to the loci identified in Fig. 1d, including Bcl6, Tcf7, Ascl2, Ccnc5, Icos, Il21 and Sh2d1a (Fig. 6c). Notably, these loci were less open in Ezh2 KO TFH cells than those in WT TFH cells (Fig. 6c). Because the EZH2-directed H3K27me3 modification is generally associated with a closed state of chromatin accessibility, we further probed whether these loci were marked by the H2K27me3 modification. Therefore, we sorted virus-specific TFH cells and T cells from the SMARTA chimeric mice on day 2 after the LCMV Armstrong infection and naive CD4+ T cells (CD4+CD25-CD62L-CD44+) from naive mice and subsequently performed H3K27me3 ChIP-Seq experiments. Strikingly, Tfh-associated genes (e.g., Bcl6, Tcf7, Ascl2 and Ccnc5) were selectively marked by the EZH2-associated H3K27me3 modification in Tfh cells, but not in T cells, during the bifurcated CD4+ T cell differentiation (Fig. 6d). Thus, although EZH2 functions as a chromatin repressor, it appeared to rewind the chromatin states of certain Tfh-associated genes to favor TFH differentiation.
Bcl-6 overexpression rescues compromised H3K27me3 modification-induced T FH cell differentiation

Consistent with the reduced accessibility of important ChARs in T FH cells caused by the EZH2 deficiency (Fig. 6c), the expression of the corresponding genes was substantially reduced, as revealed by microarray analyses (Fig. 7a). In addition to the genes analyzed in Fig. 6c (including Bcl6, Tcf7, Id3, A2sl2, Cxcr5, Icos, Il21 and Sh2d1a), we also observed the downregulation of the mRNA levels of several other T FH differentiation-associated genes, such as Tcf3, Cad40lg, Pdcd1, Egr3, Lef1, Batf and Cd28 (ref. 62) (Fig. 7a). We further confirmed our discoveries by performing reverse-transcription quantitative PCR (RT-PCR) assays (Fig. 7b). These results raised the possibility that EZH2-mediated H3K27 trimethylation increases the chromatin accessibility, primarily at T FH differentiation-associated genes, which then induce a favorable transcription program directing T FH lineage commitment.

Bcl-6 is the master regulator of T FH cell differentiation.16–18 Because virus-specific CD4+ T cells with abundant Bcl-6 and EZH2 expression tend to differentiate into the T FH cell fate (Fig. 2f–h), and lower levels of the Bcl-6 protein (Figs. 3g, k, 4c and g) and mRNA (Fig. 7a, b) and reduced chromatin accessibility (Fig. 6c) are observed in EZH2-deficient T FH cells, we hypothesized that the impaired T FH cell commitment observed in the absence of EZH2 is potentially driven by compromised Bcl-6 expression and that Bcl6 overexpression might rescue the defective T FH differentiation of activated virus-specific CD4+ T cells with decreased EZH2 activities. Naive SMARTA cells were transduced with a retrovirus encoding Bcl-6 and then treated with vehicle or the H3K27me3 inhibitor EPZ6438 in vitro for 3 days to evaluate this hypothesis. After treatment, these cells were adoptively transferred into WT recipients, which were immediately infected with the LCMV Armstrong strain (Fig. 7c). Consistent with published studies,16–18 forced Bcl-6 expression biased vehicle-treated SMARTA cells to T FH cell differentiation on day 5 postinfection (Fig. 7d, e; 42.5% vs. 76.2%). Meanwhile, a substantial reduction in the T FH cell percentage (Fig. 7c) was observed in the EZH2-deleted T FH cells originating from EZH2-deleted SMARTA cells (40%). Two months after reconstitution, the recipients were infected with LCMV Armstrong, followed by the administration of tamoxifen from days 4 to 7 after infection and an analysis on day 8. B. Expression of EZH2 in virus-specific Bcl6hiCXCR5+ T FH cells from the BM chimera WT and Ezh2ERT2Cre mice shown in a. c Flow cytometry analysis of GP66–77 tetramer-positive CD4+ T cells in the spleens of the chimeras described in a on day 8 after the LCMV Armstrong infection. Numbers adjacent to outlined areas indicate the percentage of Bcl-6hiCXCR5+ T FH cells, which were summarized in d. e Levels of Bcl-6, TCF-1, PD-1 and ICOS in the Bcl-6hiCXCR5+ T FH cells shown in e. f The BM chimeras described in a were infected with LCMV Armstrong treated with tamoxifen on days 9 to 12 after infection and analyzed on day 60. g Quantification of EZH2 expression in virus-specific CXCR5+ T FH cells originating from BM chimergic WT and Ezh2ERT2Cre mice shown in f. h Flow cytometry analysis of GP66–77 tetramer-positive CD4+ T cells in the spleens of the chimeras described in f on day 60 after the LCMV Armstrong infection. Numbers adjacent to outlined areas indicate the percentage of CXCR5+ T FH cells, which were summarized in i. NS not significant; *P < 0.05 and ***P < 0.001 (paired two-tailed t-test (b, d, e, g and I)). The data are representative of two independent experiments with at least three mice (b, d, e, g, h and I) per group.
the EPZ6438-treated group compared to that in the vehicle-treated group (Fig. 7d, e; 42.5% vs. 19.9%), consistent with our aforementioned data (Fig. 3j). Strikingly, Bcl-6 overexpression in EPZ6438-treated SMARTA cells restored the TFH cell commitment to a similar level as the forced Bcl-6 expression-amplified TFH cell commitment in the vehicle group (Fig. 7d, e; 76.2% vs. 67.9%).

