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Ezh2 Regulates Transcriptional and Posttranslational Expression of T-bet and Promotes Th1 Cell Responses Mediating Aplastic Anemia in Mice

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Acquired aplastic anemia (AA) is a potentially fatal bone marrow (BM) failure syndrome. IFN-γ-producing Th1 CD4+ T cells mediate the immune destruction of hematopoietic cells, and they are central to the pathogenesis. However, the molecular events that control the development of BM-destructive Th1 cells remain largely unknown. Ezh2 is a chromatin-modifying enzyme that regulates multiple cellular processes primarily by silencing gene expression. We recently reported that Ezh2 is crucial for the generation of BM-destructive Th1 cells through a mechanism of transcriptional and posttranslational regulation of T-bet. These results also highlight the therapeutic potential of Ezh2 inhibition in reducing AA and other autoimmune diseases. The Journal of Immunology, 2014, 192: 000–000.
the molecular pathways that control T-bet expression in Th1 cells can lead to new strategies to control AA.

Ezh2 is a histone methyltransferase that specifically catalyzes trimethylation of histone H3 at lysine 27 (H3K27me3) (19). Ezh2 forms polycomb repressive complex 2 together with other polycomb group proteins Suz12 and Eed (19), which is crucial for maintaining the cellular memory and transcriptional patterns primarily through a mechanism of silencing genes (20, 21). Several studies indicate an important role of Ezh2 and H3K27me3 in multiple lineages of effector T cells (22–25). Genome-wide mapping analysis revealed that repressive H3K27me3 marked genes associated with differentiation and maintenance of effector and memory T cells (26, 27). Recently, we demonstrated new and essential roles of Ezh2 in regulating inflammatory T cell responses in mice after allogeneic BMT (28). Loss of Ezh2 led to impaired production of alloreactive T cells that induce damage to epithelial organs (28). However, whether Ezh2 mediates pathogenic Th1 responses in AA and the mechanism of Ezh2 action in regulating Th1 cells remain unknown.

Mouse models of human AA have been established successfully (4, 29). Transfer of parent lymph node (LN) cells into haploidalike daughter recipients caused BM hypoplasia and blood pancytopenia, typical features of clinical AA. These AA mouse models have proved to be a unique approach studying pathophysiology of immune cell-mediated BMF (4, 6, 30, 31). In this report, we exploited the functional impact of Ezh2 on Th1 cell responses in vitro and in vivo. Using genetic approaches and a mouse model of human BMF, we identified a novel and critical role of Ezh2 in regulating Th1 cells mediating AA.

Materials and Methods

Mice

C57BL/6 (B6, H-2b) and B6xdBAC2 F1 (BDF1, H-2b/b; C57BL/6 × DBA/2) mice were purchased from Taconic (Rockville, MD). Cd4-Cre mice were originally derived from the Jackson Laboratory. B6/129 mice with floxed alleles of Ezh2 (Ezh2f/f) (32) were crossed to Cd4-Cre mice, before backcrossing to the B6 background (>8 generations). Age-matched and sex-matched controls were used. Experimental protocols were approved by the University of Michigan’s Committee on Use and Care of Animals.

Abs and flow cytometry analysis

Abs used for immunofluorescence staining were purchased from eBioscience, BioLegend, or BD Biosciences. Flow cytometry analyses were performed using FACScan and Canto cytometer (Becton Dickinson) as described (33).

Induction of AA

The mouse AA model was induced as described previously (30, 31). B6 LN cells (10 × 10⁶/mouse) isolated from B6 mice were transplanted into irradiated BDF1 mice (6.5 Gy). Peripheral blood was collected from the heart and the lateral tail vein. Complete blood counts were performed using a Hemavet 950 analyzer (Drew Scientific). BM cells were extracted from bilateral tibiae and femurs. Lymphocytes were isolated from inguinal, axillary, and lateral axillary LNs.

Histologic examination

The histologic examination was performed on paraffin-embedded femur pieces, fixed with 10% formalin, and colored with H&E. The histologic image was photographed with a OlympusBX41 microscope (1000.3 NA lens, original magnification x100; digital DP70 camera).

Real time RT-PCR

Total RNA was extracted from the indicated CD4+ T cell subsets using TRIzol (Invitrogen Life Technologies). cDNA was quantified through the quantitative real-time PCR technique. Real-time PCR was performed with a SYBR Green PCR mix on a Mastercycler realplex (Eppendorf). Thermostability conditions included an initial holding at 95°C for 2 min; this was followed by a three-step PCR program as follows: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 45 cycles. Transcript abundance was calculated using the ΔΔCt method (normalization with 18 s). The primer sequences are listed in Supplemental Table I.

