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

Qing Tong,*,† Shan He,‡,§ Fang Xie,† Kazuhiro Mochizuki,† Yongnian Liu,‡,§ Izumi Mochizuki,† Lijun Meng,‡,§ Hongxing Sun,§ Yanyun Zhang,§ Yajun Guo,*, Elizabeth Hexner,† and Yi Zhang†,‡

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: 5012–5022.

Aquired aplastic anemia (AA) in humans is a fatal disorder characterized by bone marrow (BM) hypoplasia and blood pancytopenia (1, 2). Clinical studies indicate that in most cases, AA is a disease caused by immune-mediated destruction of hematopoietic stem cells and hematopoietic progenitor cells (1, 2). A role for T cells in AA was first suggested by their inhibition of hematopoietic cell colony formation in cultures in vitro (2). Furthermore, CD4+ T cell clones isolated from the patients with AA have a potent ability to lyse autologous CD34+ hematopoietic cells and inhibit formation of hematopoietic cell colonies (3). Accumulating evidence indicates that CD4+ Th1 cells, which are characterized by production of high levels of IFN-γ, play important roles in mediating bone marrow failure (BMF) (2, 4–8). IFN-γ displays potent effects on suppressing hematopoiesis in vitro (2, 3). Immunosuppressive therapy and allogeneic BM transplantation (BMT) have significantly improved the survival of severe AA. However, relapse still occurs in ~35% of patients with AA when immunosuppressive therapy is withdrawn (1, 2, 9). Furthermore, graft-versus-host disease remains a major barrier to the success of allogeneic BMT (10, 11). Novel approaches are needed to improve the outcome of treatments for AA.

The transcription factor T-bet (encoded by Tbx21) is crucial for Th1 cell differentiation (12–15). T-bet is induced by TCR signaling and strongly upregulated by activation of the STAT1 transcription factor (16). T-bet binds to several enhancers and promoter of the Ifng genes, activating its transcription (12, 16). T-bet also promotes expression of the IL-12 receptor β2 chain (IL12Rβ2), resulting in greater IL-12 responsiveness and further elevated production of IFN-γ (16). In addition, T-bet prevents Th2 differentiation by inhibiting Gata3 (16). T-bet is upregulated in peripheral blood T cells from patients with AA, and it is a useful marker for predicting the responsiveness of patients to immunosuppressive therapy (17). Furthermore, experimental studies suggested that T cells lacking T-bet were defective in induction of AA in mice (6). These observations suggest that T-bet can be an attractive target for modulating Th1 cell–mediated AA. However, transcription factors are difficult drug targets (18). Thus, identifying...
Ezh2 (Ezh2fl/fl) (32) were crossed to Cd4-Cre mice, before backcrossing. Mice are listed in Supplemental Table I.

30s, 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, CD44+CD4+ naïve 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 recombinant 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 prestimulated 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 blotting. 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 (558686; 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 (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 recombinant human IL-2 (10 ng/ml; R&D Systems) and recombinant 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 × 10^6 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). T33 cells were cotransfected with pGL3-Tbx21 reporter plasmid and MigR1 viral plasmid encoding Ezh2 or empty MigR1 plasmid. 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 (Ezh2fl/fl) (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 Ezh2-deficient 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 did (Fig. 2C).

In this BMF model, only a small percentage of IL-4–producing CD4+ 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+ 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. 3C). 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+ T cells and IFN–γ–producing CD4+ 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+ 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+ 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...
FIGURE 4. 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 prestained 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 × 10^6 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.

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 IFN-γ–producing T cells in 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 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
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-\(\gamma\) also expressed high levels of Ezh2 (Fig. 5D).

To further confirm that Ezh2 associated 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
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 Il12r2b2 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 protein in these activated T-KO CD4+ T 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 with each of these viruses as controls.

We found that T-KO GFP-positive (GFP+) CD4+ T cells that were derived from cultures infected by MigR1/T-bet, which overexpressed T-bet, produced a similar percentage of IFN-γ+ T cells and WT GFP+ CD4+ T cells derived from cultures 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 culture 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 Ifng transcripts than did overexpression of STAT4 (Fig. 8D). Furthermore, T-KO CD4+ T cells expressing T-bet contained similar numbers of GFP+ cells 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, 48, 49, 27). 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). (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-producing CD4+ T cells in spleen, LN, and BM. *p < 0.05, **p < 0.01, ***p < 0.001.

