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Interaction of Ets-1 with HDAC1 Represses IL-10 Expression in Th1 Cells

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IL-10 is a multifunctional cytokine that plays a crucial role in immunity and tolerance. IL-10 is produced by diverse immune cell types, including B cells and subsets of T cells. Although Th1 produce IL-10, their expression levels are much lower than Th2 cells under conventional stimulation conditions. The potential role of E26 transformation-specific 1 (Ets-1) transcription factor as a negative regulator for Il10 gene expression in CD4+ T cells has been implicated previously. In this study, we investigated the underlying mechanism of Ets-1–mediated Il10 gene repression in Th1 cells. Compared with wild type Th1 cells, Ets-1 knockout Th1 cells expressed a significantly higher level of IL-10, which is comparable with that of wild type Th2 cells. Upregulation of IL-10 expression in Ets-1 knockout Th1 cells was accompanied by enhanced chromatin accessibility and increased recruitment of histone H3 acetylation at the Il10 regulatory regions. Reciprocally, Ets-1 deficiency significantly decreased histone deacetylase 1 (HDAC1) enrichment at the Il10 regulatory regions. Treatment with trichostatin A, an inhibitor of HDAC family, significantly increased Il10 gene expression by increasing histone H3 acetylation recruitment. We further demonstrated a physical interaction between Ets-1 and HDAC1. Coinexpression of Ets-1 with HDAC1 synergistically repressed IL-10 transcription activity. In summary, our data suggest that an interaction of Ets-1 with HDAC1 represses the Il10 gene expression in Th1 cells. The Journal of Immunology, 2012, 188: 2244–2253.

Many cell types, such as B cells, macrophages, mast cells, eosinophils, dendritic cells, and diverse subsets of T cells, produce IL-10, an immunoregulatory cytokine. IL-10 has anti-inflammatory properties and inhibits the function of macrophages and dendritic cells. IL-10 also plays critical roles in maintaining immune homeostasis and has diverse effects on numerous nonimmune cell populations, such as keratinocytes and endothelial cells (1, 2).

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CNS, conserved noncoding sequence; Ets-1, E26 transformation-specific 1; Ets-1KO, Ets-1 knockout; H3Ac, histone H3 acetylation; HDAC1, histone deacetylase 1; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IB, immunoblotting; IP, immunoprecipitated; KO, knockout; NP-40, Nonidet P-40; PLA, proximity ligation assay; qRT-PCR, quantitative RT-PCR; TsA, trichostatin A; UTR, untranslated region; WT, wild type.

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Naive CD4+ T cells can be differentiated into various effector populations, such as Th1, Th2, Th17, and regulatory T cells, when they are exposed to Ags with unique cytokine milieu. For example, Th1 cells produce IFN-γ and protect against intracellular pathogen such as virus and bacteria. Th2 cells produce IL-4, IL-5, and IL-13, and confer protection against multicellular parasitic infection (3, 4). Initially, IL-10 was reported as a typical Th2 cytokine (1). Recently, Th1 cells were also reported to produce IL-10, but only under specific circumstances (2, 5), such as in chronic and nonhealing infectious conditions (6, 7). In line with this, IL-12, IL-27, and Notch signals can also induce IL-10 production in Th1 cells (8–10). Nevertheless, the expression level of IL-10 in conventional Th1 cells still needs to be maintained in a repressed state to allow Th1 cells to exercise their effector function. Following the earlier notion, it was reported that the promoter and several putative regulatory elements within the Il10 locus were silenced in Th1 cells (11, 12). Several studies have elucidated the role of specific transcription factors, such as GATA-3 and c-Jun, together with epigenetic mechanisms, in the regulation of Il10 gene expression in Th2 cells (12–15). However, it is still unclear how the repressed state of Il10 gene expression is maintained in conventional Th1 cells.

E26 transformation-specific 1 (Ets-1) is a member of the ETS family of transcription factors, and it binds to the conserved GGAA/T sequence (16, 17). Previous studies in Ets-1 knockout (Ets-1KO) mice have demonstrated the important functions of Ets-1 in development, proliferation, and survival of NK and T cells (18–20). Furthermore, Ets-1 acts as a cofactor of T-bet and is essential for Th1 effector function and differentiation by regulating IFN-γ expression (21). Ets-1 is a negative regulator of Th17 differentiation, and Th17 cells deficient of Ets-1 express increased IL-17 and IL-17–related cytokines (22). On the contrary, Ets-1 positively regulates several cytokine genes such as Il2, Il5, and Gmcscf (23). These studies suggest that Ets-1 may modulate the
effector function of Th cells by acting as a positive or negative regulator in a context-dependent manner (24–26). In addition, Ets-1 deficiency leads to altered B cell differentiation, hyperresponsiveness to TLR9, and autoimmunity disease (22, 27). Interestingly, Ets-1KO Th1 cells produce an abnormally high level of IL-10 (21). Despite these observations, it is still unclear how Ets-1 represses the II10 gene expression in Th1 cells.

In this study, we demonstrated that Ets-1KO Th cells showed elevated IL-10 expression upon ex vivo stimulation. Th1 cells from Ets-1KO mice showed increased histone H3 acetylation (H3Ac) recruitment but reduced histone deacetylase 1 (HDAC1) binding at the II10 regulatory regions compared with their wild type (WT) counterparts. We further tested and demonstrated that a physical interaction between Ets-1 and HDAC1 cooperatively downregulated II10 gene expression in Th1 cells.

