

Research Paper

HDAC6 regulates IL-17 expression in T lymphocytes: implications for HDAC6-targeted therapies

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Abstract

The pro-inflammatory cytokine interleukin 17 (IL-17) is critically involved in immunity and inflammation. T-helper 17 and $\gamma\delta$ T cells are the predominant sources of IL-17 in the immune system. However, the mechanisms by which the expression of IL-17 is regulated in T cells remain elusive. Here, we demonstrate that loss of histone deacetylase 6 (HDAC6) in mice does not affect the generation of CD4⁺ or CD8⁺ T cells, but stimulates the development of IL-17-producing $\gamma\delta$ T cells. Our data further show that HDAC6 deficiency increases the production of IL-17 by V γ 4⁺ $\gamma\delta$ T cells in the spleen and lymph nodes. Consistent with these observations, small-molecule inhibition of HDAC6 activity in $\gamma\delta$ T cells promotes the expression of IL-17 *in vitro*. These data thus reveal that HDAC6 represses IL-17 production in T cells, providing novel insights into the role of HDAC6 in the immune system. These findings also have important implications for the clinical investigation of HDAC6-targeted therapies.

Key words: histone deacetylase 6; interleukin 17; T cell; development; knockout mouse.

Introduction

Pro-inflammatory cytokines stimulate inflammation in response to pathogen infection, as well as in response to autoantigens in autoimmune disorders. Members of the interleukin 17 (IL-17) family play important roles in inflammation, in addition to acting as key players in the development of many autoimmune diseases [1-4]. IL-17 is expressed by both innate and adaptive immunocytes. As a part of the adaptive immune response, naïve CD4⁺ $\alpha\beta$ T cells can differentiate into T-helper 17 (Th17) cells, which become mature in the peripheral organs via stimulation with specific cytokines [5, 6]. These cells are regarded as the primary source of IL-17 in autoimmune disorders.

With respect to the innate immune response, like Th17 cells, $\gamma\delta$ T cells have been shown to produce IL-17A and IL-17F, two of the most well-characterized

members of the IL-17 cytokine family [7]. Unlike Th17 cells, IL-17-producing $\gamma\delta$ T cells differentiate during embryonic development and subsequently migrate into the mucous tissues after maturity, where they interact with outside pathogens. IL-17-producing $\gamma\delta$ T cells residing in the spleen and lymph glands also appear to participate in innate inflammation. Expression of IL-17 by $\gamma\delta$ T cells plays a protective role in concanavalin A-induced liver injury [8]. Although the precise signaling pathways that regulate IL-17 expression are not very well characterized, increasing evidence suggests that different mechanisms regulate the development of Th17 and IL-17-producing $\gamma\delta$ T cells. For example, the transcription factor STAT3 (signal transducer and activator of transcription 3) is required for the development of Th17 cells, but does not appear to

play a role in the development of IL-17-producing $\gamma\delta$ T cells [9, 10]. However, little is known regarding the specific pathways that regulate IL-17 expression in $\gamma\delta$ T cells.

Histone deacetylase 6 (HDAC6) is unique among the HDACs because of its cytoplasmic localization and non-histone substrates. Several lines of evidence support the hypothesis that the deacetylase activity of HDAC6 serves as a key means for the regulation of the immune response [11]. HDAC6 plays a role in the organization of the immunological synapse through deacetylation of microtubules [12]. Furthermore, HDAC6 inhibition leads to hyperacetylation of Hsp90, thereby suppressing the activity of Foxp3⁺ T regulatory cells [13, 14]. Additionally, we have previously demonstrated that HDAC6 regulates lipopolysaccharide-induced macrophage activation in a deacetylase activity-dependent manner [15]. In the present study, we have explored the role of HDAC6 in the regulation of IL-17 expression in T lymphocytes. Results from this study provide novel insight into the mechanisms that regulate IL-17 expression and further implicate HDAC6 in the immune response.

Materials and Methods

Mice and cell culture

HDAC6 heterozygous (HZ) mice were obtained from Tso-Pang Yao (Duke University, Durham, NC, USA) and intercrossed to generate HDAC6 knockout (KO) and wild-type (WT) littermates as described previously [16, 17]. Mice were maintained in a specific pathogen-free barrier facility. All procedures for mouse care and operation were carried out according to the guidelines of experimental animals. The use of mice was approved by our Institutional Animal Care Committee. *In vitro* expansion of $\gamma\delta$ T cells was performed as previously described [18]. EL4 murine lymphoma cells were cultured at 37°C in RPMI 1640 medium containing 10% fetal bovine serum.

Flow cytometry

Primary cells isolated from WT and HDAC6 KO mice were incubated with antibodies directed against specific cell surface molecules. The cells were then fixed, permeabilized, and stained with the indicated antibodies directed against intracellular antigens as previously described [19-21]. For flow cytometry analysis, 1×10^6 cells were resuspended in phosphate-buffered saline at a final concentration of 5×10^5 cells/mL. Fluorescence was analyzed using a BD FACSCalibur instrument.

