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The transcription factor Etv5 controls T_H17 cell development and allergic airway inflammation

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Abstract

Background—The differentiation of T_H17 cells, which promote pulmonary inflammation, requires the cooperation of a network of transcription factors.

Objectives—We sought to define the role of Etv5, an Ets-family transcription factor, in T_H17 cell development and function.

Methods—T_H17 development was examined in primary mouse T cells wherein Etv5 expression was altered by retroviral transduction, small interfering RNA targeting a specific gene, and mice with a conditional deletion of Etv5 in T cells. The direct function of Etv5 on the *Il17* locus was tested with chromatin immunoprecipitation and reporter assays. The house dust mite–induced allergic inflammation model was used to test the requirement for Etv5-dependent T_H17 functions *in vivo*.

Results—We identify Etv5 as a signal transducer and activator of transcription 3–induced positive regulator of T_H17 development. Etv5 controls T_H17 differentiation by directly promoting 0*a* and *Il17f* expression. Etv5 recruits histone-modifying enzymes to the *Il17a–Il17f* locus, resulting in increased active histone marks and decreased repressive histone marks. In a model of allergic airway inflammation, mice with *Etv5*-deficient T cells have reduced airway inflammation and IL-17A/F production in the lung and bronchoalveolar lavage fluid compared with wild-type mice, without changes in T_H2 cytokine production.

Conclusions—These data define signal transducer and activator of transcription 3–dependent feed-forward control of T_H17 cytokine production and a novel role for Etv5 in promoting T cell–dependent airway inflammation.

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Keywords

T_H17 cells; transcription factor; Etv5; epigenetic modifications; allergic inflammation

Helper T cells play an essential role in adaptive immunity, with specific subsets contributing effector functions to control immunity to pathogens and regulating the development of inflammatory disease. Specific cytokines in the microenvironment mediate intracellular communication that results in the activation of signal transducer and activator of transcription (STAT) proteins and the induction of a transcriptional regulatory network. IL-6, IL-21, or IL-23 can activate STAT3, which is required for T_H17 cell differentiation and maintenance.[1,2] T_H17 cells secrete IL-17A, IL-17F, IL-21, and IL-22 and are primary mediators in controlling infection and promoting autoimmune diseases.[3'4] ΓL-17–producing $CD4^+$ T cells have been shown to be required for immunity to *Candida albicans, Mycobacterium tuberculosis, Streptococcus pneumonia*, and *Klebsiella pneumoniae*.[5–8] T_H17 cells also play a key role in the development of autoimmune inflammation, including experimental autoimmune encephalomyelitis, rheumatoid arthritis, and inflammatory bowel disease.[9–11] STAT3 activation by IL-6, IL-21, and IL-23 results in the induction of genes encoding transcription factors, including *Rorc, Maf, Irf4, and Batf*, which are essential for T_H17 cell differentiation.[12–16] Although each of these factors are critical for T_H17 cell development, [17,18] it is not clear whether additional STAT3 targets are required for T_H17 cell development and function.

ETS variant 5 (Etv5) belongs to the PEA3 subfamily of ETS transcription factors that regulate gene expression by binding to a conserved GGAA/T motif.[19] Etv5 has been shown to play important roles in coordinating limb development,[20,21] controlling gene expression in spermatogonial stem cells[22], and regulating epithelial-mesenchymal transition in many type of cancers.[19] Etv5 inhibits sonic hedgehog expression in the anterior limb bud, which is essential for the anterior-posterior patterning of the vertebrate limb[21]. Etv5 positively regulates microRNA-21, *Bcl6b*, and LIM homeobox 1, which are known to control spermatogonial stem cell self-renewal.[23] Chromosomal translocations resulting from the fusion between Etv5 and trans-membrane protease serine 2 correlate with prostate cancer.[19]

Although the role of Etv5 in developmental and cancer biology has been defined, the function of Etv5 in the immune response is still poorly understood. One report found Etv5 downstream of the IL-12–STAT4 signaling pathway, augmenting IFN-γ production in T_H1 cells.[24,25] The role of Etv5 in the development of other helper T cells has not been defined. In this report we show that STAT3-activating cytokines induce *Etv5* expression in T_H17 cells. Etv5 promotes IL-17 production in T_H17 cells in vitro and in vivo and is required in T cells for the development of allergic inflammation. Mechanistically, Etv5 directly promotes *Il17a* and *Il17f* expression. Thus Etv5 is a STAT3-induced positive regulator of T_H17 cell differentiation, promoting the development of allergen-induced airway inflammation.

METHODS

Mice

C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, Ind). Stat3$^{fl/fl}$ CD4-Cre mice were previously described.[26] Etv5$^{fl/fl}$ mice[21] were crossed withCD4-Cre transgenic mice to generate Etv5 $^{fl/fl}$ CD4-Cre^{+} mice, with Cre-negative littermates as control mice. Mice were maintained under specific pathogen-free conditions. All experiments were performed with the approval of the Indiana University Institutional Animal Care and Use Committee.

In vitro T-cell differentiation

Naive CD4^{+}CD62L^{+}T cells were positively selected from enriched CD4^{+} T cells from spleens and lymph nodes by using MACS beads and columns (Miltenyi Biotec, Auburn, Calif). Naive CD4^{+}CD62L^{+}T cells were activated with plate-bound anti-CD3 (2 μg/mL 145-2C11; Bio-XCell, West Lebanon, NH) and soluble anti-CD28 (0.5 μg/mL; BD PharMingen, San Jose, Calif) to generate T_H0 cells or with additional cytokines (all from PeproTech, Rocky Hill, NJ) and antibodies (Bio-XCell) to generate T_H1 (5 ng/mL IL-12 and 10 μg/mL anti–IL-4, 11B11), T_H2 (10 ng/mL IL-4 and 10 μg/mL anti–IFN-γ, XMG), T_H9 (20 ng/mL IL-4, 2 ng/mL TGF-β, and 10 μg/mL anti–IFN-γ; XMG), T_H17 (100 ng/mL IL-6, 10 ng/mL IL-23, 10 ng/mL IL-1β, 2 ng/mL TGF-β, and 10 μg/mL anti–IL-4, 11B11, and 10 μg/mL anti–IFN-γ, XMG), or inducible regulatory T (iTreg; 2 ng/mL TGF-β and 10 μg/mL anti–IL-4, 11B11) cells in culture conditions. Cells were expanded after 3 days with a half concentration of the original cytokines in fresh medium. Cells were harvested on day 5 for analysis.

House dust mice–induced allergic airway inflammation

Wild-type and *Etv5* mutant mice were sensitized by means of intranasal injection of 40 μg of house dust mite (HDM; Greer Laboratories, Lenoir, NC) in PBS each day for 3 consecutive days over 5 weeks. In experiments as indicated, mice were treated with 200 μg of anti–IL-17A (17F3) or IgG_1 (MOPC-21) antibodies (Bio-XCell) for 3 consecutive days over 5 weeks or 4 μg of IL-17A/F cytokine (PeproTech) on weeks 3, 4, and 5. Mice were killed 24 hours after the final intranasal challenge at week 5. The trachea was cannulated, and lungs were lavaged 3 times with 1 mL of PBS to collect bronchoalveolar lavage (BAL) cells. BAL cells and the single-cell suspension from lungs generated by using the lung dissociation kit from Miltenyi Biotec were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 2 hours followed by monensin for a total of 6 hours for cytokine analysis by means of intracellular staining. Single-cell suspension from the lungs was used for gene expression analysis with quantitative reverse transcriptase PCR (qRT-PCR). Cells from mediastinal lymph nodes were stimulated with HDM for 5 days, and cytokine production was analyzed by using ELISA. Lung tissue was analyzed after paraffin embedding and staining with hematoxylin and eosin for evaluation of the infiltration of inflammatory cells, and periodic acid–Schiff was used for evaluation of mucus production. Eosinophils, neutrophils, T cells, B cells, and mononuclear cells in the BAL fluid and lungs were characterized by cell size and expression of CD3, B220, CCR3, CD11c, and MHC class II by using flow cytometric analysis, as described previously.[27]

Retroviral expression vectors and retroviral transduction

Etv5 (Open Biosystems, Thermo Scientific, Waltham, Mass) cDNA was digested and subcloned into MSCV-YFP. Bicistronic retrovirus expressing YFP preparation of retroviral stocks was previously described.[28] $CD4^+$ T cells were transduced on day 2 with control or retrovirus vector expressing the gene of interest by using centrifugation at 2000 rpm at 25°C for 1 hour in the presence of 8 μg/mL polybrene (Sigma-Aldrich, St Louis, Mo). Viral supernatant was replaced with the former culture supernatant supplemented with 50 U/mL human IL-2. After spin infection, cells were expanded on day 3 and analyzed on day 5.