### Materials and Methods

**Mice, cells, and reagents**

C57BL/6 and BALB/c mice were purchased from Orient Bio (Gyeonggi-do, Korea). Ets-1−/−deficient (21), T-bet−/−deficient (28) and STAT4−/−deficient (29) mice were described previously. Mice were housed in specific pathogen-free barrier facilities and used in accordance with protocols approved by the Animal Care and Ethics Committees of the Gwangju Institute of Science and Technology. The HEK-293 cells were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). Cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin. Recombinant humanIL-2 and anti–IL-4 (11B11) were purchased from Sigma-Aldrich (St. Louis, MO). IL-4 was purchased from PeproTech (Rocky Hill, NY), and anti–IL-12 was purchased from Sanquin (Amsterdam, the Netherlands). Anti-CD3 (XMG1.2), and anti–IL-12 (5/7CTCCTGCACATCTGCT-3‘ and 5’-CAGGCTGAGATGAGATCCTG-3’) were provided by the National Cancer Institute, Preclinical Repository (Bethesda, MD). IL-4 was purchased from PeproTech (Rocky Hill, NY), and IL-12 was purchased from Sigma-Aldrich (St. Louis, MO). Anti-CD3 (145.2C11), anti-CD28 (37.51), anti–IFN-γ (XMG1.2), and anti–IL-12 (C17.8) were purchased from BD Biosciences (San Jose, CA). Trichostatic acid A and DMSO were obtained from Sigma-Aldrich.

**CD4+ T cell purification, differentiation, and ELISA**

CD4+ T cells were purified from the lymph nodes and spleen of 8 to 10-wk-old mice using magnetic beads (LJT34; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). For Th differentiation, the cells (5 × 106/ml) were stimulated with 1 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 under Th1-skewing (10 ng/ml IL-2 plus 10 μg/ml anti–IL-4) or Th2-skewing (10 ng/ml IL-4, 10 μg/ml anti–IL-4, and 100 U/ml recombinant human IL-2) for 6 d. ELISA for IL-10 (21). Despite these observations, it is still unclear how Ets-1 represses the II10 gene expression in Th1 cells.

### Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously (30). In brief, cells were cross-linked with formaldehyde at a final concentration 1%, lysed, and sonicated to shear DNA. After immunoprecipitation with anti–Ets-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HDAC1 (Abcam, Cambridge, MA), and anti–H3Ac (K9/14) (Millipore, Billerica, MA), or rabbit IgG (Vector Laboratories, Burlingame, CA) at 4˚C overnight, Ab/DNA complexes were eluted and cross-linking was reversed by boiling with Chelex-100 (31). After reversal of cross-links, the presence of selected DNA sequences was assessed by real-time PCR using the following primers: CNS−0.12 (5’-CTGTGATCATAGACAGCTGT-3’ and 5’-CTGTTGCGGAAATGAACTTCTG-3’), CNS+6.45 (5’-CTACATCAGGGAGCAGGCTG-3’ and 5’-CTTACCTACAGGGACCTG-3’), CNS+2.98 (5’-ACTAGGTTGAGAAGAGCTG-3’ and 5’-GATCTCGTCTTCTGCTG-3’), CNS+6.45 (5’-GTTGCATTTTCTGCAGAAGCTG-3’ and 5’-CTACATCAGGGAGCAGGCTG-3’ and 5’-CTTACCTACAGGGACCTG-3’), CCR8 promoter (5’-GTTTGAACAGTAGGCTGCT-3’ and 5’-GGTTTGAACAGTAGGCTGCT-3’), Bcl-XL 3’untranslated region (UTR) (5’-CTGAAGTCTAAACGCTTGCGG-3’ and 5’-CTGAAAGTCCACCTGCTGAG-3’). As a loading control, the qRT-PCR was done directly on DNA purified from immunocapture before immunoprecipitation. Data are presented as the amount of DNA recovered relative to the input control. Result of ChIP with isotype IgG was confirmed as a background value and showed <0.001 of relative ratio to input. IgG level was shown only when it is necessary.

### Chromatin accessibility by real-time PCR assay

Chromatin accessibility by real-time PCR assays were performed as described previously (32) with minor modifications. Cells were washed and lysed by resuspension pellets in ice-cold Nonidet P-40 (NP-40) lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM MgCl2, 0.5% NP-40, 0.15 mM spermine, 0.5 mM spermidine) and incubating on ice for 5 min. Nuclei were collected by centrifugation at 3000 rpm for 5 min at 4˚C. Isolated nuclei were washed with Mnase digestion buffer containing CaCl2 (10 mM Tris-HCl [pH 7.4], 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine), collected by centrifugation at 3000 rpm for 5 min at 4˚C, and resuspended in Mnase digestion buffer supplemented with 1 mM CaCl2. Nucleosome treatment was achieved with or without Mnase with various concentrations. Samples were divided as “cut” or “uncut” to identify MNase-treated and control untreated samples, respectively. Samples were then incubated at 37˚C for 1 min. Reactions were stopped by addition of 20 μl 100 μM EDTA/10 mM EGTA (pH 8.1) and 10 μl of 10% SDS (w/v). Genomic DNA was isolated using Genomic DNA prep kit (Salgent, Daejeon, Korea). Concentrations of final eluted DNA were accessed by means of a Bioanalyzer spectrophotometer (Shimadzu, Kyoto, Japan), and 50 ng DNA was subsequently used as a template in real-time PCR to quantify target sequences in “cut” and “uncut” samples. Each eluate was also subject to agarose gel electrophoresis to visualize the extent of Mnase digestion of genomic DNA. Primers used in the quantitative assays were validated by amplifying serially diluted genomic DNA as templates to create standard curve for each primer set and analyzed using the quantification method. Chromatin accessibility was calculated and expressed as the average fold difference of target template in “uncut” over “cut” samples. Thus, readily digested sequences (hypersensitive sites) were expected to be depleted in Mnase-treated samples and give higher chromatin accessibility values than less accessible sequences.