Cell culture

Splenic and LN CD4+ T cells were prepared by MACS purification with CD4 microbeads (Miltenyi Biotec), and the purity was usually 90–95%. Th1-skewing culture conditions were set up as previously described (34–36). In brief, CD4+CD44naive T cells (Tn) were cultured in the presence of anti-CD3 (2 µg/ml; BioLegend) and anti-CD28 (2 µg/ml; BioLegend) Abs together with recombiant human IL-2 (10 ng/ml; R&D Systems), recombinant mouse IL-12 (5 ng/ml; R&D Systems) and BM-derived dendritic cells at a ratio of 1:16. Cultured T cells were restimulated with anti-CD3 Ab (1 µg/ml; BioLegend) 5 h before intracellular staining.

Retroviral construction and T cell infection

The MigR1 retroviral vector system was described previously (35, 37). MigR1 vector was provided by Warren Pear (University of Pennsylvania), and MigR1 vector encoding T-bet or STAT4 was provided by Steve Reiner (Columbia University, New York, NY) and Takashi Usui (Kyoto University, Kyoto, Japan). MigR1 virus was produced as described (38). For retroviral infection, CD4+ T cells were pretreated with anti-CD3 and anti-CD28 Abs for 24 h, and then the retrovirus supernatant was added in the presence of 8 µg/ml polybrene (Sigma). Cells were spinoculated at 3000 rpm, 32°C for 3 h. The same retroviral infection procedure was repeated 24 h later.

Western blot analysis

Western blot was performed as described (28). Cell lysates were examined with routine Western bloting. The blots were incubated with anti-Ezh2 (612667; BD Biosciences), Stat4 (sc-486; Santa Cruz Biotechnology), T-bet (sc-21749; Santa Cruz Biotechnology), GATA-3 (S58868; BD Biosciences), or actin (ab3280; Abcam) Abs, and subsequently incubated with HRP-conjugated anti-rabbit or mouse IgG (Vector Laboratories) in TBS containing 5% nonfat dry milk and 0.05% Tween 20. The final reaction was developed with a chemiluminescent system (Pierce).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described by using EZ-Magna ChIP Kit (17-10086; Millipore) (27, 39). Sonicated extracts were precleared and incubated with Abs specific to Ezh2 (39001, active motif), H3K27me3 (PAb-069-050; Diagenode) or H3K4me3 (9751S; Cell Signaling Technology) at 4°C overnight on a 360°C rotator. The immunoprecipitated DNA was quantitated by real-time quantitative PCR. The primer sequences are listed in Supplemental Table II.

ELISA

CD4+ Tn isolated from WT B6 and T-KO mice were stimulated with anti-CD3 (2 µg/ml; BioLegend) and anti-CD28 (2 µg/ml; BioLegend) Abs together with recombiant human IL-2 (10 ng/ml; R&D Systems) and recombiant mouse IL-12 (5 ng/ml; R&D Systems). On day 7, cells were restimulated in 96-well plates with plate-bound anti CD3 Abs (1 µg/ml; BioLegend) for 5 h before collecting the supernatants. Each group contained an equal number of cells (1 × 10⁶ cells/ml). The concentrations of IFN-γ and IL-4 were measured in triplicate using recombinant mouse IFN-γ and IL-4 ELISA kits in accordance with the manufacturer’s instructions (BioLegend).

 Luciferase reporter assay

The Tbx21 promoter region ranging from +0.3 to −1.0 kb of the transcription start site (TSS) was cloned to pGL3 luciferase reporter vector to generate Tbx21-specific reporter (named pGL3-Tbx21 reporter). 3T3 cells were cotransfected with pGL3-Tbx21 reporter plasmid and MigR1 viral plasmid encoding Ezh2 or empty MigR1 vector. The cells were harvested 48 h after transfection and analyzed with the Dual Luciferase system (Promega).

Statistical analysis

Survival in different groups was compared using the log-rank test. Comparison of two means was analyzed using the two-tailed unpaired Student t test.

Results

In the absence of Ezh2, LN cells are defective in mediating AA in mice

We used a genetic approach to determine the role of Ezh2 in the regulation of T cell–mediated AA. Mice with floxed alleles of
Ezh2 (Ezh2<sup>fl/fl</sup>) (32) were crossed to B6 mice expressing Cre recombinase under control of the CD4 promoter to generate T cell–specific Ezh2 conditional knockout B6 mice (named T-KO). These T-KO mice were further backcrossed to B6 mice for 10 generations. The development of mature thymocytes and T cells in peripheral lymphoid tissues was normal in these T-KO mice, which is in agreement with previous observations (28, 32).