Transfection of small interfering RNA

Control small interfering RNA (siRNA) and siRNA targeting *Etv5* were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). T_H17 cells were transfected with control or *Etv5* siRNA on day 2 with the Amaxa Nucleofector kit (Lonza, Basel, Switzerland), rested overnight with human IL-2, and restimulated with anti-CD3 for 24 hours for gene expression and cytokine production analysis.

Luciferase reporter assay

Jurkat T cells were grown in RPMI-1640 with 10% FBS and transfected with 2 to 5 μg of the *IL17A/F* luciferase reporter plasmids (SwitchGear Genomics, Menlo Park, Calif) and control or increasing concentration of plasmid expressing Etv5 through Fugene6 reagent (Roche, Mannheim, Germany). After 24 hours, transfected cells were stimulated with PMA and ionomycin for 6 hours before analysis with the dual luciferase system (Promega, Madison, Wis).

Analysis of gene expression, protein expression, ELISA, and flow cytometry

qRT-PCR and ELISA were performed, as previously described.[29] Gene expression was normalized to housekeeping gene (β_2-microglobulin) expression. The relative gene expression was calculated by using the change-in-threshold ($-\Delta\Delta C_T$) method. Primers are listed in Table E2 in this article's Online Repository at www.jacionline.org. Nuclear lysates were extracted from activated $CD4^+$ T cells and immunoblotted with Etv5 C-20 or β-Actin C4 (Santa Cruz Biotechnology) as a control. For flow cytometry, cells were stained for surface markers and fixed with 2% paraformaldehyde for 10 minutes before analysis. For cytokine staining, $CD4^+$ T cells were stimulated with PMA and ionomycin for 2 hours followed by monesin for a total of 6 hours, fixed, permeabilized with 0.2% saponin, and stained for IL-17A–PECy7, IL-17F–phycoerythrin, or IL-13– Alexa Fluor 647 (BD PharMingen). For Etv5 intracellular staining, cells were fixed, permeabilized with 0.1% saponin, and stained for Etv5 C-20 or goat IgG (Santa Cruz Biotechnology) for 30 minutes at 4°C, followed by donkey anti-goat IgG PE (Santa Cruz Biotechnology) for an additional 30 minutes at 4°C.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed, as previously described.[24] In brief, resting T_H17 cells were cross-linked for 10 minutes with 1% formaldehyde and lysed by means of sonication. After preclearing with salmon sperm DNA, BSA, and Protein

Agarose bead slurry (50%), cell extracts were incubated with either rabbit polyclonal STAT3 C-20, Etv5 H-100, p300 N-15 (Santa Cruz Biotechnology), H3K27ac, H3K27me3 (Milli-pore, Temecula, Calif), H3K4me3 (Abcam, Cambridge, United Kingdom), or normal rabbit IgG (Millipore) overnight at 4°C. The immunocomplexes were precipitated with protein Agarose beads at 4°C for 2 hours, washed, and eluted, and cross-links were reversed at 65°C overnight. DNA was purified, resuspended in H_2O, and analyzed by using quantitative PCR. Primers are listed in Table E1 in this article's Online Repository at www.jacionline.org. The percentage input was calculated by subtracting the amount of immunoprecipitated DNA from the IgG control from the amount of immunoprecipitated DNA from the specific antibody and normalized against the amount of input DNA.

Statistical analysis

A 2-tailed Student t test or 1-way ANOVA was used to generate *P* values for all data, with a *P* value of .05 or less considered significant.

RESULTS

Etv5-deficient T cells display defects in helper T-cell differentiation

Although evidence suggests that Etv5 contributes modestly to IFN-γ production in T_H1 cells,[25] the role of Etv5 in helper T-cell development is still poorly understood. To define the function of Etv5 in T cells, we generated mice with *Etv5* deficiency in T cells (*Etv5*$^{fl/fl}$ CD4-Cre$^+$) by mating *Etv5*$^{fl/fl}$ mice[21] with mice carrying a CD4-Cre transgene (termed *Etv5* mutant mice) and confirmed the absence of *Etv5* expression in *Etv5*-deficient T cells (Fig 1, *A*). Mice with *Etv5*-deficient T cells display T-cell populations in the thymus, spleen, and lymph nodes comparable with those seen in wild-type littermate control animals and showed normal proliferation in culture (Fig 1, *B* and *C*, and data not shown). To define the role of Etv5 in helper T-cell differentiation, we first examined Etv5 expression in helper T-cell subsets. Etv5 expression was highest in resting and activated T_H1 and T_H2 cells compared with other helper cell subsets at the message and protein levels (Fig 2, *A*-*C*). Naive CD4$^+$ T cells from control and *Etv5* mutant mice were used to generate T_H1, T_H2, and T_H17 cultures, and cytokine production and gene expression were assessed. Interestingly, there were multiple defects in helper T-cell differentiation. *Etv5*-deficient T_H2 cells produced more IL-4. In contrast, *Etv5*-deficient T_H17 cells produced significantly less IL-17A compared with control cells (Fig 2, *D*). Similar results were observed at the message level in *Etv5*-deficient T cells compared with control cells (Fig 2, *E*). IFN-γ production was modestly increased in *Etv5*-deficient T_H1 cells compared with that seen in wild-type cells (Fig 2, *D* and *E*). Examination of transcription factor expression critical for the development of each effector subset demonstrated that expression of *Gata3* and *Rorc* was similar between control and *Etv5*-deficient T_H2 and T_H17 cells, respectively (Fig 2, *F*). *Etv5*-deficient T_H1 cells had higher *Tbx21* expression compared with that seen in control cells (Fig 2, *F*). Numbers of thymic-derived regulatory T (Treg) cells (defined as CD4$^+$CD25$^+$ forkhead box protein 3 [Foxp3]$^+$ cells) in both lymph nodes and spleen and *in vitro*–derived Treg cells (iTreg cells) were not significantly decreased in *Etv5*-mutant mice compared with control mice (data not shown). Thus mice that lack *Etv5* expression in T cells display altered helper T-cell differentiation *in vitro*.

Mice with *Etv5*-deficient T cells had reduced allergic inflammation

Our data suggested that Etv5 plays a repressive role in T_H1 and T_H2 cell differentiation while promoting T_H17 cell differentiation (Fig 2). HDM-induced allergic airway inflammation is T_H2 and T_H17 dependent,[30–33] and in exploring Etv5-dependent immunity, we first wanted to confirm the role of IL-17A in this model. We sensitized and challenged mice with intranasal HDM antigen and anti–IL-17A or IgG_1 antibodies for 5 weeks and assessed pulmonary inflammation (Fig 3, *A*). Mice treated with anti–IL-17A antibody had significantly lower numbers of total cells, eosinophils, and neutrophils in lung tissue and BAL fluid compared with control mice (Fig 3, *B*). Consistent with the decrease in neutrophil numbers, expression of *Cxcl2*, an IL-17– induced gene, but not *Cxcl1*, was reduced in lung tissue from mice treated with anti–IL-17A antibody compared with control lung tissue (Fig 3, *C*). Our results demonstrated that IL-17A is important in mediating allergic airway inflammation on HDM allergen exposure.

We next wanted to test the role of Etv5 *in vivo* by inducing allergic airway inflammation in control and *Etv5* mutant mice. *Etv5* mutant mice had significantly decreased numbers of total cells and specific cell populations in lung tissue compared with control mice (Fig 3, *D*). Analysis of the cellular composition after BAL showed similar results (Fig 3, *E*). Histologic examination also demonstrated diminished inflammatory cell infiltrates in the lungs and decreased mucus production in the airways of *Etv5* mutant mice compared with control mice (Fig 3, *F*). Consistent with histologic analysis, the expression of genes involved in goblet cell metaplasia (*Muc5ac* and *Clca3*) was reduced in lungs of *Etv5* mutant mice compared with that seen in control mice (Fig 3, *G*). Given that we observed the greatest fold reduction (>5-fold) in infiltrating neutrophils in *Etv5* mutant mice compared with control mice (Fig 3, *D* and *E*), we wanted to examine the expression of neutrophil-attractant chemokines in total lung RNA. Consistent with the decrease in neutrophil numbers, expression of *Cxcl2*, an IL-17–induced gene, was reduced in lung tissue from *Etv5* mutant mice compared with that seen in control lung tissue (Fig 3, *G*). In contrast, there was no difference in *Cxcl1* expression or in expression of the T_H2-induced chemokines *Ccl17* and *Ccl22* (Fig 3, *G*).