### Immunoprecipitation and immunoblotting

Immunoprecipitation was performed using a polyclonal Ab recognizing HDAC1 (Abcam), Ets-1 (Santa Cruz Biotechnology), or rabbit IgG (Vector Laboratories, Burlingame, CA) following protein A-Sepharose (Millipore) incubation overexpressed HEK-293 or primary Th1 total lysates. To determine levels of HDAC1 protein, we prepared total lysate from Th1 and Ets-1KO Th1 cells in RIPA buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 1% NP-40) using protease inhibitor cocktail mixture (Roche, Mannheim, Germany) for 30 min on ice. Samples were loaded with a Page Ruler Prestained Protein ladder (Fermentas, Glen Burnie, MD) on a 10% SDS-PAGE gel. The proteins were electroblotted onto a nitro-
cellulose membrane (Bio-Rad, Richmond, CA). After blocking for 1 h in 5% skim milk, the membranes were incubated with mAb of Ets-1 (Santa Cruz Biotechnology), HDAC1 (Millipore), and β-actin (Abcam) in 3% blocking buffer overnight at 4°C. The blots were developed using a 1/5000 diluted anti-mouse HRP (Abcam) and visualized.

**Intracellular cytokine staining**

Cells were stimulated with 1 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 plus 10 μg/ml brefeldin A (Sigma-Aldrich) for 6 h. Cells were harvested, washed with PBS, and fixed in fixation/permeabilization buffer (eBioscience, San Diego, CA) for 0.5 h. Cells were washed and resuspended in 100 μl permeabilization buffer (eBioscience). For intracellular detection of IL-10, anti–IL-10–PE (eBioscience) or isotype control Ab (eBioscience) was added and incubated for 0.5 h at 4°C. After incubation, cells were washed, resuspended in 1 ml PBS, and analyzed by flow cytometry (Beckman Coulter, Brea, CA).

**Proximity ligation assay**

WT Th1 and Ets-1KO Th1 cells were differentiated and were incubated in 8S Multicell (CTRL Life Science, Gwangju, Korea) coated with Poly-l-Lys in PBS for overnight at 4°C. For fixation, 4% paraformaldehyde was added and incubated for 10 min. 0.25% Triton X-100 was used for permeabilization. Proximity ligation assay was performed using the Rabbit PLUS and Mouse MINUS Duolink in situ proximity ligation assay (PLA) kits with Ab of Ets-1 (rabbit) and HDAC1 (mouse) (OINK Bioscience, Uppsala, Sweden) according to the manufacturer’s protocol. Subsequently, slides were dehydrated, air-dried, and embedded in DAPI-containing mounting medium. Fluorescence was detected using a Fluoview microscope (Olympus, Center Valley, PA).

**Computational analysis**

Genomic sequences spanning the Il10 gene were analyzed using the Web-based alignment software, VISTA browser 2.0 (33), to identify CNSs.

**Statistical analysis**

Data are the mean ± SD of at least three independent experiments, unless differently specified in the text. A Student t test was used to calculate the statistical significance of the experimental data. The level of significance was set at *p < 0.05 and **p < 0.01. Significance was indicated only when appropriate.

**Results**

**Increased IL-10 expression in Ets-1–deficient Th cells**

To elucidate the molecular mechanism of Ets-1–mediated Il10 gene repression in Th1 cells, we compared IL-10 expression in Th1 cells differentiated from WT or Ets-1KO mice. Cells were left without stimulation or stimulated with anti-CD3/anti-CD28; then IL-10 mRNA and protein levels were measured by qRT-PCR, ELISA, and flow cytometry. Th1 cells from Ets-1KO showed >8-fold increase in IL-10 mRNA expression compared with WT Th1 cells (Fig. 1A). Next, we measured the IL-10 protein level by ELISA to check whether protein level is also increased in Ets-1KO cells. Indeed, a significant increase of IL-10 (9-fold) was observed in Ets-1KO cells compared with WT Th1 cells (Fig. 1B). We further analyzed protein expression by performing intracellular cytokine staining. The IL-10+ cells constituted ∼9% of Ets-1KO cells compared with 1.7% in WT Th1 (Fig. 1C). Similarly, Ets-1KO Th2 cells also expressed more IL-10 than WT Th2 cells even though WT cells already produced a high level of IL-10.