To assess whether conditionally deleting Ezh2 in T cells affected their ability to mediate AA, we transferred donor LN cells derived from WT and T-KO B6 mice into irradiated (6.5 Gy) BDF1 recipients. In this setting, transfer of donor LN cells causes severe BM destruction and blood pancytopenia in these haploidentical recipients, which closely reflects the pathogenesis of human AA (4, 6, 30). As expected, BDF1 mice receiving WT B6 LN cells developed BM hypoplasia and severe blood pancytopenia after LN cell infusion compared with control mice receiving only total body irradiation, with all of them dying from the disease within 12 d after transfer (Fig. 1A). Histologic examination showed the destruction of BM and lack of hematopoietic cell islands in the BM of these recipients (Fig. 1B). In contrast, transfer of T-KO LN cells did not cause severe AA in these BDF1 recipients (Fig. 1A, 1B). As compared with control mice receiving total body irradiation, there was no significant reduction of BM cellularity and peripheral blood WBCs in these BDF1 mice receiving T-KO LN cells (Fig. 1C, 1D). Importantly, all T-KO LN cell recipients survived without clinical signs and histologic evidence of AA (Fig. 1A, 1B). Thus, T cells required Ezh2 to mediate AA.

Ezh2 is required for the development of Th1 cells inducing AA in mice

Previous studies have demonstrated that Th1 cells are crucial for inducing BMF in this model of experimental AA (2, 3). To examine the effects of Ezh2 deficiency on Th1 cell development in vivo, we harvested donor T cells from the spleen, LN, and BM of these mice 10 d after transfer of WT or T-KO LN cells. We found that loss of Ezh2 led to a significant reduction in the percentage and number of Th1 cells in the spleen, LN, and BM (Fig. 2A, 2B). Real-time RT-PCR analysis showed that alloantigen-activated T-KO T cells expressed dramatically lower levels of Ifng (5-fold), Stat4 (2.5-fold), and Tbx21 (4-fold) transcripts than their WT counterparts (Fig. 2C).

In this BMF model, only a small percentage of IL-4–producing CD4<sup>+</sup> T cells occurred in the spleen, LN, and BM of BDF1 mice receiving WT LN cells (Fig. 2A, 2B), suggesting that the inflammatory stimuli produced during the AA process predominantly induces Th1 cell differentiation in vivo. Ezh2 deficiency did not result in significant changes in the ability of CD4<sup>+</sup> T cells to produce IL-4 protein as assessed by flow cytometry (Fig. 2A). Furthermore, loss of Ezh2 had no significant effect on the expression of Th2 cell genes (e.g., Il4, Il5, Il13, Stat6, and Gata3; Fig. 2C). There was a moderate reduction in Rorγt and an increase in Foxp3 (Fig. 2C). However, these moderate changes in genes expression did not lead to the reduction in Il17 expression (Fig. 2C) and increase in regulatory T cells (Tregs; data not shown).

Collectively, these results suggest that during the AA process, Ezh2 is critically involved in regulating the development of Th1 cells, but has little effect on Th2 or Th17 cells.

Data from our previous studies and others indicate that Ezh2 deficiency can lead to impaired expansion and survival of activated T cells (28, 40). It is possible that the inability of T-KO LN cells to cause AA could result from decreased expansion and survival of BM-destructive Th1 cells. To test this possibility, we tracked the longitudinal proliferation and differentiation of WT and T-KO LN cells that were injected into sublethally irradiated BDF1 recipients. Compared with WT LN cells, T-KO LN cells produced significantly less in frequency of both CD4<sup>+</sup> T cells and IFN-γ–producing CD4<sup>+</sup> T cells in the spleen, LN, and BM 6 and 10 d after transfer (Fig. 3A, 3B). Because WT LN cells caused lethal AA in all BDF1 recipients by day 12 (data not shown), we were prevented from further comparing the difference between WT and T-KO LN cells at later time points. However, in BDF1 mice receiving T-KO LN cells, although there was a marked increase in numbers of total donor CD4<sup>+</sup> T cells in the spleen, LN, and BM at day 43 after transfer compared with that at day 6 (Fig. 3C), the frequency of IFN-γ–producing CD4<sup>+</sup> T cells was not increased in parallel (Fig. 3B). These data suggest that the lack of both differentiation and expansion of Th1 cells may be responsible for the inability of T-KO T cells to mediate AA early...
during disease process, whereas impaired Th1 differentiation of T-KO T cells could account for reduced AA during later stage.