Real-time quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from EL4 cells using the

TriPure isolation reagent (Roche) according to the manufacturer's protocol. cDNA was prepared using the M-MLV reverse transcriptase (Promega) and Oligo(dT) primers (Takara). The following specific primers were used for the PCR reactions: Hes1 forward: 5'-ACACCGGACAAACCAAAGAC-3', Hes1 reverse: 5'-ATGCCGGGAGCTATCTTTCT-3'; SOX13 forward: 5'-CCCTATTTCTCTCCAGACTGTTCTTT-3', SOX13 reverse: 5'-GCTGGTTAAGTTATTCA TCATTATCTTCTT-3'; SOX4 forward: 5'-GAACGCCTTTATGGTGTGGT-3', SOX4 reverse: 5'-GAACGG AATCTTGTCTGCTGT-3'; IL-17A forward: 5'-TGAA GGCAGCAGCGATCA-3', IL-17A reverse: 5'-GGAAG TCCTTGGCCTCAGTGT-3'; IL-17F forward: 5'-CGC CATTACAGCAAGAAATCC-3', IL-17F reverse: 5'-CTC CAACCTGAAGGAATTAGAACAG-3'. β -actin served as a reference gene for normalization.

Luciferase assays

Cells harvested from a 10-cm culture dish were transfected by electroporation with a pGL3 firefly luciferase reporter plasmid containing the IL-17 promoter sequence. Cells were co-transfected with the pRL-TK renilla luciferase reporter plasmid to control for the transfection efficiency. Luciferase activity was measured, and luciferase activity was reported as the firefly/renilla luciferase ratio as described [22, 23].

Statistics

Analysis of statistical significance was performed by the Student's t-test for comparison between two groups and by the ANOVA test for multiple comparisons.

Results

Loss of HDAC6 does not affect the development of CD4⁺ or CD8⁺ T lymphocytes

Although several reports have revealed an important role for HDAC6 in T lymphocyte function, a link between HDAC6 and the development of T lymphocytes remains largely unknown. We explored the role of HDAC6 in the development of T lymphocytes by analyzing the populations of CD4⁺ and CD8⁺ T lymphocytes in both lymphoid and non-lymphoid tissues using flow cytometry. In agreement with the previous study, HDAC6 deficiency had no obvious effect on the development of CD4⁺/CD8⁺, CD4⁺, or CD8⁺ T lymphocytes in the thymus (Figure 1A-D). Furthermore, the loss of HDAC6 did not significantly alter the distribution of the CD4⁺ or CD8⁺ T lymphocytes in peripheral tissues, including the spleen, lymph nodes, liver, and blood (Figure 1A-D).