We next assessed cytokine production in the lung tissue and BAL fluid of control and *Etv5* mutant mice with HDM-induced allergic inflammation. Consistent with *in vitro* analyses, there was a significantly decreased frequency and number of IL-17A–producing $CD4^+$ T cells from the lung and BAL cells of *Etv5* mutant mice compared with that seen in control mice (Fig 4, *A*-*C*, and data not shown). No change was observed in the frequency of IL-13–producing $CD4^+$ T cells from the lung and BAL cells, although cell numbers were decreased in *Etv5* mutant mice compared with those seen in control mice, which is concomitant with the decrease in overall inflammation in the lung (Fig 4, *A*-*C*). We further confirmed that the concentration of IL-17A, but not IL-13 and IL-4, was significantly reduced in the BAL fluid of *Etv5* mutant mice compared with that seen in control mice (Fig 4, *D*). The frequencies of Treg cells (identified as $CD4^+$ $Foxp3^+$) in the lung and BAL cells were similar between control and *Etv5* mutant mice (data not shown). We next assessed the generation of helper T-cell responses in the periphery by stimulating mediastinal lymph node cells with HDM extract. IL-17A production was significantly reduced in *Etv5* mutant mice compared with

that seen in control mice, although there was no difference in the amount of IL-13 or IL-4 (Fig 4, *E*).

To determine whether loss of IL-17 is the critical defect in *Etv5* mutant mice, we treated HDM-sensitized *Etv5* mutant mice with either PBS or IL-17A and IL-17F together and assessed pulmonary inflammation (Fig 5, *A*). IL-17A/F–treated *Etv5* mutant mice had increased numbers total cell, eosinophil, and neutrophil numbers in lung tissue and BAL fluid compared with those seen in the PBS group (Fig 5, *B*). In addition, *Cxcl2*, but not *Cxcl1*, gene expression was increased when *Etv5* mutant mice were treated with IL-17A/F cytokines compared with values seen in control mice (Fig 5, *C*). Collectively, these results suggested that the critical role of Etv5 *in vivo* in response to an allergen in experimental airway inflammation is the regulation of IL-17–secreting T cells.

STAT3-activating cytokines induce *Etv5* expression

Given that Etv5 positively regulates IL-17A *in vitro* and in HDM-induced allergic inflammation, we wanted to further elucidate the ability of Etv5 to promote T_H17 cell differentiation. We first assessed the kinetics of *Etv5* gene expression during T_H17 cell differentiation. We observed an increase in *Etv5* expression 48 hours after activation that gradually decreased over the subsequent 3 days of differentiation (Fig 6, *A*). Because STAT4 regulates Etv5 expression in T_H1 cells,[25] we tested whether STAT3 induced Etv5 expression in T_H17 cultures. Stimulation of wild-type T_H17 cells with IL-6 or IL-23 to activate STAT3 or IL-12 to activate STAT4 led to increased Etv5 mRNA and protein expression compared with that seen in unstimulated cells (Fig 6, *B* and *C*). To further confirm that Etv5 is a STAT3 target gene, we treated activated control and *Stat3*-deficient T cells with or without IL-6 for 48 hours to induce receptor expression. Cells were harvested, rested overnight, and restimulated with IL-6, IL-12, or IL-23 to assess *Etv5* expression. As expected, IL-6, IL-23, or IL-12 induced *Etv5* expression in IL-6–primed $CD4^+$ T cells (Fig 6, *D*). However, IL-6– and IL-23–induced, but not IL-12–induced, expression of *Etv5* was STAT3 dependent (Fig 6, *D*). To determine whether STAT3 could directly bind to conserved STAT-binding sites (consensus STAT3-binding sequence $TTN_{4-5}AA$[34]) in the mouse and human *Etv5* genes (Fig 6, *E*), differentiated wild-type T_H17 cells were stimulated with STAT3-activating cytokines, and binding was examined using ChIP assay. In T_H17 cells STAT3-activating cytokines, but not IL-12, resulted in STAT3 binding to the *Etv5* promoter, with greater amounts at the distal than the proximal binding sites (Fig 6, *F*). The decreased STAT3 binding after IL-12 stimulation is likely due to displacement by STAT4 (Fig 6, *F*). These results suggested that STAT3-activating cytokines, including IL-6 and IL-23, promote *Etv5* expression in T_H17 cells.

Etv5 directly activates the *Il17a-Il17f* locus in T_H17 cells

To further define the function of Etv5 in T_H17 cell programming, we ectopically expressed Etv5 in T_H17 cells and assessed cytokine production. Ectopic *Etv5* expression in T_H17 cells resulted in increased IL-17A and IL-17F production compared with that seen in control cells (Fig 7, *A* and *B*). To test whether Etv5 alone could induce IL-17 production in $CD4^+$ T cells, *Etv5* was ectopically expressed in nonpolarized $CD4^+$ T cells (T_H0 cells). Ectopic *Etv5* expression in T_H0 cells was able to induce IL-17A production compared with that seen in

control cells (Fig 7, *C*). Consistent with these observations, reduced *Etv5* expression in T_H17 cells by transfecting T cells with siRNA targeting *Etv5* (Fig 7, *D*) or using *Etv5*-deficient T cells (Fig 7, *E*) resulted in decreased IL-17A and IL-17F levels compared with those seen in control cells. IL-21 production was decreased in the absence of Etv5, but GM-CSF and IL-10 production was not significantly different in control cells from that seen in cells that lack Etv5 expression (Fig 7, *D* and *E*). We next examined the expression of transcription factors that are required for T_H17 cell differentiation.[13,15,16,35,36] Surprisingly, reduced *Etv5* expression did not alter gene expression of *Rorc, Batf, Maf*, and *Irf4* (Fig 7, *F* and *G*). Because Etv5 did not affect any of the known regulators of IL-17A–IL-17F expression, we tested whether Etv5 directly regulated expression of the I*l17a-Il17f* locus. The ChIP assay using differentiated T_H17 cells revealed that Etv5 bound at several sites across the I*l17a*-I*l17f* locus, including the promoters and several conserved noncoding sequences (Fig 8, *A* and *B*). Jurkat T cells transfected with *IL17A* and *IL17F* luciferase reporters and a plasmid encoding Etv5 demonstrated that Etv5 promotes the transcriptional activity of the *IL17A* and *IL17F* promoters but not a nuclear factor of activated T cells promoter, in a dose-dependent manner (Fig 8, *C*). To define the modifications of histones correlated with active (H3K4 trimethylation and H3K27 acetylation) and repressed (H3K27 trimethylation) gene expression at the *Il17a-Il17f* locus, we performed ChIP assays from control and *Etv5*-deficient T_H17 cells. We further observed decreased H3K4 methylation and H3K27 acetylation and increased H3K27 methylation across the *Il17a-Il17f* locus in the absence of Etv5 (Fig 8, *D*). In addition, the association of histone acetyltransferase p300 was decreased at the *Il17a-Il17f* locus in *Etv5*-deficient T_H17 cells compared with that seen in control cells (Fig 8, *D*). These results suggested that Etv5 directly promotes IL-17 production, recruits histone-modifying enzyme, and mediates epigenetic changes at the *Il17a–Il17f* locus in T_H17 cells.

DISCUSSION

The development of specialized helper T-cell subsets requires a network of transcription factors that promote the expression and production of specific cytokines. T_H17 development requires the activity of STAT3-activating cytokines and the induction of STAT3 target genes, including *Rorc, Batf*, and *Irf4*, in T_H17 cell differentiation. In this report we demonstrate that Etv5 is downstream of STAT3 in directly promoting *Il17a/f* expression and mediating changes in histone modifications at the *Il17a/f* locus. In the HDM-induced allergic airway inflammation model, *Etv5* mutant mice had reduced lung inflammation, mucus production in the airway, and IL-17 production compared with control mice.