**Ezh2 promotes in vitro Th1 cell differentiation in cultures under Th1-skewing conditions**

To further examine the importance of Ezh2 in regulating Th1 cell differentiation, we highly purified CD4+ Tn from WT and T-KO B6 mice and cultured them under Th1-skewing conditions without anti–IL-4 Ab. One day after activation in Th1-skewing cultures, both WT and T-KO CD4+ Tn produced barely detectable IFN-γ as assessed by intracellular cytokine staining (Fig. 4A). Three days after culture, there were 3.7-fold less in frequency of IFN-γ-producing CD4+ T cells in the culture of T-KO CD4+ T cells than that of WT CD4+ T cells (Fig. 4A). This impaired ability of T-KO CD4+ T cells to produce IFN-γ persisted throughout a period of 7 d during culture (Fig. 4A). ELISA further confirmed that T-KO CD4+ T cells secreted ∼2.5-fold less IFN-γ than WT CD4+ T cells did 7 d after culture (Fig. 4B), when Th1 cells fully developed (12, 35). These data confirmed the observations in our preceding experiments in vivo (Fig. 2) that Th1 cell differentiation was impaired in the absence of Ezh2.

It has been shown that Ezh2 is associated with Vav1 protein (40), which is a protein important for mediating proximal TCR signaling in T cell activation (41). To rule out the possibility that the
Ezh2 associates with Th1 gene loci

Development of Th1 cells involves a complex mechanism (15). Th1 cell differentiation is initiated by IFN-γ upregulation of T-bet, which specifically activates Ifng transcription (15). IL-12 activation of STAT4 is also important to promote Th1 cell differentiation (13–15). To understand the mechanism by which Ezh2 promoted the development of Th1 cells, we first used ChIP assay to examine the presence of Ezh2 and histone methylation markers on Th1 gene loci (e.g., Ifng, Tbx21, and Stat4). CD4+ Tn were isolated from normal WT B6 mice and cultured under Th1-skewing conditions for 7 d. Chromatin was prepared from both CD4+ Tn and Th1 cells. As expected, the amount of H3K27me3 was markedly reduced at the promoter regions of Ifng, Tbx21, and Stat4 gene loci (Fig. 5A). In contrast, H3K4me3, which is a permissive histone methylation marker associated with gene activation (27, 43), was increased at the promoter region of these genes (Fig. 5A). These data are in agreement with previous observations that H3K27me3 strongly marked the promoter, intergenic, and 3’UTR regions of Tbx21 in CD4+ Tn, whereas differentiated Th1 cells had reduced H3K27me3 but increased H3K4me3 at the Tbx21 regulatory regions (22, 27).

Interestingly, there was no significant reduction in the amount of Ezh2 at both regulatory and promoter regions of these Th1 genes in differentiated Th1 cells compared with CD4+ Tn, with moderately decreased induction of Th1 cells by Ezh2 inhibition might be associated with impaired activation of T cells in cultures, we examined the effects of Ezh2 deficiency on the activation and division of T-KO CD4+ T cells cultured under Th1-skewing conditions. We found that in the absence of Ezh2, CD4+ Tn were normally activated as evidenced by the upregulation of T cell activation markers CD25, CD44, and CD69 (Fig. 4C). Furthermore, like WT CD4+ T cells, T-KO CD4+ T cells underwent extensive cell division as evidenced by their dilution of fluorescence dye CFSE in cultures (Fig. 4D). These data suggest that in the absence of Ezh2, CD4+ T cells can be normally activated to undergo cell division and expansion upon stimulation with Th1-skewing conditions.

Previous studies suggest that IL-4 can reduce Th1 cell differentiation (35, 42). We found that in vitro activated T-KO T cells produced more IL-4–producing T cells in frequency than their WT counterparts (Fig. 4A). It is possible that increased production of IL-4 in T-KO T cells might account for their impaired Th1 cell differentiation. To test this possibility, we added neutralizing anti–IL-4 Ab into the cultures. Indeed, blockade of IL-4 efficiently inhibited the production of IL-4 by both WT and T-KO CD4+ T cells (Fig. 4E). Seven days after cultures, there was no marked difference in frequency of IL-4–producing T cells between activated WT and T-KO CD4+ T cells (Fig. 4E). In contrast, neutralizing IL-4 in cultures did not improve the ability of T-KO CD4+ T cells to produce IFN-γ (Fig. 4E). Furthermore, we confirmed that both WT and T-KO CD4+ T cells derived from Th1-skewing cultures expressed similar levels of GATA3 protein and mRNA (Fig. 4F). All these results suggest that Ezh2 promotes Th1 cell differentiation through a mechanism independent of IL-4 and GATA3.