- Intriguingly, we identified a previously unknown role for Ezh2 in protecting proteasome-mediated degradation of T-bet protein in Th1 cells. Ezh2 deficiency led to increased degradation of T-bet protein in Th1 cells. Addition of the proteasome inhibitor MG-115 restored the expression of T-bet protein in Ezh2-deficient Th1 cells. This effect of Ezh2 on protecting T-bet from proteasome-mediated degradation differs from the effect of Ezh2 on repressing gene transcription (19). T-bet is crucial for controlling appropriate Th1 cell differentiation through reversing and establishing new epigenetic states in T cells (52, 53).

- For example, Ezh2 methylates STAT3 in cancer cells, leading to enhanced STAT3 activity (55). For example, Ezh2 methylates STAT3 in cancer cells, leading to enhanced STAT3 activity (55). Given the presence of high amounts of Ezh2 at the promoter regions of Ifng gene locus in Th1 cells, we speculated that Ezh2 could play critical roles in stabilizing T-bet protein at this gene locus, thereby facilitating the T-bet regulation of Th1 cell differentiation. Formal experiments are needed to address this point and to determine the mechanisms whereby Ezh2 protects T-bet from proteasome-mediated degradation in Th1 cells.

- Accumulating evidence indicates that T-bet can be an effective target for the prevention and treatment of immune-mediated AA. Abnormal expression of T-BET has been shown in human patients with AA (17). T cells from patients with AA who were refractory to immunosuppressive treatment expressed high levels of T-BET. In contrast, patients with AA who responded to the therapy expressed high levels of T-BET.
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. Aberrant 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 hematopoiesis 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 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.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


52, 5202
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<th>Gene name</th>
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<td></td>
<td>Reverse: CGGCTACCACATCCAAGG</td>
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<td><strong>Foxp3</strong></td>
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Supplemental Table 2. Primers for ChIP

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| Ifng (-28kb) | Forward AACTGCTTATGCTGGATTTGAGAT  
Reverse CTCCTATGCTTATTTGGCTGGTCTTA |
| Ifng (-6kb) | Forward CCCAGTGAGTGCTTTAAAATTTCT  
Reverse CTGGATGGTTTGGAGGATAATGT |
| Ifng (-0.4kb) | Forward CGGGGCTGTCTCATCGTGC  
Reverse CTCGGGATTACGTATTTTCACAA |
| Ifng (+20kb) | Forward GGCCTGATTACACACCCAAACT  
Reverse CCAAGAAGAAGCCAGAAATCAA |
| Ifng (+46kb) | Forward GCAGCCCGGAGCAAGAGT  
Reverse CCAATATTACCTTCAGTGACAGCA |
| Tbx21 (-20kb) | Forward CCTGGAGCGGTTAGAGACTG  
Reverse TCCCCAGTCCCTAGAAAATCA |
| Tbx21 (-0.5kb) | Forward GGGCTAGAGGACAAGCATT  
Reverse TCCTGTATGCCCAGGCTTTG |
| Tbx21 (-0.1kb) | Forward GGGCTAGAGGACAAGCATT  
Reverse TCCTGTATGCCCAGGCTTTG |
| Tbx21 (+0.1kb) | Forward GTCTATTAGGTCCCTAGAAAATCA  
Reverse TCCTGTATGCCCAGGCTTTG |
| Tbx21 (+20kb) | Forward GTCTATTAGGTCCCTAGAAAATCA  
Reverse TCCTGTATGCCCAGGCTTTG |
| Stat4 (-10kb) | Forward GTCAGACCGGCGGAGGTGG  
Reverse TGCTTACTGAGCATGGACAGT |
| Stat4 (-0.4kb) | Forward GTCTATTAGGTCCCTAGAAAATCA  
Reverse TCCTGTATGCCCAGGCTTTG |
| Stat4 (0kb) | Forward GGTGGCAACATTATCTGCTTCA  
Reverse TCTTTAGATTGTGAATCAATAGCA |
| Stat4 (+0.2kb) | Forward GAAAATTTGATATGTACCCTAATATG  
Reverse TTTCTCACTCCCTGGAATCTTAGAC |
| Stat4 (+10kb) | Forward GGGGCATTTTTGAGTTCTTT  
Reverse ACCAGGCACACACACACAGT |