The requirement for IL-17 in asthma is not completely defined. Increased IL-17A and IL-17F levels are associated with severe asthma in patients.[37] IL-17 produced from activated $CD4^+$ T cells induces the expression of macrophage- and neutrophil-attracting chemokines that recruit inflammatory cells into the airways.[38] However, in mouse models IL-17 has differential effects during sensitization or challenge, and the requirement for IL-17 varies among model systems.[32,39–42] Importantly, the HDM adjuvant–free model is dependent on IL-17 in addition to T_H2 cytokines.[30–33] IL-17 production and neutrophil recruitment in the lung are increased after HDM sensitization and challenge.[43] We observed a significant decrease in IL-17 production in the lung, BAL fluid, and peripheral lymphoid organs, as

well as diminished neutrophil-attracting chemokine levels and neutrophil recruitment to the lung in *Etv5* mutant mice compared with that seen in control mice. Although *Cxcl1* and *Cxcl2* are IL-17– inducible genes, only the latter was affected when IL-17 levels were reduced in *Etv5*-mutant mice or blocked with anti–IL-17A antibody. The result suggested *Cxcl2* expression was regulated differentially compared with *Cxcl1* in this model. Indeed, in a model of sterile inflammation, IL-17 controls neutrophil trafficking by preferentially regulating the expression of *Cxcl2* but not *Cxcl1*.[44] In addition, it has been demonstrated that *Cxcl1* expression is associated with systemic inflammation and that *Cxcl2* expression is limited to localized inflamed tissue.[45]

Although HDM sensitization and challenge elicits T_H2 responses,[30,31,43] our data demonstrated that IL-17 was the most abundant cytokine produced in the lung, suggesting IL-17 production from T cells is critical for inflammation in this model. In the absence of Etv5 in T cells, there is diminished inflammation and reduced IL-17 concentrations, without any corresponding effects on the amounts of T_H2 cytokine production. In addition to decreased neutrophil recruitment to the lung, eosinophil numbers were also reduced. Because IL-17A and IL-17F are shown to have differential effects on eosinophil recruitment during antigen-induced airway inflammation,[41] further studies are needed to determine whether IL-17 is responsible for the reduction in eosinophil numbers in the lung in this model. Importantly, we demonstrate that blocking IL-17A in the HDM model decreases cellular infiltrates similarly to that observed in mice lacking Etv5 in T cells. Moreover, intranasal administration of IL-17A/F to HDM-challenged Etv5 mutant mice results in a recovery of inflammation in the lung. Our results suggest that T_H17 production of IL-17 provides a unique contribution to the inflammatory milieu in the development of allergic pulmonary inflammation.

Our data show that Etv5 deficiency in T cells does not alter expression of T_H17 transcription factors or other T_H17 cytokines, suggesting a restricted effect on expression of the *Il17a-Il17f* locus. This is unique among transcription factors that promote T_H17 development, including retinoic acid–related orphan receptor γt, interferon regulatory factor 4, and B-cell activating transcription factor-like, which regulate multiple components of the T_H17 genetic program.[12,13,15,16,46] It is also important to note that Etv5 has a critical role in T_H17 cytokine production despite expression of Etv5 not being specific for T_H17 cells. This highlights that the amount of expression of a transcription factor is not always the most important determinant of function. Our results further document that transcription factors that are not restricted to a particular subset still play obligate roles in defining cellular phenotype in cooperation with lineage-specific factors.

These results demonstrate that Etv5 is a novel STAT3-induced transcription factor that directs expression of the *Il17a-Il17f* locus in T_H17 cells. This is consistent with a recent report that Etv5 also contributes to IL-17 production in γδ T cells and suggests that Etv5 might have common functions among multiple IL-17– secreting T-cell populations.[47] Our results further suggest that in addition to lineage regulators, such as *Rorc, Irf4*, and *Batf*, which regulate multiple genes in a subset, there are additional transcription factors that are part of the helper T-cell subset differentiation program that have a more restricted and

specific function. Thus Etv5 represents a unique contributing factor that positively regulates inflammatory responses in T_H17 cells and promotes allergic airway inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BAL	Bronchoalveolar lavage
ChIP	Chromatin immunoprecipitation
Foxp3	Forkhead box protein 3
HDM	House dust mite
iTreg	Inducible regulatory T
PMA	Phorbol 12-myristate 13-acetate
qRT-PCR	Quantitative reverse transcriptase PCR
siRNA	Small interfering RNA
STAT3	Signal transducer and activator of transcription 3
Treg	Regulatory T

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Key messages

- Etv5 directly promotes IL-17 production in T_H17 cells.
- Mice that lack Etv5 in T cells display decreased airway inflammation compared with that seen in wild-type mice.

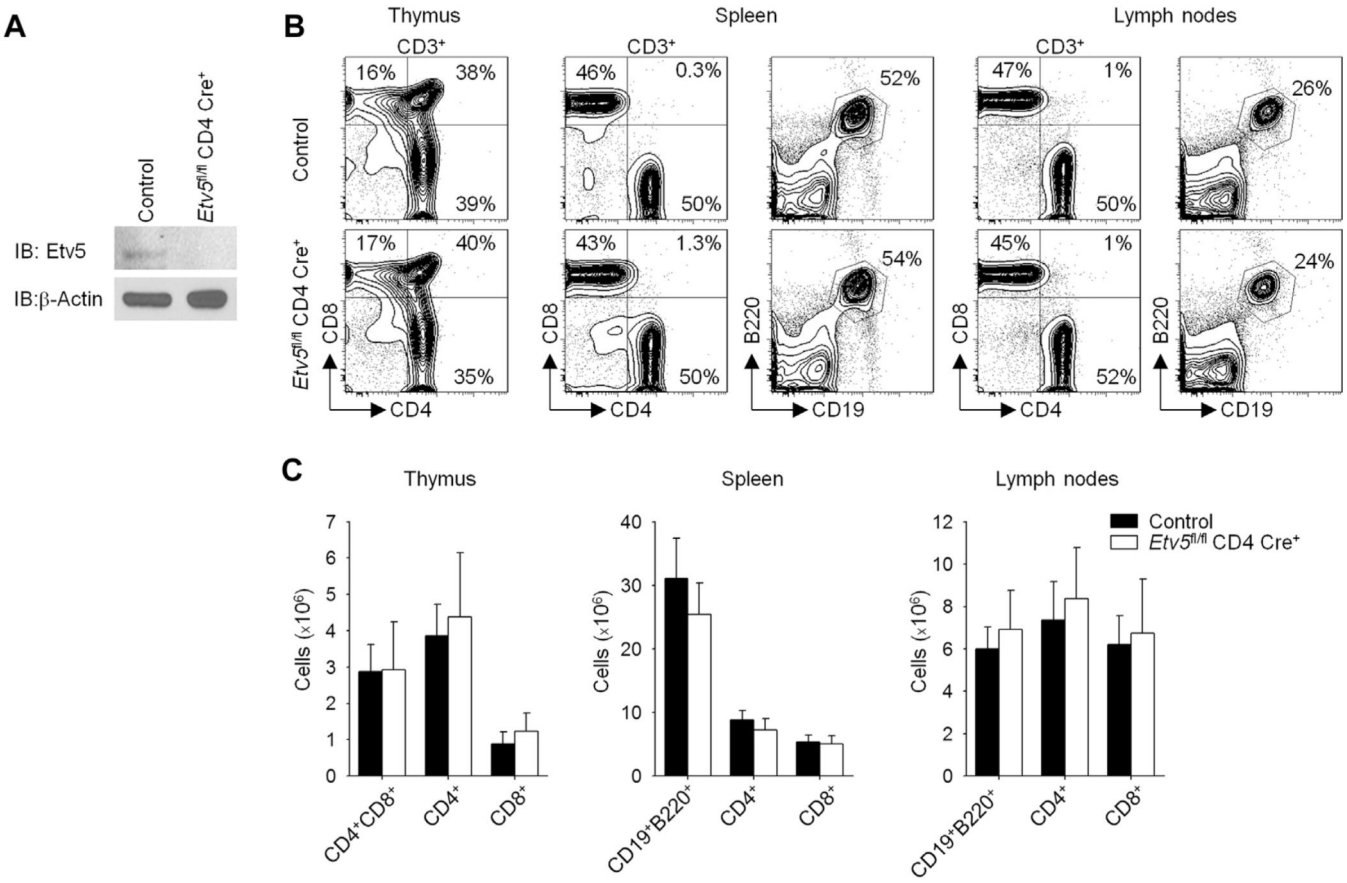

FIG 1.

Characterization of mice with *Efv5*-deficient T cells. **A**, Naive $CD4^+CD62^+$ T cells from control and $Etv5^{fl/fl}$ CD4-Cre^+ mice were activated with anti-CD3 and anti-CD28 for 5 days. Nuclear lysates were extracted and immunoblotted for Etv5 and β-actin as a control. **B** and **C**, Total cells were isolated from the thymus, spleen, and lymph nodes of control and *Etv5* mutant mice and stained for cell-surface markers, with the percentage of positive cells (Fig 1, *B*) and cell numbers (Fig 1, *C*) shown. Data are representative of 2 independent experiments with similar results (Fig 1, *A* and *B*) or are means ± SEMs of 3 mice per group and representative of 2 independent experiments with similar results (Fig 1, *C*).