We further tested whether ex vivo isolated Ets-1KO CD4+ T cells also expressed higher levels of IL-10 transcript than WT cells. Like in Th1 cells, ex vivo CD4+ T cells isolated from Ets-1KO mice expressed a much higher level of IL-10 transcript compared with WT CD4+ T cells under unstimulated (>8-fold increase) and stimulated (>5-fold increase) conditions (Fig. 2A). A significant increase in IL-10 protein level (2-fold) was also observed in Ets-1KO cells compared with WT CD4+ T cells (Fig. 2B). Intracellular cytokine staining showed that ∼0.5% of WT Th cells was stained positively for intracellular IL-10 ex vivo, whereas near 3% of Ets-1KO cells were positive for IL-10 (Fig. 2C). These results collectively indicate that Ets-1 plays a negative role in Il10 gene regulation in both Th1 and Th2 cells.

**Changes in histone modification and chromatin accessibility upon Ets-1 deficiency**

To find out the underlying molecular mechanism of Ets-1–mediated Il10 gene repression in Th1 cells, we analyzed whether Ets-1 deficiency caused any changes in histone architecture and epigenetic modifications around the Il10 regulatory regions. We and others previously reported the involvement of epigenetic regulation in IL-10 expression (11–14). We have previously measured the levels of recruited H3Ac and H4Ac in this region compared with other regulatory regions. We focused on four CNSs of the Il10 locus identified with VISTA: promoter (−0.12), intron regions (+1.75 and +2.98), and 3′ end (+6.45) (Fig. 3A). To measure the relative amount of H3Ac recruited to the promoter and intronic regions of the Il10 locus, we performed ChIP with Ab specific for H3Ac. Consistent with the published data, a significant increase of H3Ac level was observed in WT Th2 cells compared with WT Th1 cells at the promoter (−0.12) and introns (+1.64 and +2.98) (Fig. 3B). However, the CNS+6.45 region did not show any difference in H3Ac levels between WT Th1 and Th2 cells. This observation is in agreement with the previous report showing no histone acetylation (H4Ac) in this region compared with other regions of the Il10 locus (13). Interestingly, Ets-1KO Th1 cells displayed a pattern of H3Ac recruitment similar to that of WT Th2 cells. We further tested whether increased H3Ac recruitment and
higher IL-10 expression in Ets-1KO Th1 cells are associated with changes in the accessibility of chromatin architecture around the Il10 locus. Nuclei were prepared from Th1 cells differentiated from WT and Ets-1KO CD4^+ T cells and treated with MNase. The chromatin accessibility was measured by qRT-PCR. Ets-1KO Th1 cells showed a significantly increased chromatin accessibility than WT Th1 cells in the IL-10 promoter region, but not in CNS+1.65 and CNS+2.98 regions (Fig. 3C). The chromatin accessibility for the promoter region of HPRT and H19 genes was also tested as control regions for open and closed accessibility, respectively. These results suggest that an increased IL-10 expression in Ets-1KO Th1 cells is correlated with enhanced chromatin accessibility and H3Ac enrichment.

**Downregulation of HDAC1 recruitment in Ets-1–deficient Th1 cells**

Acetylation of histone is regulated by HDACs (35). The class I deacetylase HDAC1 and HDAC2 are highly expressed in thymus and spleen, and HDAC1-associated factors, such as Ikaros, Aiolos, and Sin3A, play important roles during T cell development (36, 37). To examine whether the increased H3Ac recruitment and more accessible chromatin structure at the Il10 locus in Ets-1KO Th1 cells are related with the loss of HDAC1 recruitment, we analyzed the level of in vivo HDAC1 binding to Il10 regulatory loci by ChIP analysis. Previous studies including our own have showed that higher HDAC1 recruitment is inversely correlated to IL-10 expression (11, 12). In this study, we also confirmed the involvement of HDAC1 recruitment in differential IL-10 expression in Th1 and Th2 cells. Th1 cells expressing much lower IL-10 level than Th2 cells showed a preferential HDAC1 enrichment in promoter (−0.12) and intron regions (+1.65 and +2.98) (Fig. 4A). HDAC1 enrichment at the Il10 regulatory regions, however, was significantly reduced in Ets-1KO Th1 cells to the comparable level of WT Th2 cells (Fig. 4A).

To further elucidate the role of HDAC in IL-10 repression, we tested the effect of HDAC inhibitor, trichostatin A (TsA), on IL-10 expression level and histone acetylation status in Th1 cells. CD4^+ T cells obtained from WT and Ets-1KO were differentiated into Th1 cells. At day 4 of differentiation, cells were treated with TsA or DMSO (control) and then harvested 2 d later. TsA treatment significantly increased IL-10 expression up to 8-fold as measured by qRT-PCR (Fig. 4B). TsA treatment also increased IL-10 protein levels as measured by ELISA and intracellular cytokine staining (Fig. 4B, 4C). To test whether the increase of IL-10 expression by TsA treatment was also affected by changes in the recruitment of H3Ac to the regulatory elements of the Il10 locus, we performed ChIP analysis. Indeed, TsA treatment significantly increased H3Ac recruitment at CNS−0.12, CNS+1.65, and CNS+2.98 (Fig. 4D). These results collectively suggest that Ets-1 deficiency reduced the recruitment of HDAC1 in Il10 regulatory elements, which leads to increased histone acetylation and favorable chromatin architecture for upregulation of IL-10 expression.