Ezh2 promotes in vitro Th1 cell differentiation in cultures under Th1-skewing conditions. Naive CD4+ T cells isolated from WT B6 and T-KO mice were stimulated with anti-CD3 and anti-CD28 Abs under Th1-skewing condition. Cells were collected at the indicated time for analysis. (A) Dot plots (upper panel) and graphs (lower panel) show the fraction and MFI of IFN-γ– or IL-4–producing cells. (B) ELISA assays show the level of IFN-γ and IL-4 in the culture medium at day 7 after restimulation with anti-CD3 Ab. Each group contained an equal number of T cells (1 × 106 cells/ml). (C) Naive CD4+ T cells isolated from WT B6 or T-KO mice were prestained with CFSE and stimulated with anti-CD3 and anti-CD28 Abs. Two days after culture, cells were collected for the analysis of T cell activation markers. (D) Histograms show the cell divisions at the indicated time after culture. (E) Naive CD4+ T cells isolated from WT B6 and T-KO mice were stimulated with anti-CD3 and anti-CD28 Abs under Th1-skewing condition together with anti-IL-4 Ab (10 μg/ml) for 7 d. Dot plots (left panel) and graphs (right panel) show the fraction of IFN-γ– or IL-4–producing cells. (F) Naive CD4+ T cells before (Tn) and after cultured under Th1-skewing condition for 7 d (Th1) were harvested and lysed for Western blot analysis (upper panel). The relative expression level of each protein was indicated under the band, which was determined by densitometry analysis. Seven days after culture, the indicated gene expression was analyzed in WT and T-KO cells (lower panel). Data are representative of two independent experiments. Error bars indicate mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
increased amount of Ezh2 at the promoter region of Stat4 (Fig. 5B). Real-time RT-PCR showed that Ezh2 was positively correlated with expression of these Th1 genes (Fig. 5C). All CD4+ T cells producing IFN-γ also expressed high levels of Ezh2 (Fig. 5D).

To further confirm that Ezh2 associates with Th1 gene loci, we prepared chromatin from WT and T-KO CD4+ T cells activated under Th1-skewing conditions for 7 d. ChIP analysis revealed a significantly reduced amount of Ezh2 and H3K27me3 at the promoter regions of these gene loci in T-KO Th1 cells compared with WT Th1 cells (Fig. 6A–C). Thus, despite the reduction of H3K27me3 at the promoter regions of these Th1 genes, its catalyzing enzyme Ezh2 remains associated with these regions. Notably, T-KO Th1 cells showed a higher amount of H3K4me3 at the promoter regions of these Th1 gene loci than WT Th1 cells did (Fig. 6D). This suggests that loss of Ezh2 leads to the switch to a permissive histone methylation signature for Th1 genes, which is

**FIGURE 5.** Ezh2 associates with the promoter regions of type-1 gene loci. Freshly isolated WT CD4+ T cells (Tn) and WT CD4+ T cells after 7 d culture in Th1-skewing conditions (Th1) were processed for ChIP using Abs specific for H3K27me3, H3K4me3, Ezh2, and control IgG. (A) The graphs show the ChIP–quantitative PCR (qPCR) for H3K27me3 or H3K4me3 binding to the promoter region of Ifng, Tbx21, or Stat4. (B) Schematic representation of the mouse Ifng, Tbx21, or Stat4 locus (upper panel). Open rectangles indicate the transcriptional start site. Closed triangles show the locations of real-time qPCR primer pairs used in the ChIP assay, which are indicated in relative kb to the transcriptional start site. The graphs (lower panel) show the ChIP-qPCR for Ezh2 binding, with a series of primer pairs shown above covering the regulatory and promoter region of Ifng, Tbx21, or Stat4 locus. (C) Graphs show the relative expression of indicated genes measured by real-time PCR in freshly isolated WT CD4+ T cells (Tn) and WT CD4+ T cells after 7 d culture in Th1-skewing conditions (Th1). (D) Dot plots show the fraction of IFN-γ- and Ezh2-expressing cells in freshly isolated WT CD4+ T cells (Tn) and WT CD4+ T cells after 7 d culture in Th1-skewing conditions (Th1). Data are representative of three independent experiments. Error bars indicate mean ± SD. **p < 0.01, ***p < 0.001.

**FIGURE 6.** Ezh2 specifically binds to promoter regions of Th1 type gene loci. WT and T-KO CD4+ T cells collected 7 d after culture under Th1-skewing conditions and processed for ChIP using Abs specific for Ezh2, H3K27me3, and H3K4me3. (A) A schematic of the primer regions in the mouse Ifng, Tbx21, or Stat4 locus. (B–D) The graphs show the relative amount of Ezh2, H3K27me3, H3K4me3, and IgG at the regions of Ifng, Tbx21, or Stat4 locus.
favorable for their activation. However, our results showed that loss of Ezh2 impaired Th1 cell development (Fig. 2C).