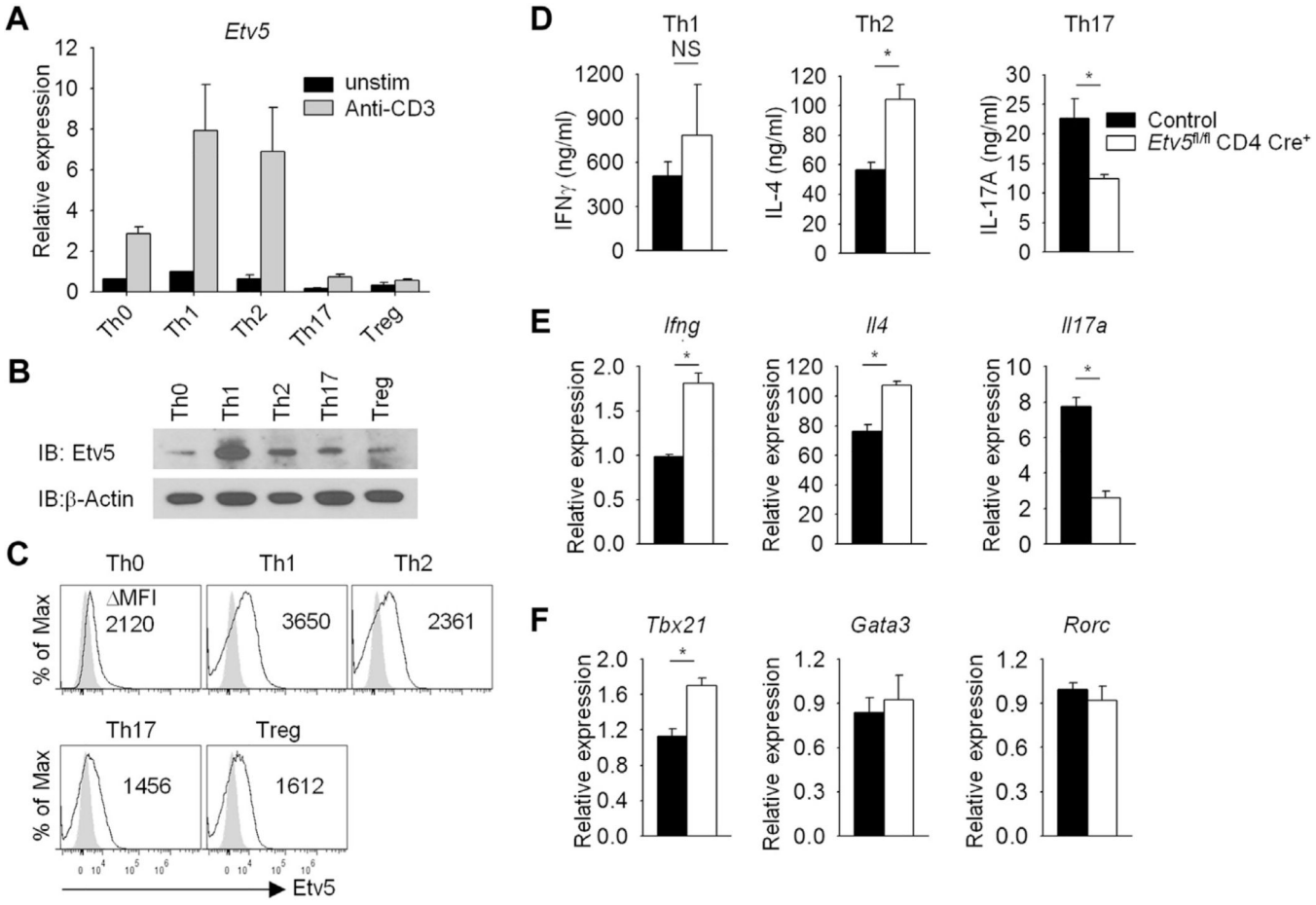

FIG 2.

Helper T-cell differentiation in the absence of Etv5 in T cells. Naive control and *Etv5*-deficient $CD4^+CD62L^+$ T cells were activated (T_H0) or cultured under T_H1, T_H2, T_H17, and Treg cell polarizing conditions. Etv5 expression was measured in helper T-cell subsets by using qRT-PCR before and after 6 hours of anti-CD3 stimulation **(A)**, immunoblotting **(B)**, or intracellular staining **(C)** before anti-CD3 stimulation. Δ Mean fluorescence intensity was calculated by subtracting the background from the signal of Etv5 antibody. T_H1, T_H2, and T_H17 cells were used for assessing cytokine production by means of ELISA after 24 hours of anti-CD3 stimulation **(D)** and gene expression analysis after (*Ifng, Il4*, and *Il17a*; **E**) or before (*Tbx21, Gata3*, and *Rorc*; **F**) 6 hours of anti-CD3 stimulation by means of qRT-PCR. Data are means ± SEMs of 4 independent experiments (Fig 2, *D-F*) or means ± SDs of replicate samples (Fig 2, *A*) and representative of 3 independent experiments with similar results (Fig 2, *A-C*). **P* < .05. *NS*, Not significant.

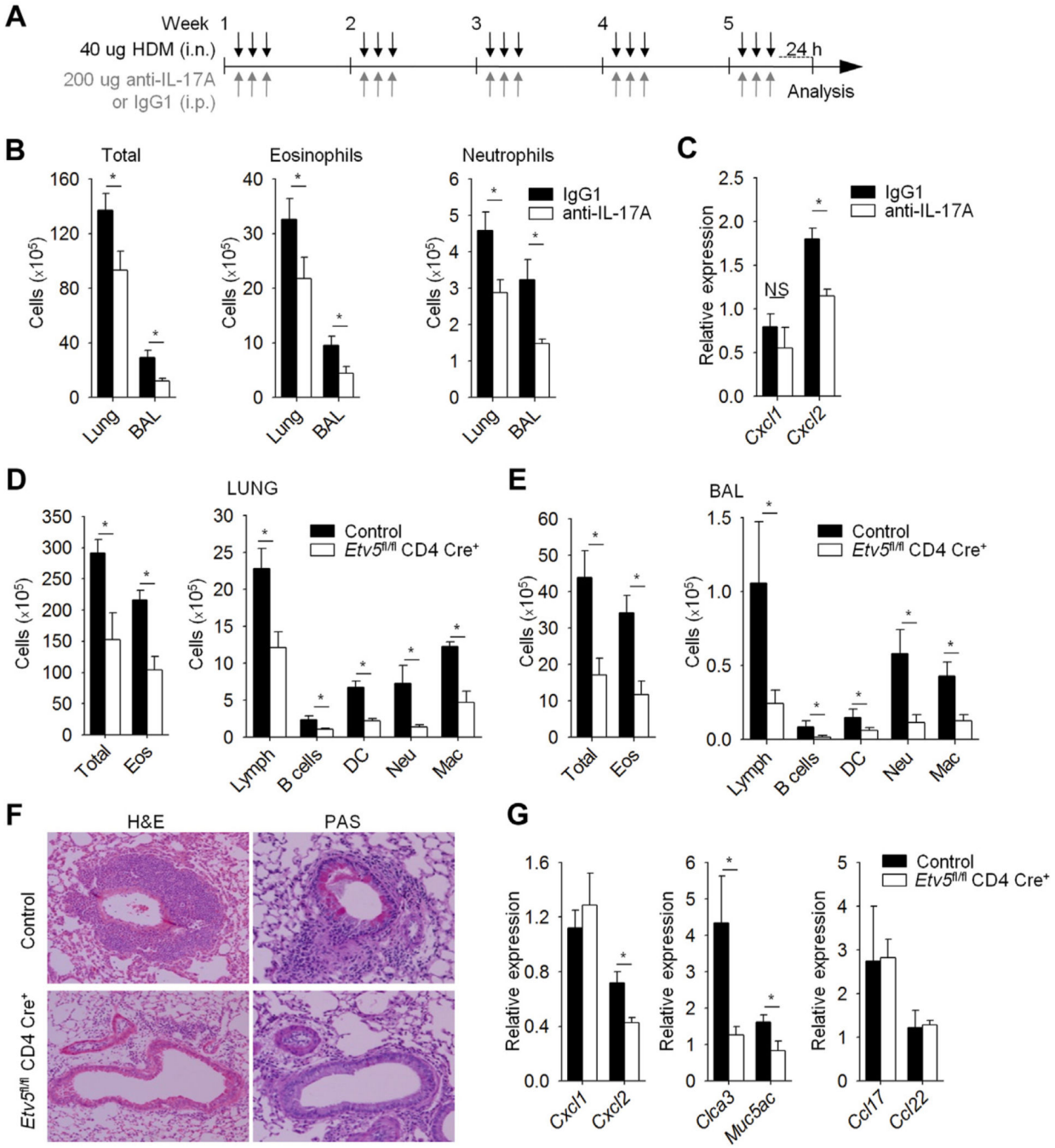

FIG 3.