Taken together, the EZH2-associated H3K27me3 modification is a crucial positive determinant of Bcl-6 expression, which further directs TFH cell differentiation.

**DISCUSSION**

During the initial phase of an acute viral infection, activated virus-specific CD4+ T cells differentiate into either Th1 or TFH cells.15,55,56 Bcl-6 is required to specify the TFH fate choice,16–18 and Blimp-1 antagonizes this effect by suppressing Bcl-6 expression and Bcl-6-mediated transcriptional activity.16 Thus, the Bcl-6–Blimp-1 balance coordinates the early TFH commitment. The preserved and elevated TCF-1 expression in activated virus-specific CD4+ T cells induces Bcl-6 expression but inhibits Blimp-1 expression, and thus, it plays a critical role in guiding the TFH commitment pathway.15 Currently, little is known about the epigenetic mechanism that stabilizes these TF networks during early TFH fate determination. In this study, we defined a distinct chromatin state in virus-specific TFH cells compared to virus-specific Th1 cells in response to an acute infection. Importantly, early fate-committed TFH cells exhibited higher expression of the histone methyltransferase EZH2 in response to an acute viral infection and

**Fig. 6** Role of EZH2 in the remodeling of TFH lineage-associated chromatin accessibility during viral infection. a Numbers of chromatin peaks with differential accessibility (FDR < 0.05; FC > 1.5) in naïve CD4+ T cells (N), WT TFH cells and KO TFH cells. b PCA plot of the peak accessibilities in naïve CD4+ T cells (green), WT TFH cells (red) and KO TFH cells (blue). Each dot represents a replicate of the group. c ATAC-Seq signal profiles of TFH lineage-associated gene loci in naïve CD4+ T cells (N), WT TFH cells and KO TFH cells. d Tracks of H3K27me3 ChIP-Seq data from TFH lineage-associated gene loci in naïve CD4+ T cells (N), TFH cells and Th1 cells. The data were obtained from two independent experiments with one biological replicate (pooled from at least five mice per group) in each experiment (a–c) and from one experiment with one biological replicate (d)
across H3K27me3 modification compared with that of their TH1 counterparts. The ablation of EZH2 by genetic deletion in virus-specific CD4+ T cells substantially reduced the early TFH commitment by disrupting the TFH-specific chromatin state. Specifically, EZH2 is required for the stabilization of chromatin accessibility and transcription pattern of Bcl6, which is essential for the TFH fate commitment. Thus, our results highlighted the importance of EZH2, a widely recognized chromatin repressor, in the priming of TFH cell fate commitment by reinforcing Bcl-6 expression in activated TFH precursors.

TFH differentiation is characterized as a stepwise process:1 early fate commitment (2 days after an acute infection, occurring at the T–B border), maturation (3–4 days after an acute infection, occurring in B-cell follicle and pre-GC) and memory formation (~1–2 months after an acute infection). In our study, the chromatin identity of virus-specific TFH cells was discerned from virus-specific TH1 cells within the first 2 days after viral infection. In particular, chromatin remodeling of a cluster of TFH lineage differentiation-associated genes, including Bcl6, Tcf7, Id3, Ascl2, Cxcr5, Icos, Il21 and Sh2d1a, was observed in virus-specific TFH cells. This distinct
The histone methyltransferase EZH2 primes the early differentiation of... X Chen et al.

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ADDITIONAL INFORMATION

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