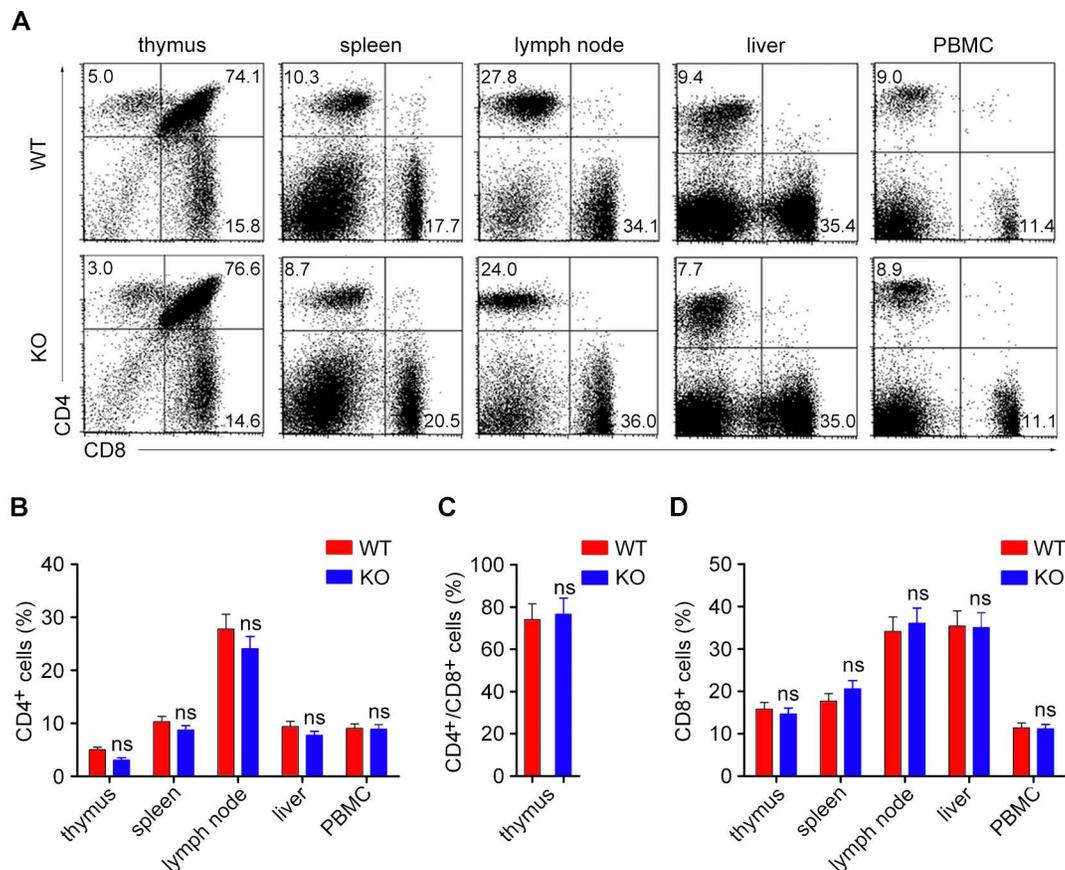


Figure 1. HDAC6 deficiency does not affect the development of CD4⁺ or CD8⁺ T cells. (A) Primary lymphocytes were isolated from the thymus, spleen, lymph nodes, liver, and PBMC (peripheral blood mononuclear cells) of WT and HDAC6 KO mice. CD4⁺, CD8⁺, and CD4⁺/CD8⁺ subpopulations of T cells (selected by gating CD3⁺ cells) were analyzed by flow cytometry. (B–D) Quantification of the percentages of CD4⁺ (B), CD4⁺/CD8⁺ (C), and CD8⁺ (D) cells in WT and HDAC6 KO mice based on two-color flow cytometric analysis as shown in (A). Data are representative of three independent experiments. Error bars indicate mean \pm SEM. ns, not significant.

HDAC6 deficiency results in an increase in the population of IL-17-producing $\gamma\delta$ T cells

We next compared the IL-17-producing and interferon- γ (IFN- γ)-producing subpopulations of $\gamma\delta$ T and CD4⁺ T cells. Flow cytometry revealed that the number of IL-17-producing CD4⁺ T cells in the spleen was slightly increased in HDAC6 KO mice, while the number of IL-17-producing $\gamma\delta$ T cells was dramatically increased in HDAC6 KO mice (Figure 2A and B). In contrast, the number of IFN- γ -producing $\gamma\delta$ T and CD4⁺ T cells was not significantly affected by HDAC6 deficiency (Figure 2A and B). These results suggest a key role for HDAC6 in the regulation of IL-17 production by $\gamma\delta$ T cells.

We next analyzed the subpopulations of IL-17-producing $\gamma\delta$ T cells by staining with anti-V γ 1 or anti-V γ 4 antibodies. Consistent with previous studies, our results showed that the V γ 4⁺ subset represented the major source of IL-17-producing $\gamma\delta$ T cells in both spleens and lymph glands (Figure 2C and D). Deletion of HDAC6 also increased IL-17 production in these V γ 4⁺ $\gamma\delta$ T cells (Figure 2C and D).

The deacetylase activity of HDAC6 is required for its regulation of IL-17 production by $\gamma\delta$ T cells

We then sought to determine whether the deacetylase activity of HDAC6 is involved in its regulation of IL-17 production by $\gamma\delta$ T cells. We isolated $\gamma\delta$ T cells from the spleen of mice and expanded the cells *in vitro*. Consistent with the *in vivo* findings, treatment of the *in vitro* expanded $\gamma\delta$ T cells with tubacin, a selective inhibitor of HDAC6 [24], significantly increased IL-17 production (Figure 3A and B). Interestingly, deletion or inhibition of HDAC6 decreased the amount of IFN- γ -producing $\gamma\delta$ T cells *in vitro* (Figure 3A and C), which was not significantly affected by HDAC6 deficiency *in vivo* (Figure 2A and B), reflecting the different sensitivity of this subpopulation of $\gamma\delta$ T cells to HDAC6 deficiency in the *in vitro* and *in vivo* systems. Collectively, these results indicate that the role of HDAC6 in the regulation of IL-17 production by $\gamma\delta$ T cells is dependent on its deacetylase activity.