Etv5 mutant mice have reduced HDM-induced allergic airway inflammation. **A**, Wild-type mice were immunized (intranasally *[i.n.]*) with HDM for 5 weeks to induced allergic inflammation and treated with anti–IL-17A or IgG_1 antibodies (intraperitoneally *[i.p.]*). **B**, Total and inflammatory cell counts in the lungs and BAL fluid of HDM-induced airway inflammation in wild-type mice. **C**, Total cells from lungs of wild-type mice were used for gene expression analysis by means of qRT-PCR. **D-G**, Control and *Etv5* mutant mice were sensitized and challenged (intranasally) with HDM for 5 weeks to induce allergic

inflammation. Inflammatory cells in the lung tissue and BAL fluid of control and *Etv5* mutant mice were as follows: *DC*, dendritic cells; *Eos*, eosinophils; *Lymph*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils. Fig 3, *F*, Cell infiltration in the lungs and mucus in the airways of control and *Etv5* mutant mice were evaluated by means of hematoxylin and eosin (*H&E*) and periodic acid–Schiff (*PAS*) staining. Fig 3, *G*, Total cells from lungs of control and *Etv5* mutant mice were used for gene expression analysis by means of qRT-PCR. Data are means ± SEMs of 5 to 6 mice per group (Fig 3, *A*-*G*) and representative of 2 independent experiments with similar results. $*P < .05$. *NS*, Not significant.

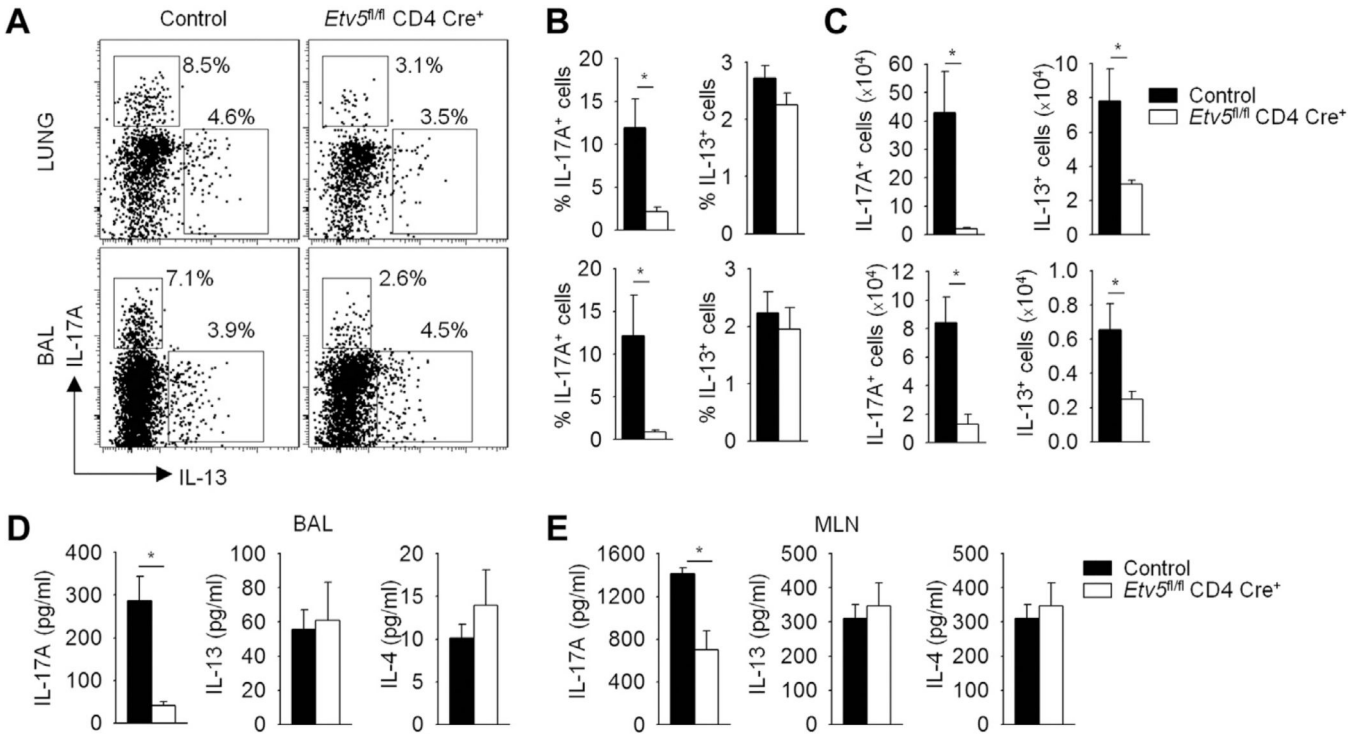

FIG 4.

Etv5 regulates T_H17 cells in HDM-induced allergic airway inflammation. **A-C**, Lung and BAL cells from HDM-induced allergic airway inflammation in control and *Etv5* mutant mice (from Fig 3) were stimulated with PMA and ionomycin for 6 hours to assess cytokine production (Fig 4, *A-C*) by using intracellular staining, with the average percentage of positive cells (Fig 4, *B*) and cell numbers (Fig 4, *C*) shown. **D** and **E**, Cells from mediastinal lymph nodes (*MLN*) were stimulated with HDM for 5 days. BAL fluid (Fig 4, *D*) and cell-free supernatant (Fig 4, *E*) were used to assess cytokine production by using ELISA. Data are means ± SEMs of 6 mice per group and representative of 2 independent experiments with similar results. $*P < .05$.