**Physical interaction between Ets-1 and HDAC1**

We then set to determine how deficiency of Ets-1 led to a reduced level of HDAC1 recruitment in the Il10 regulatory regions. We found that mRNA level of HDAC1 was not altered by Ets-1 deficiency (data not shown). However, HDAC1 protein level was slightly decreased in Ets-1KO Th1 cells compared with WT counterpart (Fig. 5A). However, this difference was subtle and could not account for the difference in HDAC1 recruitment shown in Fig. 4A. We therefore postulated that Ets-1 interacted directly with and recruited HDAC1 to the Il10 locus. To test whether there is a physical interaction between Ets-1 and HDAC1, we performed communoprecipitation experiment in HEK-293 cells that were transfected with Ets-1 and HDAC1 expression constructs. HDAC1 protein was detected by anti-HDAC1 immunoblotting (IB) in immunoprecipitate brought down by Ets-1 Ab (Fig. 5B, top panel). Conversely, immunoprecipitation with anti-HDAC1 Ab, but not control IgG, also pulled down Ets-1 protein (Fig. 5B, bottom panel). In addition, we were able to demonstrate a physical interaction between endogenous Ets-1 and HDAC1 proteins in vivo in primary Th1 cells by immunoprecipitation assay (Fig. 5C). To further confirm the colocalization of Ets-1 and HDAC1, we performed an in situ PLA (38). Because a PLA signal can be detected when the proteins of interest are in close proximity, this technique enabled us to detect direct protein–protein (Ets-1 and HDAC1) interaction in CD4^+ T cells. Association of endogenous Ets-1 and HDAC1 in primary Th1 cells was observed and the interaction took place in the nucleus (Fig. 5D, upper row). The specificity of this interaction was confirmed by the absence of association between Ets-1 with HDAC1 in Ets-1KO Th1 cells (Fig. 5D, bottom row).

**Functional synergism of Ets-1 and HDAC1 to repress IL-10 expression**

To test the relationship between Ets-1 recruitment and the differential IL-10 expression profile, we performed an Ets-1 ChIP assay in Th1 (IL-10^{lo}) and Th2 (IL-10^{hi}) cells, using an Ets-1–specific Ab. Specific binding of Ets-1 to the IL-10 regulatory elements was confirmed using Ets-1KO mice as a negative control in Th1 cells (Fig. 6A). No significant difference was observed in
the level of Ets-1 enrichment between Th1 and Th2 cells (Fig. 6B). We further examined the functional significance of the interaction between Ets-1 and HDAC1 in the repression of Il10 gene expression. We performed in vitro reporter assay using IL-10 reporter constructs containing a minimal IL-10 promoter and the diverse CNSs (30). Overexpression of Ets-1 alone failed to repress the IL-10 reporter activity of CNS−0.12 (Fig. 6C, upper panel, fourth bar). Interestingly, Ets-1 alone actually induced reporter

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**FIGURE 3.** Accessible chromatin configuration at the Il10 regulatory regions in Ets-1KO Th1 cells. (A) CNSs in the Il10 loci of mouse and human are shown. The mouse genomic sequence is used as the base sequence on the x-axis. CNS regions are named according to their distance in kilobases from the IL-10 transcription start site. (B) ChIP was performed using WT and Ets-1KO Th1 cells with anti-H3Ac or isotype IgG Ab, and the levels of precipitated DNA were measured by qRT-PCR with primers specific for each indicated CNS region. (C) Nuclei isolated from WT or Ets-1KO Th1 cells were left untreated or subjected to MNase treatment, as described in Materials and Methods. Extracted genomic DNA was then subjected to RT-PCR using specific primers for indicated regulatory regions. Graphs depict the relative chromatin accessibility normalized to undigested samples and show mean ± SEM, n = 3. All data are presented as means with SD from three independent experiments. *p < 0.05, **p < 0.01.

**FIGURE 4.** Increased IL-10 expression upon treatment of HDAC inhibitor, TsA. (A) ChIP was performed using WT and Ets-1KO Th1 cells with anti-HDAC1 or isotype IgG Ab, and levels of precipitated DNA were measured by qRT-PCR with primers specific for each indicated CNS region. All data are presented as means with SD from three independent experiments. (B) IL-10 mRNA and protein level were analyzed by qRT-PCR and ELISA. (C) Intracellular IL-10 protein level was measured by flow cytometry analysis after intracellular cytokine staining with IL-10 Ab or control isotype IgG. (D) ChIP was performed on nontreated or TsA-treated cells with anti-H3Ac or isotype IgG Ab, and levels of precipitated DNA were measured by qRT-PCR with primers specific for each indicated CNS region. All data are presented as means with SD from three independent experiments; intracellular cytokine staining data are representative of three separate experiments. *p < 0.05, **p < 0.01.
activity of CNS+1.65 and CNS+2.98. However, dose-dependent overexpression of HDAC1 significantly decreased Ets-1–induced IL-10 reporter activity (Fig. 6C, fifth and sixth bars). These results suggest that Ets-1–mediated repression of IL-10 expression is dependent on HDAC1.