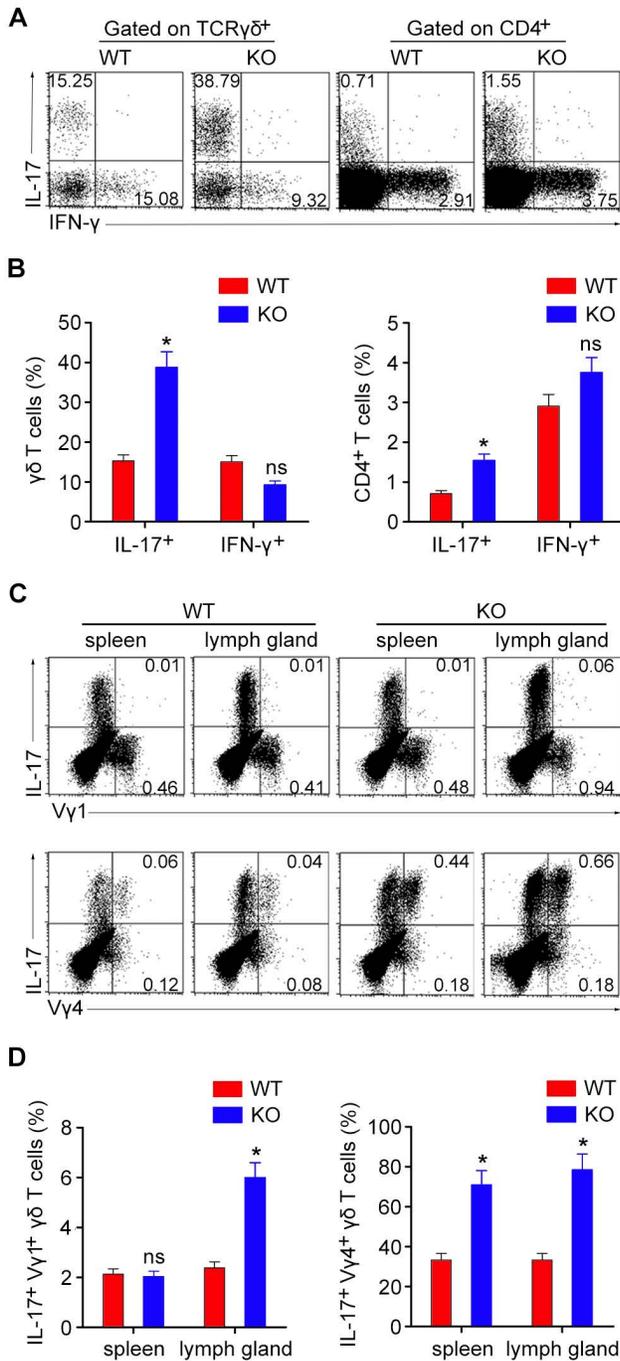


Figure 2. HDAC6 deficiency increases the development of IL-17-producing $\gamma\delta$ T cells. (A) Lymphocytes isolated from the spleen of WT and HDAC6 KO mice were stimulated with PMA and ionomycin, and the expression of IFN- γ and IL-17 was analyzed in $\gamma\delta$ T and CD4⁺ T cells. (B) Quantification of the IFN- γ - and IL-17-producing subpopulations of $\gamma\delta$ T and CD4⁺ T cells based on flow cytometry as shown in (A). (C) Lymphocytes isolated from the spleen or lymph glands of WT and HDAC6 KO mice were stimulated with PMA and ionomycin, and the expression of IL-17 in V γ 1⁺ or V γ 4⁺ $\gamma\delta$ T cells was analyzed by gating CD3⁺ cells. (D) Quantification of IL-17-producing subpopulations of V γ 1⁺ or V γ 4⁺ $\gamma\delta$ T cells based on flow cytometry as shown in (C). Data are representative of three independent experiments. Error bars indicate mean \pm SEM. * $p < 0.05$; ns, not significant.

HDAC6 deficiency promotes the development of IL-17-producing $\gamma\delta$ T cells in the thymus

Development of $\gamma\delta$ T cells primarily occurs in the thymus during mouse embryonic development. To assess the role of HDAC6 in the development of $\gamma\delta$ T cells in the thymus, we isolated thymocytes from newborn WT, HDAC6 HZ, and HDAC6 KO mice. Immunostaining and flow cytometric analysis revealed that the levels of thymic IL-17-producing $\gamma\delta$ T cells were increased in HDAC6 HZ and KO mice (Figure 4A and B). These results, together with the data shown above (Figure 3), suggest that HDAC6 may regulate the fate of $\gamma\delta$ T cells by determining whether they differentiate into IL-17-producing or IFN- γ -producing cells.