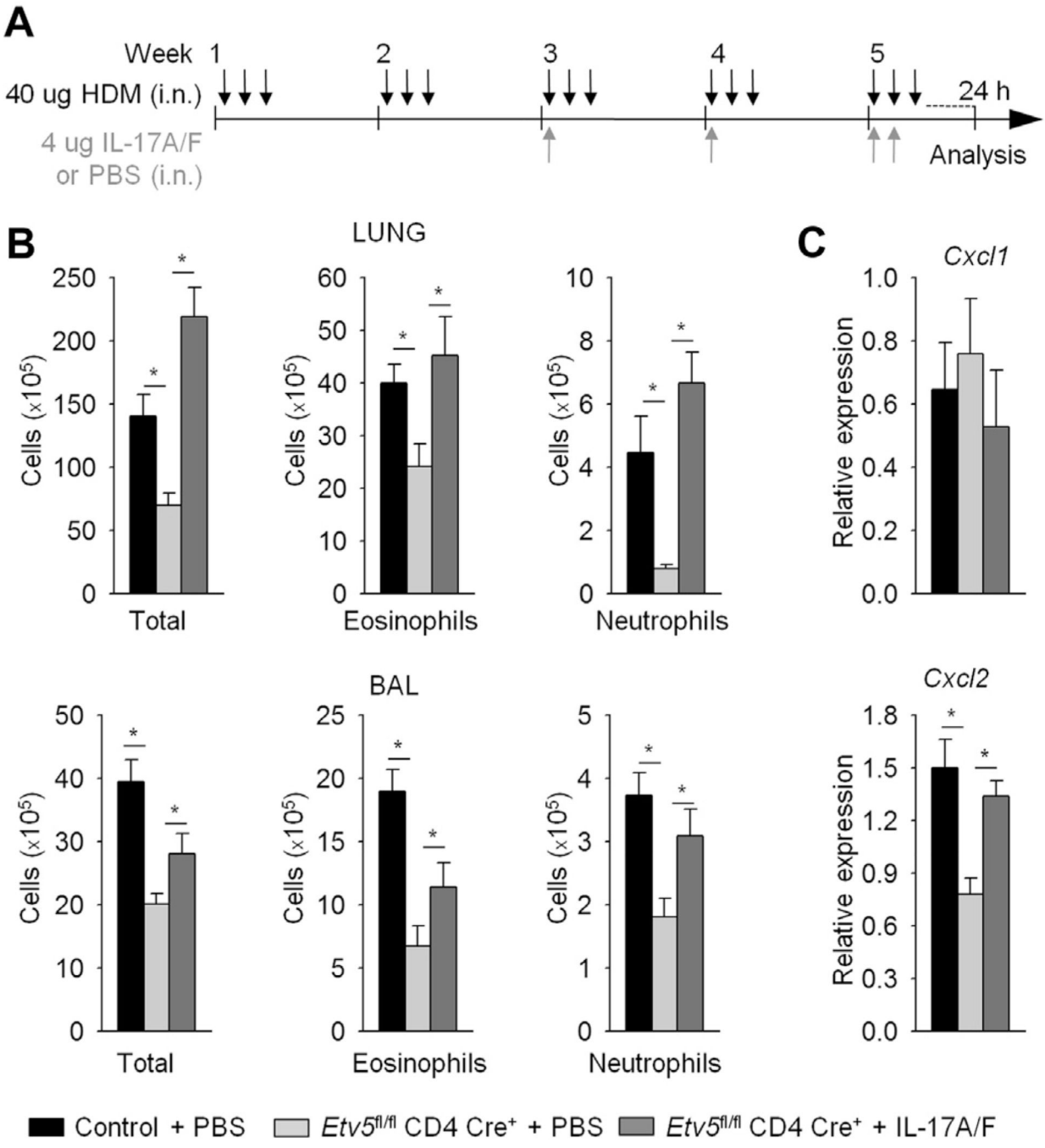

FIG 5.

IL-17 cytokine contributes to HDM-induced allergic airway inflammation. Control and *Etv5* mutant mice were immunized (intranasally *[i.n.]*) with HDM for 5 weeks to induce allergic inflammation. **A**, Mice were treated with IL-17A/F cytokine or PBS on weeks 3, 4, and 5. **B**, Total and inflammatory cells (eosinophils and neutrophils) in the lung tissue and BAL fluid of control and *Etv5* mutant mice. **C**, Total cells from lungs of control and *Etv5* mutant mice were used for gene expression analysis by means of qRT-PCR. Data are means ± SEMs of 5

mice per group and representative of 2 independent experiments with similar results. *$P < .$ 05.

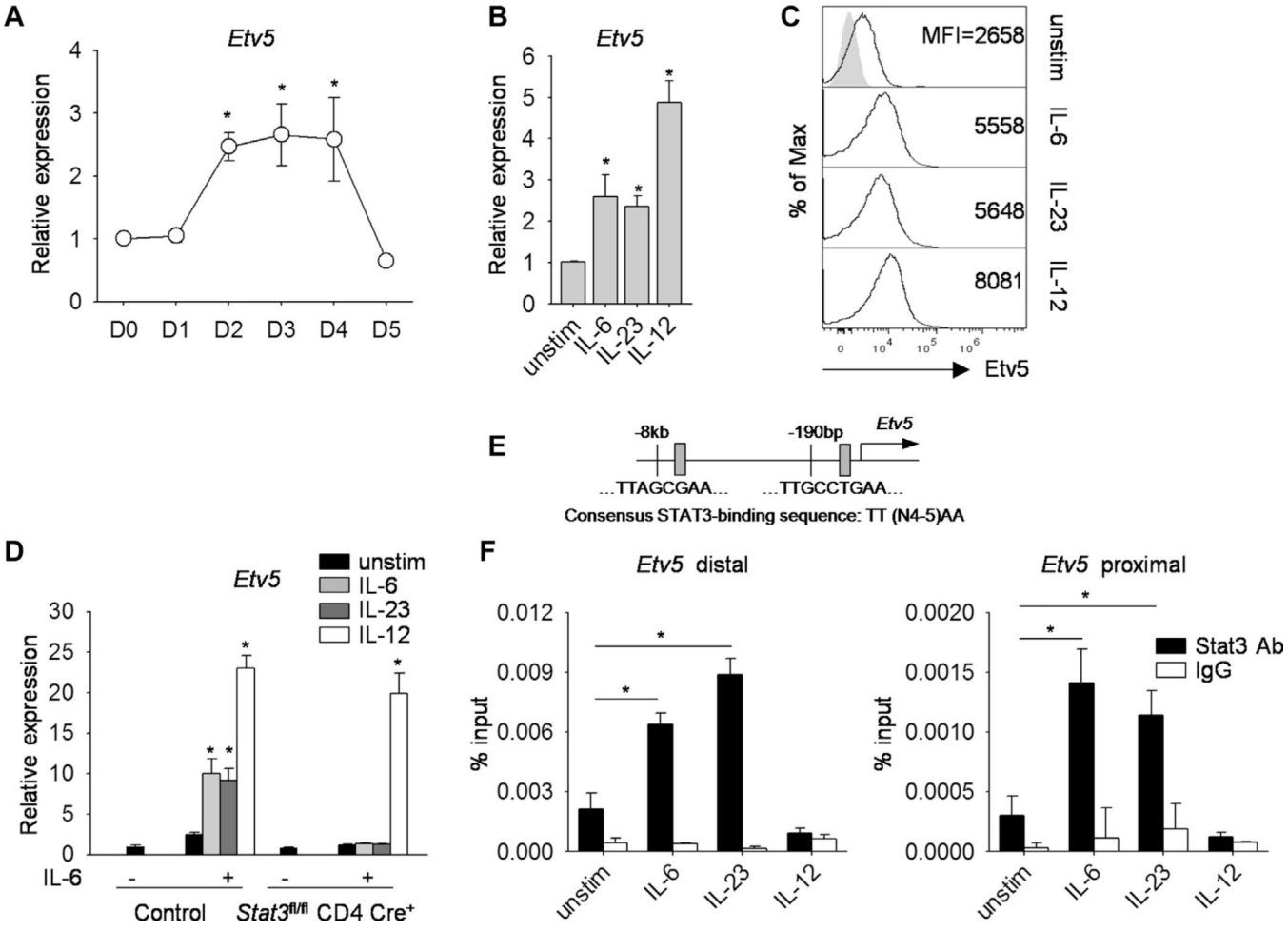

FIG 6.

Efv5 is regulated by STAT3-activating cytokines in T_H17 cells. Naive wild-type $CD4^+CD62L^+$ T cells were cultured under T_H17-polarizing conditions. **A**, Kinetics of *Etv5* gene expression during T_H17 cell differentiation. **B** and **C**, T_H17 cells were stimulated with IL-6, IL-23, and IL-12 for 2 hours before gene expression analysis by means of qRT-PCR (Fig 6, *B*) or protein expression by means of intracellular staining (Fig 6, *C*). **D**, Naive control and *Sfaf3*-deficient $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence or absence of IL-6 for 48 hours, rested overnight, and restimulated with IL-6, IL-12, or IL-23 for 2 hours before gene expression analysis by using qRT-PCR. **E**, Schematic of *Etv5* promoter-containing STAT3-binding sites. **F**, Cells prepared as in Fig 6, B, were used for ChIP analysis with STAT3 antibody and IgG as a control. Data are means ± SDs of replicate samples and representative of 3 independent experiments with similar results (Fig 6, *A*-*F*). $*P < .05$.