**Ezh2 regulates T-bet at both the transcriptional level and posttranslational level**

To determine how Ezh2 regulated the expression of T-bet and STAT4 in Th1 cells, we examined the expression of T-bet and STAT4 mRNA in WT and T-KO CD4+ T cell 7 d after culture under Th1-skewing conditions. CD4+ Tn were assessed as controls. Seven days after culture under Th1-skewing conditions, T-KO CD4+ T cells expressed ∼1.8-fold less T-bet mRNA than their WT counterparts did (Fig. 6A). STAT4 mRNA was slightly decreased in activated T-KO CD4+ T cells (Fig. 7A). In addition, Ezh2 deficiency had no effect on the expression of Th1-related Ifngr1 and Il12rb2 genes (Fig. 7B), two critical signaling molecules upstream of T-bet and STAT4, respectively (15). These data together with our observations that Ezh2 was positively associated with Tbx21 gene (Figs. 5, 6) indicate that Ezh2 promotes the expression of T-bet at the transcriptional level.

To assess whether Ezh2 directly activated Tbx21 transcription, we cotransfected 3T3 cells with pGL3-Tbx21 reporter and MigR1 viral plasmid encoding Ezh2 or empty MigR1 plasmid. These 3T3 cells were harvested 48 h after transfection and analyzed with the Dual Luciferase system. Overexpression of Ezh2 resulted in moderate induction of Tbx21 reporter activity (Fig. 7C). This finding indicates that Ezh2 can directly activate Tbx21 transcription.

We further verified whether loss of Ezh2 led to reduction of STAT4 and T-bet protein in Th1 cells. In a comparison with activated WT CD4+ T cells, there was only minimal reduction of STAT4 and T-bet protein in Th1 cells. In a comparison with activated WT CD4+ T cells, there was only minimal reduction of STAT4 and T-bet protein in Th1 cells (Fig. 7D). Most notably, T-KO CD4+ T cells showed ∼4-fold and 10-fold less T-bet protein at day 3 and day 7 after culture, respectively, than their WT counterparts did (Fig. 7D). This dramatic reduction of T-bet protein in T-KO Th1 cells appeared not to be completely supported by moderate reduction of T-bet mRNA in these cells. We reasoned that loss of Ezh2 might lead to increased degradation of T-bet protein in Th1 cells. To test this reasoning, we treated WT and T-KO Th1 cells with the proteasome inhibitor MG115 (44). The addition of the proteasome inhibitor MG-115 restored the expression of T-bet protein, but not STAT4 protein, in Ezh2-deficient Th1 cells (Fig. 7E), suggesting that T-bet protein is more susceptible than STAT4 to proteasome-mediated degradation in Th1 cells lacking Ezh2. Altogether, Ezh2 promotes T-bet expression at transcriptional and posttranslational levels, largely with the latter. These results identify a novel and important role for Ezh2 to regulate Th1 cells.

**Introduction of T-bet into T-KO CD4+ T cells fully rescues their differentiation into Th1 cells**

To determine whether the downregulation of T-bet caused the impairment of Th1 cell development, we used MigR1 virus bicistronically encoding T-bet and GFP (named MigR1/T-bet) to infect T-KO CD4+ T cells cultured under Th1-skewing conditions. T-KO CD4+ T cells infected with MigR1 encoding STAT4 and GFP (named MigR1/STAT4) or GFP alone (named MigR1/GFP) were assessed in parallel. Expression of GFP allowed us to track cells expressing T-bet or STAT4. WT CD4+ T cells were also infected by either MigR1/T-bet or MigR1/GFP (Fig. 8A, 8B). Interestingly, compared with WT GFP+CD4+ T cells infected with MigR1/GFP or MigR1/STAT4, T-KO GFP+CD4+ T cells expressing STAT4 had significantly lower frequency of IFN-γ+ T cells (Fig. 8A, 8B).