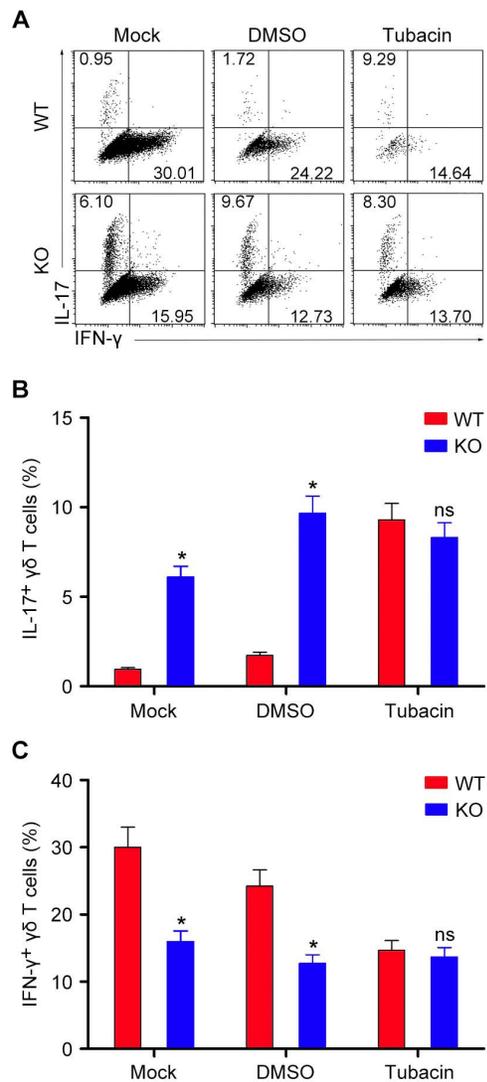


Figure 3. The deacetylase activity of HDAC6 is required for its regulation of IL-17 production by $\gamma\delta$ T cells. (A) $\gamma\delta$ T cells isolated from the spleen of WT and HDAC6 KO mice were cultured for 6 days and treated with 1 μ M tubacin or an equal volume of the vehicle DMSO. IFN- γ - and IL-17-producing subpopulations of $\gamma\delta$ T cells were then analyzed by flow cytometry. (B and C) Quantification of the percentages of IL-17-producing (B) and IFN- γ -producing (C) $\gamma\delta$ T cells. Data are representative of three independent experiments. Error bars indicate mean \pm SEM. * $p < 0.05$; ns, not significant.

HDAC6 regulates IL-17A and IL-17F mRNA expression

To examine whether HDAC6 regulates IL-17 mRNA expression in $\gamma\delta$ T cells, we stimulated the *in vitro* expanded $\gamma\delta$ T cells with PMA and ionomycin and then analyzed the levels of the IL-17A and IL-17F mRNAs by real-time qRT-PCR. We found that the mRNA levels of both IL-17A and IL-17F were remarkably increased upon the loss of HDAC6 in $\gamma\delta$ T cells (Figure 5A).

To confirm the effect of HDAC6 on IL-17 expression, we used EL4 murine lymphoma cells, which have been employed as a model to study IL-17 production by T cells [25]. EL4 cells were stimulated

with PMA and ionomycin, and the levels of the IL-17A and IL-17F mRNAs were quantified by real-time qRT-PCR. Stimulation of EL4 cells with PMA and ionomycin increased the expression of IL-17A and IL-17F (Figure 5B). Therefore, we next explored the role of HDAC6 in the regulation of IL-17A and IL-17F transcription in activated EL4 cells by treating cells with the HDAC6 inhibitor tubacin. We found that tubacin increased both IL-17A and IL-17F expression in a concentration-dependent manner (Figure 5C and D). Similar results were achieved with tubastatin A (Figure S1), another HDAC6-selective inhibitor [26].

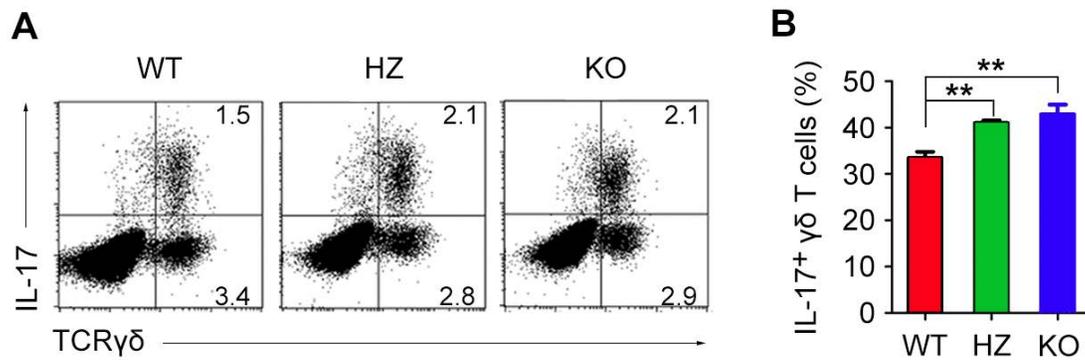


Figure 4. HDAC6 regulates the development of IL-17-producing $\gamma\delta$ T cells in the thymus. (A) Thymic lymphocytes were isolated from newborn WT, HDAC6 HZ, and HDAC6 KO mice. IL-17 expression in $\gamma\delta$ T cells was analyzed by flow cytometry after stimulation with PMA and ionomycin. (B) Quantification of the percentage of IL-17-producing $\gamma\delta$ T cells in WT, HDAC6 HZ, and HDAC6 KO mice. Data are representative of three independent experiments. Error bars indicate mean \pm SEM. ****** $p < 0.01$.

