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The ETS Family Transcription Factors Etv5 and PU.1 Function in Parallel To Promote Th9 Cell Development

Byunghee Koh,[*,†,1] Matthew M. Hufford,[*,1] Duy Pham,[*,†,1] Matthew R. Olson,[*] Tong Wu,[‡] Rukhsana Jabeen,[*] Xin Sun,[§] and Mark H. Kaplan[*,†]

The IL-9–secreting Th9 subset of CD4 Th cells develop in response to an environment containing IL-4 and TGF-β, promoting allergic disease, autoimmunity, and resistance to pathogens. We previously identified a requirement for the ETS family transcription factor PU.1 in Th9 development. In this report, we demonstrate that the ETS transcription factor ETS variant 5 (ETV5) promotes IL-9 production in Th9 cells by binding and recruiting histone acetyltransferases to the *Il9* locus at sites distinct from PU.1. In cells that are deficient in both PU.1 and ETV5 there is lower IL-9 production than in cells lacking either factor alone. In vivo loss of PU.1 and ETV5 in T cells results in distinct effects on allergic inflammation in the lung, suggesting that these factors function in parallel. Together, these data define a role for ETV5 in Th9 development and extend the paradigm of related transcription factors having complementary functions during differentiation. *The Journal of Immunology*, 2016, 197: 2465–2472.

A network of transcription factors governs the development of the effector Th subsets that dominate during immunity to infectious disease and inflammation. The first transcription factors important for differentiation are part of the signal relay connecting the extracellular environment to changes in gene expression, which include STAT and SMAD proteins (1–3). These first signals lead to the expression of additional transcription factors that promote and maintain the expression of genes associated with a Th cell lineage that is specialized to function during specific inflammatory responses. One of the most recently described subsets of Th cells, Th9 cells, promote allergic disease, autoimmunity, tumor immunity, and immunity to parasites and develop in response to a cytokine environment containing TGF-β and IL-4 (4). Downstream of IL-4, Th9 development requires early signals from STAT6, inducing the additional transcription factors BATF and IFN regulatory factor 4 (IRF4) (5–7). The TGF-β signal requires multiple SMAD proteins, but also the SMAD-independent induction of PU.1 (5, 8–11).

The ETS transcription factor PU.1 is responsible for the development of multiple lineages of myeloid cells and contributes to the function of hematopoietic stem cells (12). During T cell development, PU.1 expression is extinguished, but it is reexpressed in peripheral T cells, with varying expression among Th subsets (8, 13). In Th2 cells, PU.1 interferes with the function of GATA3 and IRF4, resulting in diminished Th2 cytokine production (13–15). PU.1 also limits expression of CD40L and IL-21, attenuating the function of T follicular helper cells (16). In Th9 cells, PU.1 promotes the expression of IL-9 by recruiting the Gcn5 histone acetyltransferase to the *Il9* locus, and also promotes the expression of additional genes enriched in Th9 cells (6, 8, 9). Although PU.1 is clearly an important transcription factor for the development of Th9 cells, IL-9 production is not entirely eliminated in the absence of PU.1. Because ETS family factors often have overlapping functions, this suggested that another ETS factor might be able to regulate *Il9* gene expression in the absence of PU.1.

ETS variant 5 (ETV5) is an ETS family member that has largely been examined for roles in development. ETV5 coordinates limb development, controls spermatogonial gene expression, and regulates epithelial-mesenchymal gene expression (17–20). ETV5 was also identified as an IL-12–induced Th1 gene, although recent work suggests it has only a minimal role in IFN-γ production, contrary to the original report (21, 22). We reported that Etv5 is cytokine inducible in a STAT-dependent manner, and that Etv5-deficient T cells have diminished Th17 cells but slightly increased Th2 development in vivo and in vitro (22). Based on the induction of Etv5 by the Th cell polarizing cytokines IL-12 and IL-6, it seemed possible that Etv5 impacted other Th subsets. In this report, we tested the contribution of ETV5 to Th9 development.