FIGURE 5. Physical interaction of Ets-1 with HDAC1. (A) Total cell lysates of WT or Ets-1KO Th1 cells were prepared and total HDAC1 levels were assayed by Western blot. Ab to β-actin was used as a loading control. (B) Flag-Ets-1 and HDAC1 constructs were transiently transfected into HEK-293 cells. Transfected cells were harvested after 24 h and lysates were IP with Ets-1 or isotype IgG Ab. Coprecipitated HDAC1 was analyzed by IB with HDAC1 Ab. IP of Ets-1 was confirmed by IB with Ets-1 Ab. Conversely, transfected cells were harvested after 24 h and lysates were IP with HDAC1 or isotype IgG Ab. Coprecipitated Ets-1 was analyzed by IB with Ets-1 Ab. IP of HDAC1 was confirmed by IB with HDAC1 Ab. (C) Primary Th1 cells were used for immunoprecipitation with Ets-1 Ab, then detected with HDAC1 Ab. Ponceau S–stained IgG H chain was shown as a loading control (IgG). Immunoprecipitation using rabbit IgG (isotype) was used as a negative control for coimmunoprecipitation. (D) Detection of Ets-1 and HDAC1 interaction in primary WT Th1 and Ets-1KO Th1 cells using Duolink with two primary Abs. Duolink signals are shown in red, and DAPI was used for staining of nuclei. Stained cells were examined by confocal microscopy (original magnification ×60). Data were representative of three independent experiments.

FIGURE 6. Suppression of IL-10 expression by functional cooperation between Ets-1 and HDAC1. (A) ChIP was performed using WT and Ets-1KO Th1 cells with anti–Ets-1 or isotype IgG Ab, and the levels of precipitated DNA were measured by qRT-PCR with primers specific for each indicated CNS region. (B) Ets-1 recruitment in the Il10 regulatory regions in Th1 and Th2 cells. ChIP assay was performed using primary Th1 and Th2 cells. Quantitative real-time PCR was performed on each CNS region as indicated. Data are presented as means with SD from three independent experiments. (C) HEK-293 cells were transfected with the indicated IL-10 CNS reporter constructs together with Ets-1 or HDAC1 alone, or Ets-1 plus HDAC1 in combination. Luciferase activity is expressed relative to the expression of a cotransfected Renilla luciferase plasmid (pRL-TK) as a control for transfection efficiency. Relative luciferase units are expressed as the fold difference relative to the control value. Data shown are the average of at least three independent experiments. *p < 0.05, **p < 0.01.

Role of T-bet or STAT4 in Ets-1–mediated IL-10 repression
A previous report showed that Ets-1 deficiency could decrease the level of STAT4 and T-bet expression (21). However, the exact role of T-bet or STAT4 in regulating IL-10 expression still remains undefined. Thus, we tested whether the increased IL-10 expression
in Ets-1–deficient Th1 cells is a result of a defect in Th1 cell differentiation and whether T-bet or STAT4 deficiency is linked to differential Ets-1 recruitment to the IL-10 regulatory sites. The effect of T-bet or STAT4 deficiency on IL-10 expression was analyzed. CD4+ T cells from T-betKO and STAT4KO were differentiated into Th1 cells, and IL-10 levels were measured by RT-PCR and ELISA. In vitro differentiated Th1 cells from T-betKO and STAT4KO did not show any significant differences in the levels of IL-10 production, mRNA (data not shown), and protein (Fig. 7A, 7D). As expected, T-betKO and STAT4KO T cells showed significantly reduced IFN-γ, whereas IL-4 level was up-regulated (Supplemental Fig. 1). Next, we performed Ets-1 ChIP analysis to test the effect of T-bet or STAT4 deficiency on the recruitment of Ets-1 and HDAC1 to the IL-10 regulatory sites. T-bet or STAT4 deficiency slightly increased Ets-1 binding to CNS−0.12 and CNS+1.65, but not CNS+2.98. However, no significant difference was observed in the level of HDAC1 recruitment to the IL-10 regulatory sites, which is well correlated with the similar level of IL-10 expression, regardless of T-betKO and STAT4KO deficiency (Fig. 7A, 7D). This result suggests that T-bet or STAT4 may not directly regulate Ets-1–mediated IL-10 repression in Th1 cells.

Increased IL-24 and CCR8 expression in Ets-1–deficient Th1 cells

We also tested whether the Ets-1 and HDAC1 interaction could also downregulate the expression of Th2 cell-associated molecules in Th1 cells. Similar to IL-10 expression in Th1 cells, IL-24 level is significantly lower in Th1 than Th2 cells (39). Chemokine receptor CCR8 is also preferentially expressed in Th2, but not Th1 cells (40). Because both targets have relatively low expression level in Th1 cells, we tested the effect of Ets-1 deficiency on the expression level of IL24 and Ccr8 mRNA. Ets-1KO Th1 cells showed an increase in both IL24 and Ccr8 expression level (Fig. 8A). To further confirm the physical association of Ets-1 and HDAC1 to the promoter of IL24 and Ccr8 loci, we performed Ets-1 and HDAC1 ChIP experiments. Bcl2l1 3′ UTR region was used as a negative binding region for Ets-1 and HDAC1 (41). Significant enrichment of Ets-1 and HDAC1 was observed in the promoter region of IL24 and Ccr8 compared with the negative Bcl2l1 locus (Fig. 8B, 8C). In addition, Ets-1 deficiency significantly increased the level of H3Ac recruitment to the IL24 and Ccr8 promoter regions (Fig. 8D). These results collectively suggest that interaction of Ets-1 with HDAC1 could repress the expression of Th2 cell-associated molecules, such as Il10, Il24, and Ccr8, in Th1 cells by affecting histone modification of regulatory elements.

Discussion

The main purpose of this study was to elucidate the molecular mechanism of Ets-1–mediated Il10 gene repression in Th1 cells. We demonstrated that Ets-1 negatively regulates IL-10 expression through functional and physical cooperation with HDAC1 in Th1 cells. Ets-1 deficiency resulted in increased H3Ac recruitment and chromatin accessibility, whereas decreasing HDAC1 recruitment in the Il10 regulatory regions. Physical interaction between Ets-1 and HDAC1 cooperatively repressed IL-10 transcription in Th1 cells.