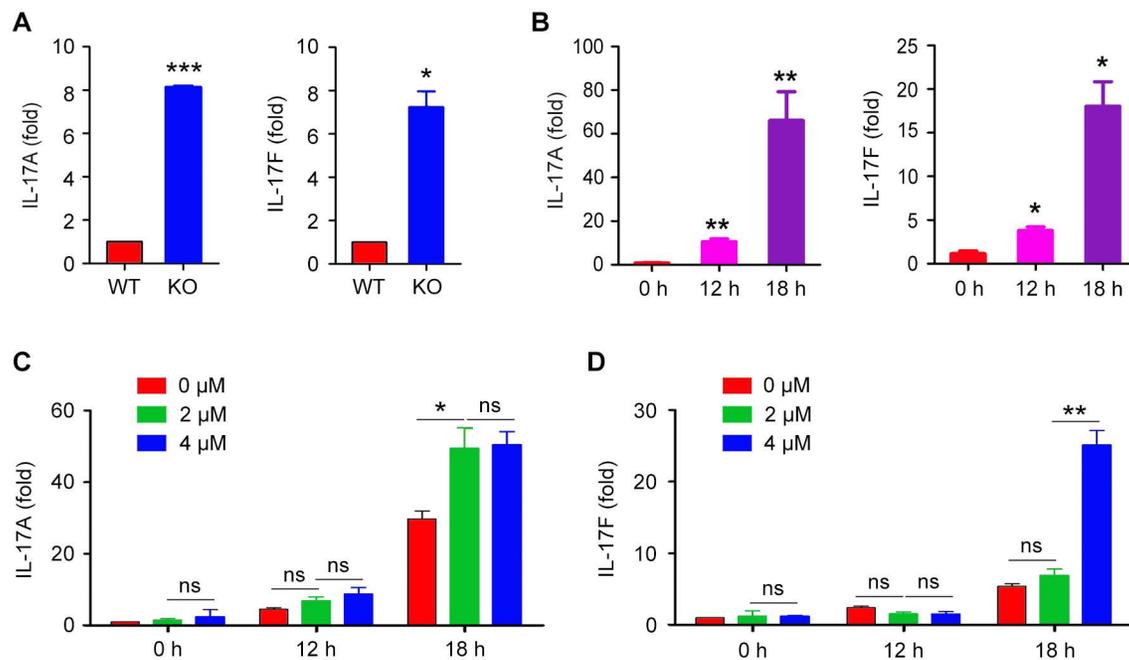


Figure 5. Effects of HDAC6 on IL-17 mRNA expression. (A) qRT-PCR analysis of IL-17A and IL-17F mRNA levels in WT and HDAC6 KO $\gamma\delta$ T cells expanded *in vitro* for 6 days and incubated with PMA and ionomycin for 6 hours. (B) qRT-PCR analysis of IL-17A and IL-17F mRNA levels in EL4 cells incubated with PMA and ionomycin for 0, 12, or 18 hours. (C and D) qRT-PCR analysis of IL-17A and IL-17F mRNA levels in EL4 cells treated with 0, 2, or 4 μ M tubacin for 4 hours, followed by incubation with PMA and ionomycin for 0, 12, or 18 hours. Data are representative of three independent experiments. Error bars indicate mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Effects of HDAC6 on IL-17-associated transcription factors and IL-17 promoter-dependent luciferase activity

Hes1, SOX4, and SOX13 are key regulators for the development of IL-17-producing $\gamma\delta$ T cells. To determine whether these transcription factors are involved in IL-17 production, we analyzed the levels of Hes1, SOX4, and SOX13 mRNAs using real-time qRT-PCR. We found that the loss of HDAC6 decreased the expression of SOX4 in $\gamma\delta$ T cells, but did not significantly affect the expression of Hes1 or SOX13 (Figure 6A). PMA and ionomycin decreased the expression of these transcription factors in EL4 cells (Figure 6B). Inhibition of HDAC6 with tubacin or tubastatin A did not rescue Hes1, SOX4, and SOX13 expression (Figure 6C and Figure S2). These results suggest that alternative mechanisms may underlie the

regulation of IL-17 production by HDAC6.

We next analyzed whether the thymus-specific isoform of retinoic acid receptor-related orphan receptor γ (ROR γ t), a transcription factor critical for the differentiation program of pro-inflammatory Th17 cells [27], is involved in the regulation of IL-17 production. Activity of the firefly luciferase under control of the IL-17 promoter could be increased by the expression of ROR γ t alone (Figure 6D). Similarly, increased ROR γ t expression also increased IL-17 promoter-dependent luciferase activity (Figure 6E). Furthermore, consistent with our findings that HDAC6 represses IL-17 expression in $\gamma\delta$ T cells, inhibition of HDAC6 with tubacin also significantly increased the IL-17 promoter-dependent luciferase activity (Figure 6F).