Materials and Methods

Mice

C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). *Etv5*^{fl/fl} CD4-Cre[+] mice (18, 22) were crossed with *Sfpi1*^{fl/fl} mice (23), and Cre[−] littermates were used as control mice. *Stat6*^{−/−}, *Batf*^{fl/fl}, and *Irf4*^{fl/fl} mice were previously described (6, 24, 25). 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 the enriched CD4^{+} T cells from spleen and lymph nodes using MACS beads and columns (Miltenyi Biotec). Naive CD4^{+}CD62L^{+} T cells were activated with

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CNS, conserved noncoding sequence; ETV5, ETS variant 5; IRF4, IFN regulatory factor 4; PAS, periodic acid–Schiff; qRT-PCR, quantitative RT-PCR; Treg, regulatory T cell.

plate-bound anti-CD3 (2 μg/ml 145-2C11; BioXCell) and soluble anti-CD28 (0.5 μg/ml; BD Pharmingen) to generate Th0 or with additional cytokines (all from Peprotech) and Abs (BioXCell) to generate Th1 (5 ng/ml IL-12 and 10 μg/ml anti–IL-4 [11B11]), Th2 (10 ng/ml IL-4 and 10 μg/ml anti–IFN-γ [XMG]), Th9 (20 ng/ml IL-4; 2 ng/ml TGF-β; and 10 μg/ml anti–IFN-γ [XMG]), Th17 (100 ng/ml IL-6; 2 ng/ml TGF-β; 10 ng/ml IL-23; 10 ng/ml IL-1β; 10 μ/ml anti–IFN-γ [XMG]; and 10 μg/ml anti–IL-4 [11B11]), and regulatory T cell (Treg) (50 U human IL-2; 2 ng/ml TGF-β; 10 μg/ml anti–IFN-γ [XMG], and anti–IL-4 [11B11]). Cells were expanded after 3 d with half concentration of the original cytokines in fresh medium. Cells were harvested on day 5 for analysis. The *Etv5* retroviral vector was previously described (22) and cells were transduced during differentiation as performed previously (26).

OVA-induced allergic airway inflammation

Mice were sensitized with OVA adsorbed to alum and challenged intranasally with aqueous OVA as previously described (8). Mice were euthanized by CO_2 inhalation. Bronchoalveolar lavage fluid was obtained by making an incision in the trachea and subsequently flushing the airways three times with a single use of 1 ml of sterile PBS. We spun down the BAL fluid–associated cells and collected supernatants for ELISA. Lungs were perfused via the right ventricle of the heart with 3 ml of PBS to remove blood lymphocytes from the vasculature. Some lung tissue was isolated for histology or RNA expression. Residual lung tissue was minced and enzymatically digested with type II collagenase (37˚C for 30 min; Worthington), followed by passing through a steel screen. RBCs in the cell suspension were lysed using ammonium chloride. Cells were counted using a hemacytometer after exclusion of dead cells using trypan blue dye.

Analysis of gene expression, ELISA, and flow cytometry

Quantitative RT-PCR (qRT-PCR) and ELISA were performed as previously described (26). Gene expression was normalized to housekeeping gene expression (β₂-microglobulin). The relative gene expression was calculated by the change-in-threshold ($-\Delta\Delta C_T$) method. For cytokine staining, $CD4^+$ T cells were stimulated with PMA and ionomycin for 2 h followed by monensin for a total of 6 h, fixed, permeabilized with 0.2% saponin, and stained for IL-17A–PE/Cy7, IFN-γ–FITC, or IL-4–Alexa Fluor 647 (BD Pharmingen) or IL-9–PE (Biolegend).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed as described previously (27). In brief, resting Th cells were cross-linked for 10 min with 1% formaldehyde and lysed by sonication. After preclearing with salmon sperm DNA, BSA, and Protein Agarose bead slurry (50%), cell extracts were incubated with either rabbit polyclonal Etv5 H-100, p300 N-15, GCN5 H-75 (Santa Cruz), H3ac, H4K16ac, H4K5ac, H4K8ac, or normal rabbit IgG (all from Millipore) overnight at 4˚C. The immunocomplexes were precipitated with protein agarose beads at 4˚C for 2 h, washed, eluted, and cross-links reversed at 65˚C overnight. DNA was purified, resuspended in H_2O, and analyzed by quantitative PCR. Primers were described previously (5, 8). Unless stated otherwise, 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 Ab and normalized against the amount of input DNA.