Unlike subset-specific Th cytokines, such as IFN-γ and IL-4, IL-10 is relatively promiscuous and can be produced by both Th2 and Th1 cells. Although Th2 cells express a much higher amount of IL-10 (11, 12), Th1 cells also express IL-10 under certain conditions (2, 5–10) through unknown mechanisms. We have previously shown that Ets-1–deficient Th1 cells produced larger amounts of IL-10 than WT Th1 cells (21). In this study, we further confirmed a significant increase of IL-10 expression in ex vivo isolated Ets-1KO CD4 T cells (Fig. 2). Ets-1 is a transcription factor and has a dual function, acting as both a transcriptional activator and repressor (16, 42, 43). In most cases, Ets-1 positively regulates the expression of its target genes (23). However, Ets-1 can also suppress its target gene through interacting with repressive factors like EAP1/Daxx (44). Recently, Hollenhorst et al. (45) reported an enrichment of Ets-1 in regulatory elements of the Il10 locus through ChIP-seq analysis in human Jurkat T cell. We also found in vivo binding of Ets-1 in the regulatory regions of the Il10 gene including promoter, intron 3, and intron 4 regions (Fig. 6A). However, the amount of Ets-1 binding was similar in Th1 (IL-10low) and Th2 (IL-10high) cells (Fig. 6B). How does Ets-1 binding selectively repress IL-10 expression in Th1 cells? We assumed that Ets-1 may regulate IL-10 expression by altering the local chromatin architecture. Indeed, chromatin accessibility was significantly increased in Ets-1KO Th1 cells, mainly at the IL-10 promoter region (Fig. 3C), in comparison with WT Th1 cells. We also tested the H3Ac level in Il10 regulatory regions because H3Ac is one of the well-characterized active marks of histone

**FIGURE 7.** Role of T-bet or STAT4 on Ets-1–mediated IL-10 repression in Th1 cells. (A and D) CD4+ T cells were isolated from WT and T-betKO or STAT4KO mice. After 6 d of Th1 differentiation, cells were stimulated with anti-CD3/anti-CD28. IL-10 expression was analyzed by ELISA. (B and E) ChIP was performed using WT and T-betKO or STAT4KO Th1 cells with anti–Ets-1 or isotype IgG Ab. (C and F) ChIP was performed using WT and T-betKO or STAT4KO Th1 cells with anti-HDAC1 or isotype IgG Ab, and the levels of precipitated DNA were measured by qRT-PCR with primers specific for each indicated CNS region. Results showed mean ± SEM, n = 3. All data are presented as means with SD from three independent experiments. *p < 0.05.
FIGURE 8. Repression of Il24 and Ccr8 expression by Ets-1 and HDAC1. (A) RNA was isolated from WT and Ets-1KO Th1 cells under unstimulated conditions after 6 d of differentiation. Il24 and Ccr8 mRNA were analyzed by qRT-PCR, and Bcl2l1 was used as a negative control. (B) ChIP was performed using WT Th1 cells with anti-Ets-1 or anti-HDAC1 Ab. Promoter regions of IL-24 and Ccr8 were used for enrichment. (C) ChIP was performed using WT and Ets-1KO Th1 cells with anti-H3Ac or isotype IgG Ab (and the levels of precipitated DNA were measured by qRT-PCR with primers specific for each indicated region). Results showed mean ± SEM; n = 3. All data are presented as means with SD from three independent experiments. *p < 0.05, **p < 0.01.

modification in gene expression (34, 35, 46, 47). Ets-1KO Th1 cells showed a significantly higher H3Ac level around the IL10 regulatory regions (Fig. 3B). These results suggest that higher expression level of IL-10 in Ets-1KO Th1 cells is closely linked with enhanced histone acetylation status (11, 13).

DNA binding proteins often interact with and recruit HDAC proteins to exert their suppressive activity on target gene expression (46). The increased chromatin accessibility together with enriched H3Ac level in Ets-1KO Th1 cells suggest the involvement of differential recruitment of silencing complexes. HDAC proteins are the main regulators of histone deacetylation (35). Previously, we reported that HDAC1 was enriched in the promoter and introns 3 and 4 of the Il10 gene in D5 cells (Th1 clone) (11). Recently, Villagra et al. (48) also reported that HDAC1 regulated IL-10 expression in APCs. In this study, we found a significant decrease of HDAC1 recruitment in Il10 regulatory regions in Ets-1KO Th1 cells compared with WT Th1 cells (Fig. 4A). Treatment of TsA, an HDAC inhibitor, significantly enhanced IL-10 expression in Th1 cells by decreasing HDAC1 recruitment (Fig. 4B–D). These results suggest the involvement of histone deacetylase activity in the repression of Il10 expression. The effects of TsA in this experimental system may be direct, because the TsA treatment did not affect Ets-1 expression level but increased the level of IL-10 expression. However, the effect of TsA might also be indirect, because TsA can affect the expression of other genes, which in turn can regulate IL-10 expression.