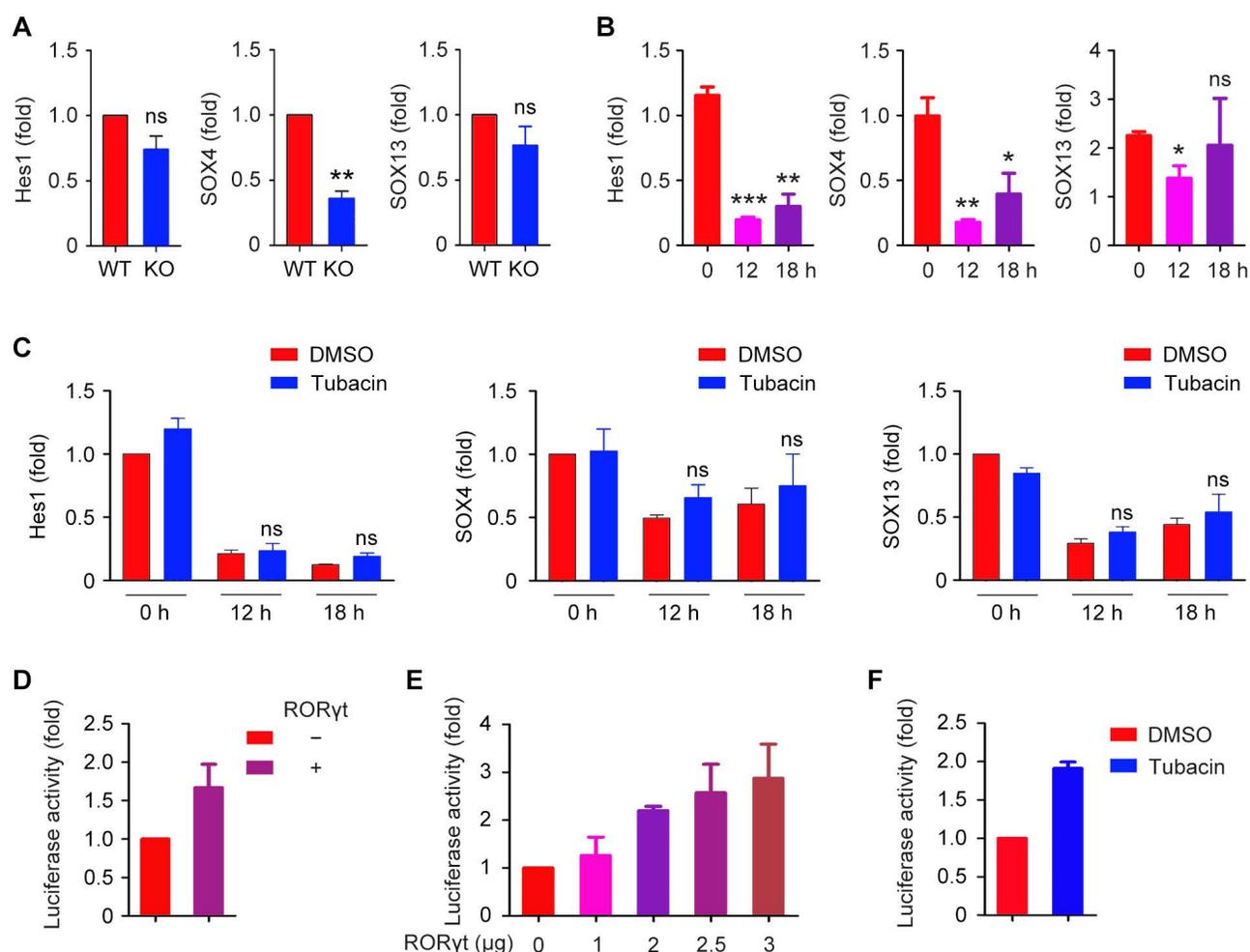


Figure 6. Effects of HDAC6 on IL-17-associated transcription factors and IL-17 promoter-dependent luciferase activity. (A) qRT-PCR analysis of Hes1, SOX4, and SOX13 mRNA levels in WT and HDAC6 KO $\gamma\delta$ T cells expanded *in vitro* for 6 days and incubated with PMA and ionomycin for 6 hours. (B) qRT-PCR analysis of Hes1, SOX4, and SOX13 mRNA levels in EL4 cells incubated with PMA and ionomycin for 0, 12, or 18 hours. (C) qRT-PCR analysis of Hes1, SOX4, and SOX13 mRNA levels in EL4 cells treated with DMSO or 2 μ M tubacin for 4 hours, followed by incubation with PMA and ionomycin for 0, 12, or 18 hours. (D) Quantification of the relative luciferase activity in cells transfected with the firefly luciferase reporter under control of the IL-17 promoter, along with an ROR γ t expression plasmid. (E) Quantification of the relative luciferase activity in EL4 cells transfected with the firefly luciferase reporter under control of the IL-17 promoter, together with increasing amounts of the ROR γ t expression plasmid. (F) Quantification of the relative luciferase activity in EL4 cells transfected with the firefly luciferase reporter under control of the IL-17 promoter and treated with DMSO or 2 μ M tubacin. Error bars indicate mean \pm SEM. * p < 0.05; ** p < 0.01; *** p < 0.001; ns, not significant.