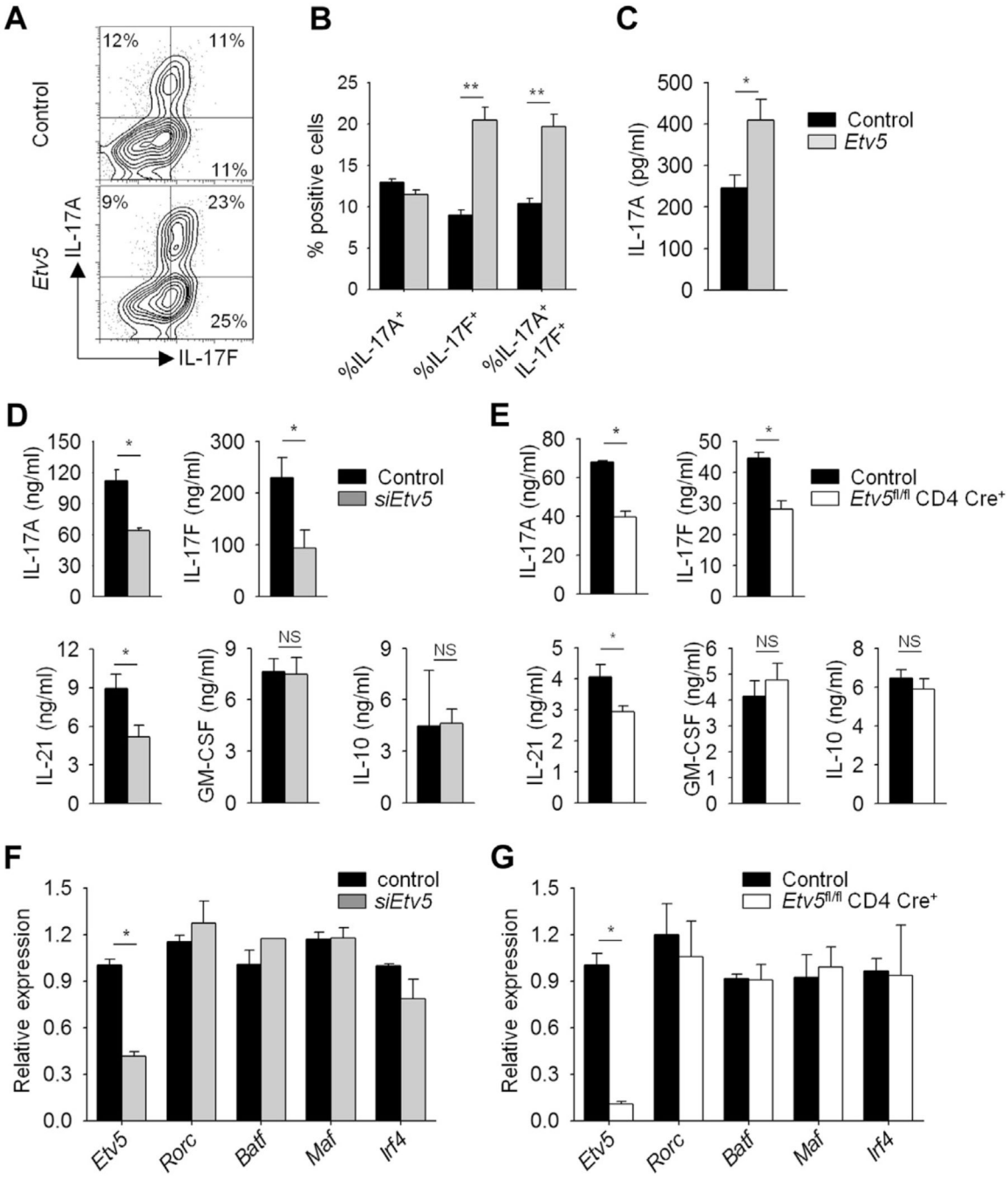

FIG 7.

Etv5 promotes cytokine production in T_H17 cells. **A-C**, Naive $CD4^+CD62L^+$ T cells were isolated from wild-type mice and differentiated under neutral conditions (T_H0) or T_H17 culture conditions. On day 2, cells were transduced with either control or Etv5-YFP (Etv5)–expressing retrovirus. On day 5, T_H17 cells were stimulated with PMA and ionomycin for 6 hours before intracellular staining for cytokine production (Fig 7, *A*), with the percentage positive cells shown in Fig 7, *B*. Data are gated on YFP^+cells. Fig 7, *C*, Cytokine production from sorted YFP^+ T_H0 cells was assessed by using ELISA after 24 hours of stimulation with

anti-CD3. **D** and **F**, Wild-type T_H17 cells were transfected with control or siRNA-specific Etv5, rested overnight, and restimulated with anti-CD3 to assess cytokine production by means of ELISA (Fig 7, *D*) and gene expression by using qRT-PCR (Fig 7, *F*). **E** and **G**, Control and *Etv5*-deficient T_H17 cells were restimulated with anti-CD3 to assess cytokine production by using ELISA (Fig 7, *E*) and gene expression by using qRT-PCR (Fig 7, *G*). Data are means ± SEMs of 4 independent experiments. **P* < .05 and ***P* < .01. *NS*, Not significant.

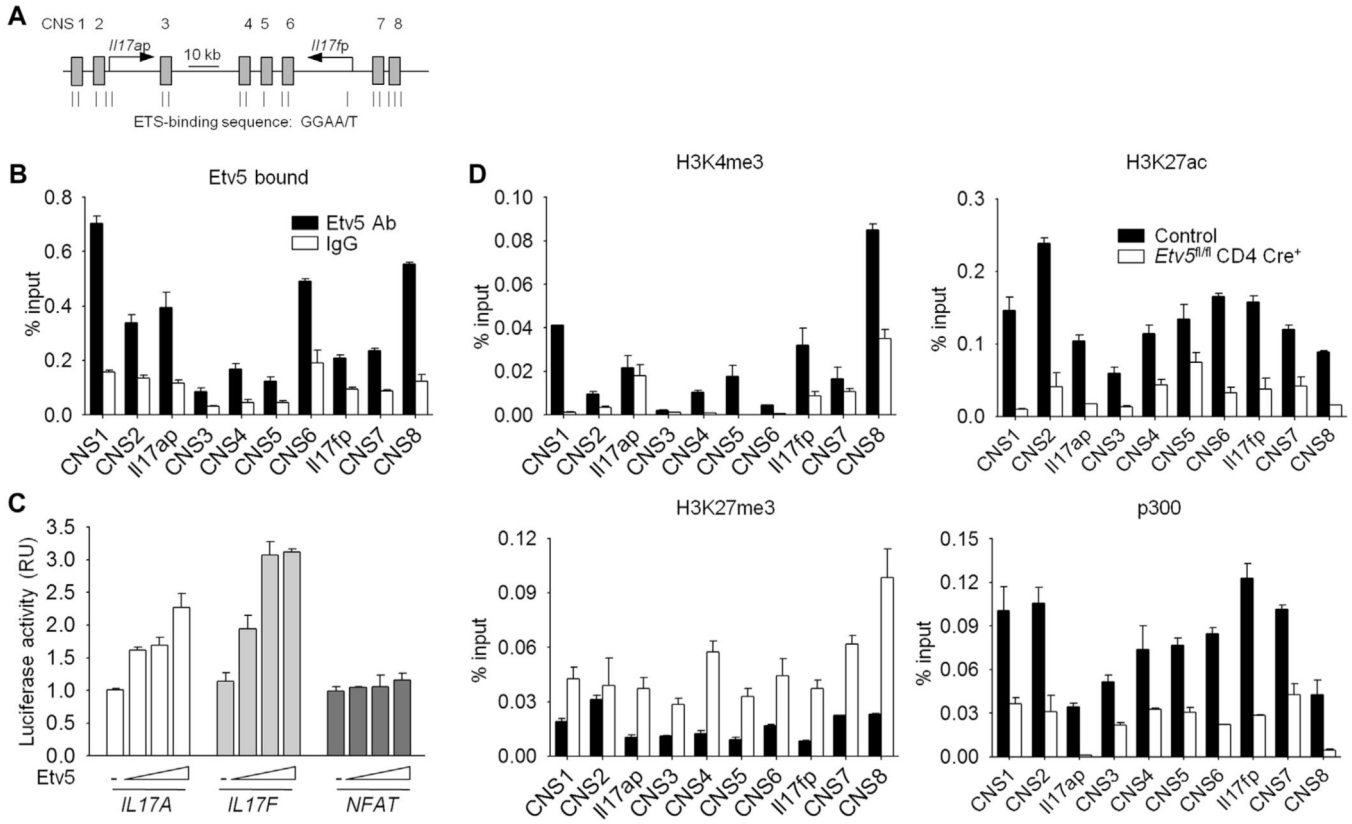

FIG 8.

Etv5 binds the *Il17a-Il17f* locus in T_H17 cells. **A**, Schematic of the *Il17a-Il17f* locus containing ETS-binding sites. **B** and **D**, Naive $CD4^+CD62L^+$ T cells were isolated from control and *Etv5* mutant mice and differentiated under T_H17 culture conditions. ChIP analyses were performed by using differentiated control T_H17 cells to examine transcription factor binding (Fig 8, *B*) or differentiated control and *Etv5*-deficient T_H17 cells to assess histone modification and the association of histone-modifying enzyme (Fig 8, *D*) at the *Il17a–Il17f* locus. **C**, Luciferase activity in Jurkat T cells transfected with increased concentrations of plasmid encoding Etv5 along with *IL17A, IL17F*, or *NFAT* luciferase reporters and then activated for 6 hours with PMA and ionomycin. Data were normalized to control samples. Data are means ± SDs of replicate samples and representative of 3 to 4 independent experiments with similar results. *RU*, Relative unit.