**FIGURE 7.** Ezh2 regulates T-bet at both the transcriptional level and posttranslational level. (A) Graphs show the gene expression of Stat4 and Tbx21 in freshly isolated WT or T-KO CD4+ T cells (Tn) and cells after 7 d culture in Th1-skewing conditions (Th1). (B) The graph shows the relative expression of indicated genes measured by real-time PCR in WT or T-KO CD4+ T cells after 7 d culture in Th1-skewing conditions. (C) The schematic (left panel) shows the construction of Tbx21 promoter region ranging from +0.3 to −1.0 kb of the TSS. 3T3 cells were cotransfected with pGL3-Tbx21 reporter plasmid and an empty vector control or Ezh2 in combination. Luciferase reporter activity was normalized to the activity obtained for the cotransfected Renilla control. The graph (right panel) represents the relative light units (RLU). (D) Western blot shows the expression of Ezh2, Stat4, and T-bet after 3 or 7 d of Th1-skewing differentiation conditions. The relative expression level of each protein was indicated under the band, which was determined by densitometry analysis. (E) Western blots show the expression of T-bet and STAT4 in freshly isolated WT or T-KO CD4+ T cells (Tn) and cells after 7 d culture in Th1-skewing conditions (Th1) with or without the treatment of MG115 (2 μM) for 6 h. Data are representative of two independent experiments. Error bars indicate mean ± SD. **p < 0.01.
Furthermore, T-KO CD4+ T cells expressing STAT4 contained ∼40% less IFN-γ+ T cells than T-KO CD4+ T cells expressing T-bet (Fig. 8A, 8B). These data suggest that viral expression of T-bet fully rescues the ability of Ezh2-deficient CD4+ T cells to differentiate into Th1 cells, whereas overexpression of STAT4 only partially improves Th1 cell differentiation of activated T-KO T cells.

To validate these observations, we highly purified GFP-positive T-KO T cells from these cultures (Fig. 8C) and confirmed the overexpression of T-bet and STAT4 in these T-KO CD4+ T cells, respectively, using real-time RT-PCR (Fig. 8D). Furthermore, overexpression of T-bet in T-KO T cells induced significantly more IFN-γ transcripts than did overexpression of STAT4 (Fig. 8D). Phosphorylation of STAT4 is critical for IFN-γ production and Th1 cell differentiation (13, 45). Western blot analysis showed that MigR1/STAT4-infected T-KO T cells expressed 7.5-fold more STAT4 protein and 1.2-fold more phosphorylated STAT4 than their WT counterparts did (data not shown). Thus, the reduction of T-bet in activated T-KO CD4+ T cells is a major contributor to their impaired development of Th1 cells.

We finally examined whether retroviral expression of T-bet could rescue the ability of Ezh2-deficient T cells to induce AA. Both CD4+ and CD8+ T cells were highly purified from T-KO B6 mice and activated in vitro for 24 h, followed by infection with MigR1/T-bet, MigR1/STAT4, and MigR1/GFP, respectively. Thirty-six hours later, these infected T cells were harvested, assessed using flow cytometry for the expression of GFP, and transferred into sublethally irradiated BDF1 mice. Each mouse received unfractonated T cells that contained similar numbers of GFP+ cells (0.8 × 10^6 CD4+ T cells plus 0.6 × 10^6 CD8+ T cells). BDF1 mice receiving T-KO T cells transduced by either T-bet or STAT4 survived >24 d after transfer, whereas all BDF1 mice receiving WT T cells infected by control MigR1/GFP died of AA within 14 d (Fig. 9A and data not shown). This suggests that transfer of this amount of T-KO T cells expressing T-bet or STAT4 did not cause lethal AA. However, as compared with control T-KO T cells infected by MigR1/GFP, MigR1/T-bet–infected T-KO T cells caused marked reduction of BM cells and peripheral blood cells (i.e., platelets, RBCs, WBCs, and neutrophils) 24 d after transfer (Fig. 9A, 9B). Furthermore, GFP+ MigR1/T-bet-infected KO T cells (which expressed T-bet) had ∼4-fold more in frequency of IFN-γ-producing T cells than control GFP+ T-KO T cells infected by MigR1/GFP (Fig. 9D). In contrast, transfer of MigR1/STAT4-infected T-KO T cells resulted in moderate decrease of BM cells, but not blood pancytopenia (Fig. 9A, 9B). Notably, GFP+ MigR1/STAT4-infected T-KO T cells (which expressed STAT4) failed to produce high levels of IFN-γ (Fig. 9C, 9D). Thus, Ezh2 regulation of T-bet is important for production of Th1 cells mediating AA.

Discussion

Our findings elucidate a new and critical role of Ezh2 in controlling pathogenic Th1 cell responses during AA process, which has not been previously identified. Ezh2 is absolutely required for CD4+ Th1 cells to mediate fatal AA. Ezh2 inhibition led to dramatic reduction of BM-destructive Th1 cells in vivo, decreasing BM-infiltrating Th1 cells, and protecting mice from BMF. Decreased capability of Ezh2-deficient T cells to differentiate into Th1 cells resulted from reduced expression of T-bet mRNA and protein, as retroviral expression of T-bet in Ezh2-deficient CD4+ T cells fully rescued their differentiation into Th1 cells. In contrast, ectopic expression of STAT4 only partially restored the ability of Ezh2-deficient CD4+ T cells to produce IFN-γ. Interestingly, although Ezh2 is known to act primarily as a gene silencer, it promoted the expression of T-bet gene in Th1 cells via a mechanism of directly activating Tbx21 gene promoter. Furthermore, Ezh2 was required to prevent proteasome-mediated degradation of T-bet protein in Th1 cells. This constellation of Ezh2 actions induced the optimal production of Th1 cells destructing BM cells, highlighting the therapeutic potential of Ezh2 inhibition in controlling AA.