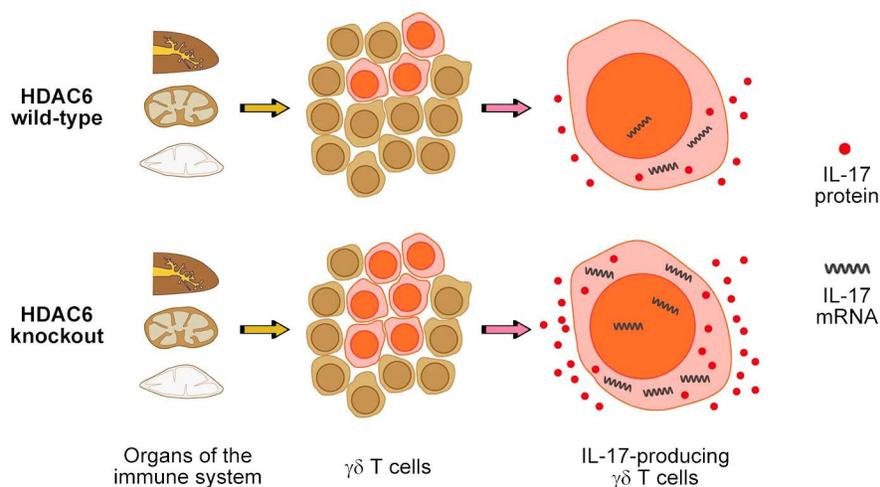


Figure 7. A working model for HDAC6 function in the regulation of IL-17 expression in $\gamma\delta$ T cells.

Discussion

Our study demonstrates that deletion of HDAC6 in mice or inhibition of its deacetylase activity promotes IL-17 expression in $\gamma\delta$ T cells (Figure 7). Although a comprehensive understanding of the mechanisms underlying the regulation of IL-17 expression in distinct subsets of T cells remains elusive, our findings demonstrate that HDAC6 plays a crucial role in the regulation of IL-17 expression (Figure 7).

Considering the similar role of HDAC6 in the regulation of IL-17 expression in $\gamma\delta$ T and EL4 cells examined in this study, we propose that the underlying mechanism is likely to be conserved and could potentially overlap known pathways by which HDAC6 regulates gene expression. For example, HDAC6 has previously been reported to play a role in the regulation of β -catenin and extracellular signal-regulated kinase (ERK) signaling pathways [28-30], as well as in the inactivation of Runt-related transcription factor 2 (Runx2), which has been shown to promote the expression of IL-17 [31]. These studies provide a plausible mechanism to explain the effect of HDAC6 activity on IL-17 expression that should be explored in future studies.

HDAC6-selective inhibitors are currently under investigation in multiple clinical settings, including cancer and neurodegenerative diseases [11, 32-34]. Our findings suggest that the side effects of HDAC6 inhibition on immunity should be considered as a part of clinical trials. Both IL-17A and IL-17F are known to play protective roles in the host defense, and T lymphocytes expressing IL-17 may recruit neutrophils to tissues undergoing inflammation [35]. Thus, increased IL-17A and IL-17F expression induced by HDAC6 inhibition may promote the clearance of

extracellular bacterial or fungal infections. However, in addition to these pro-inflammatory roles of IL-17 in host immune responses, increased IL-17 production could also result in elevated risk for autoimmune diseases.

Results from this study demonstrate that loss of HDAC6 promotes the accumulation of IL-17-producing $\gamma\delta$ T cells. This subset of T lymphocytes has previously been reported to play a protective role in concanavalin A-induced hepatitis [8]. Thus, it is possible that inhibition of HDAC6 could promote the differentiation of IL-17-producing $\gamma\delta$ T cells during embryonic development, resulting in an increase in the population of IL-17-producing $\gamma\delta$ T cells in peripheral immune organs. These results suggest that drugs targeting HDAC6 might be investigated for a protective role in hepatitis.

Supplementary Material

Supplementary figures.

<http://www.thno.org/v07p1002s1.pdf>

Abbreviations

IL-17, interleukin 17; Th17, T-helper 17; HDAC6, histone deacetylase 6; WT, wild-type; HZ, heterozygous; KO, knockout; qRT-PCR, quantitative reverse transcription-PCR; IFN- γ , interferon- γ ; ROR γ t, thymus-specific isoform of retinoic acid receptor-related orphan receptor γ ; ERK, extracellular signal-regulated kinase; Runx2, Runt-related transcription factor 2.

Acknowledgements

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Competing Interests

The authors have declared that no competing interest exists.

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