Ets-1 deficiency did not cause any significant differences in HDAC1 mRNA levels (data not shown), although a slight decrease of HDAC1 protein level was observed in Ets-1KO Th1 cells (Fig. 5A). However, this difference in the level of HDAC1 protein is subtle and, in our view, is insufficient to explain the marked reduction in HDAC1 recruitment to the Il10 gene seen in Ets-1KO Th1 cells. Overexpression of Ets-1 alone failed to suppress IL-10 reporter activity rather than activate reporter activities of CNS+1.65 and CNS+2.98 (Fig. 6C). However, HDAC1 coexpression efficiently suppressed Il10 reporter activity, suggesting the important role of HDAC1 as a repressor complex of the Ets-1 complex. Our data collectively suggest that Ets-1 negatively regulates IL-10 expression by affecting histone modification states and HDAC1 recruitment. Ets-1-mediated recruitment of HDAC1 likely is also subject to regulation by yet to be characterized mechanisms.

It is known that IFN-γ can suppress the expression of Th2 cytokines, including IL-10, raising the possibility that overproduction of IL-10 by Ets-1KO Th1 cells may be a secondary effect, because of the reduced IFN-γ level. Upon Ets-1 deficiency, a decreased level of STAT4 and T-bet expression was also observed (21). In this study, we tested whether T-bet or STAT4 deficiency could mediate differential Ets-1 and HDAC1 recruitment to the IL-10 regulatory sites. However, no quantitative differences were observed in the level of IL-10 expression by T-bet or STAT4 deficiency in Th1 compared with WT Th1 cells (Fig. 7A, 7D). In addition, it has been reported that T-bet deficiency did not cause any effect on IL-10 production by NK cells (49). According to another report in Th1 cells, STAT4 may positively regulate IL-10 production, rather than repress it, under the control of IL-12 stimulation (10). Furthermore, T-bet or STAT4 deficiency did not cause statistically significant changes in the level of Ets-1 and HDAC1 recruitment to the IL-10 regulatory sites, suggesting that T-bet or STAT4 may not be directly involved in Ets-1-mediated IL-10 repression in Th1 cells.

Function of Ets-1 is mainly dependent upon its interaction with other binding partners (16, 42, 43, 50). For example, Ets-1 cooperates with transcriptional coactivator CREB binding protein and the related p300 protein to upregulate its target genes (51). In contrast, Ets-1 cooperates with EAP1/Daxx to repress its target genes (44). Therefore, Ets-1-mediated recruitment of HDAC1 might be heavily influenced by its binding partners. In this study, we demonstrated that HDAC1 is the major partner of Ets-1–mediated IL-10 repression (Fig. 5). However, other repressor proteins could also composite as an Ets-1 repressor complex. To identify the Ets-1 interacting protein partners, we have recently performed mass spectrometry using the Ets-1 immunoprecipitated (IP) protein complex. We confirmed the interaction of Ets-1 with HDAC1, mSin3a, and DNA methyltransferase 3a and 3b (C.G. Lee and S.-H. Im, unpublished data). Those molecules could form an Ets-1 repressor complex and affect the epigenetic status of DNA methylation (52, 53) and histone modifications, resulting in an inactive chromatin configuration at the Ets-1 target gene loci.
However, it is still unclear whether the formation of the Ets-1 and HDAC1 complex is a general mechanism to suppress Ets-1 target genes. To test this idea, we analyzed two target genes, Il24 and Ccr8, that are known to have higher expression in Th2 compared with Th1 cells. Like IL-10, Ets-1 deficiency significantly increased the expression level of Il24 and Ccr8 mRNA in Th1 cells by increasing H3Ac levels in the promoter regions (Fig. 8D). Recently, we have performed an expression microarray to observe the transcriptional changes between WT Th1 and Ets-1KO Th1 cells. More than 800 genes were identified as potential Ets-1 target genes. Among them, 312 genes were upregulated (data not shown). Currently, we are investigating whether the formation of an Ets-1 and HDAC1 complex is also involved in the repression of these newly identified Ets-1 target genes.

Another possible scenario is that the ability of Ets-1 to recruit HDAC1 is regulated by its posttranslational modifications, which may differ in Th1 and Th2 cells. Phosphorylation is a well-known posttranslational modification of the ETS family of transcription factors (17). Phosphorylation of Ets-1 can enhance or attenuate its transcriptional activity (54–56). However, neither activating nor inhibitory phosphorylation of Ets-1 had much impact on Th cytokine production (57). Acetylation of Ets-1 often results in the inhibitory phosphorylation of Ets-1 had much impact on Th cytokine production (54–56). However, neither activating nor inhibitory phosphorylation of Ets-1 was shown to affect the expression of Ets-1 target genes. Therefore, it is not clear whether the recruitment of Ets-1 and/or HDAC1 is involved in the repression of newly identified Ets-1 target genes.

In summary, our work advances our understanding of how Ets-1 suppresses IL-10 expression. Our findings are also clinically relevant. Single nucleotide polymorphisms at the Ets1 locus were recently shown to be associated with a greater risk for systemic lupus erythematosus (SLE) (59, 60). Patients with SLE often overproduce IL-10, the level of which correlates with disease activity (61, 62). However, the molecular mechanism mediating the overproduction of IL-10 is poorly understood. It will be of great interest to investigate whether the recruitment of Ets-1 and/or HDAC1 to the Il10 locus is altered and whether the interaction between Ets-1 and HDAC1 is disrupted in SLE patients.

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


An additional funding source has been added to the grant support footnote. The corrected footnote is shown below. The footnote has been corrected in the online version of this article, which now differs from the print version as originally published.

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