Because Ezh2 specifically catalyzes the repressive maker H3K27me3, a corollary belief is that Ezh2 may be required for repressing cytokine gene expression (22, 46, 47). CD4+ Tn showed moderate levels of H3K27me3 in the promoter regions of Ifng and Tbx21 gene loci (46, 47, 48). Upon Th1 differentiation, H3K27me3 was reduced in the promoter regions of Th1 genes (e.g., Ifng, Tbx21, and Stat4), whereas H3K4me3 was upregulated in these gene loci (27). We confirmed and further extended these
Twenty-four days after LN-cell infusion, donor CD4+ T cells were isolated from the spleen, LN, and BM to measure the production of IFN-γ. Total BM cellularity was calculated assuming that bilateral tibia and femurs contain 25% of total marrow cells (mean ± SD; n = 6–8 mice per group). (A) Peripheral blood was collected 24 d after LN cell infusion and the cell numbers of RBCs, WBCs, platelets, and neutrophils were calculated (mean ± SD; n = 6–8 mice per group). (B) Twenty-four days after LN cell infusion, donor CD4+ T cells were isolated from the spleen, LN, and BM to measure the production of IFN-γ. The plots show the percentage of donor CD4+ T cells in spleen, the percentage of GFP- and GFP+ cells, and the fraction of IFN-γ-producing cells in GFP- and GFP+ cells. (D) Graphs show the percentage (upper panel) and the number (lower panel) of donor IFN-γ+CD4+ T cells in spleen, LN, and BM. *p < 0.05, **p < 0.01, ***p < 0.001.

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low levels of T-BET in their circulating blood cells (17). Experimental studies further indicate that genetic inhibition of T-bet leads to decreased induction of AA in mice (6); however, so far, there is no pharmacologic approach available to modulate T-bet. Furthermore, transcription factors are difficult drug targets (18). Given that Ezh2 plays critical roles in the regulation of T-bet mRNA and protein in Th1 cells, we proposed that targeting Ezh2 may prove advantageous to modulate T-bet and its controlled pathogenic T cell inflammation.

Ablerrant expression of Ezh2 has been associated with many types of malignant diseases such as prostate cancer, breast cancer, and lymphomas (19, 56, 57). Data from our previous studies indicate that Ezh2 is markedly increased in activated T cells (58, 59). Inhibition of Ezh2 leads to the reduction of graft-versus-host disease in mice of allogeneic BMT (28). In these mouse models of allogeneic BMT, infusion of donor BM cells fully rescues hematomyelosis and thymopoiesis in lethally irradiated recipients (10, 11), which prevents us from precisely assessing the impact of T cells in mediating BMF. Our experiments using the AA mouse model and in vitro cultures provide compelling evidence for a T cell–intrinsic contribution of Ezh2 to the expression of Th1–associated transcription factors and cytokines. Inhibition of Ezh2 in T cells through conditional deletion suppressed Th1 cell differentiation and development of BMF. As several Ezh2-specific inhibitors have recently been discovered for experimental treatment of cancer (60–63), it will be important to test whether these Ezh2-specific inhibitors may control AA in experimental models. In summary, we have identified the critical role of Ezh2 in regulating Th1 responses during AA and the mechanism by which Ezh2 promotes Th1 cell responses mediating BMF in mice. Our findings open new perspectives to study the importance of Ezh2 in regulating the development of other lineages of CD4+ T cells (e.g., Th2, Th17, Th9, and regulatory T cells). Indeed, a recent study found that Ezh2 is involved in repressing the development of BMF. As several Ezh2-specific inhibitors have recently been discovered for experimental treatment of cancer (60–63), it will be important to test whether these Ezh2-specific inhibitors may control AA in experimental models.

In summary, we have identified the critical role of Ezh2 in regulating Th1 responses during AA and the mechanism by which Ezh2 promotes Th1 cell responses mediating BMF in mice. Our findings open new perspectives to study the importance of Ezh2 in regulating the development of other lineages of CD4+ T cells (e.g., Th2, Th17, Th9, and regulatory T cells). Indeed, a recent study found that Ezh2 is involved in repressing the development of Th9 cells (64). Furthermore, it will be important to investigate whether deregulated Ezh2 expression and activity are associated with inflammatory disorders in humans, such as autoimmune diseases and chronic infections.

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Disclosures

The authors have no financial conflicts of interest.