Histology

Lungs were perfused with PBS, excised from the thoracic cavity, inflated with 4% neutral-buffered formaldehyde, and fixed overnight at room temperature. Tissues were embedded in paraffin, sectioned, and stained with H&E or via the periodic acid–Schiff (PAS) method. Lung histology was scored by a reader with experimental conditions masked. Lung pathology was scored on H&E-stained slides from a range of 0 to 4 as follows: 0 = no visible inflammatory infiltrate; 1 = patchy and/or light infiltrate into conducting airways; 2 = widespread and/or intense infiltrate into conducting airways with little involvement in alveoli spaces; 3 = widespread/intense infiltrate into conducting airways with patchy and/or light involvement in alveolar spaces; and 4 = widespread/intense infiltrate into all aspects of the lung architecture. Goblet cell hyperplasia was scored by the average proportion of PAS staining in 20 randomly selected airways from two independent reads with a scoring range from 0 (no PAS^+ epithelial cells) to 1 (100% PAS^+ epithelial cells).

Statistical analysis

Statistical analyses were performed using Prism5 software (for Macintosh; GraphPad Software). A two-tailed Student *t* test or one-way ANOVA was used to generate *p* value data as specified in the figure legends. A post hoc Tukey test was used for multiple comparisons. A *p* value <0.05 was considered statistically significant.

Results

Etv5 promotes Il9 production

Our recent results demonstrated that ETV5 promotes IL-17 production and is induced in response to STAT-activating cytokines (22). However, ETV5 had limited functions in Th1 cells and seemed to repress IL-4 production. The repression of IL-4 was reminiscent of the phenotype observed in T cells that lack PU.1 expression (15). Thus, we were interested in determining the effects of ETV5 on Th9 development. To test this, we isolated naive T cells from *Etv5*fl/fl mice that were either negative or positive for CD4-Cre (referred to in the figures as *Etv5*Δ^CD4) and cultured them under Th2 or Th9 skewing conditions. By intracellular cytokine staining, we observed a diminished percentage of IL-9–producing cells in Th2 and Th9 cultures lacking expression of ETV5 (Fig. 1A). We also observed an increased percentage of IL-4⁺ cells in Th2 and Th9 cultures lacking ETV5 (Fig. 1A). This observation was consistent with decreased IL-9 and increased IL-4 in the supernatants of Th2 and Th9 cultures, and with mRNA expression of these cytokines (Fig. 1B, 1C). We further observed that additional Th2 cytokines, including IL-5 and IL-13, were increased in the absence of ETV5 (Fig. 1B).

To directly demonstrate the function of ETV5, we transduced developing Th9 cells with retroviruses containing either no cDNA or expressing ETV5. Ectopic expression of ETV5 in Th9 cells increased the production of IL-9 and further decreased the minimal production of IL-4 (Fig. 1D). Similarly, ectopic expression of ETV5 in Th2 cells repressed IL-4 production and increased the percentage of cells positive for IL-9 (Fig. 1D). Thus, ETV5 regulates production of IL-9.

Etv5 gene expression is induced after T cell activation. We compared the expression of Etv5 during the development of Th2, Th9, and inducible Tregs, and observed that although there is no induction of Etv5 during Treg development, Etv5 is dynamically regulated in Th2 and Th9 cells (Fig. 2A). We observed a rapid increase in Th2 cells and a slower increase in Th9 cells that ultimately fell to similar levels after 5 d in culture (Fig. 2A, 2B). After stimulation of Th9 cells after 5 d of culture, *Etv5* mRNA is rapidly induced by 4 h (Fig. 2C). Consistent with regulation dissimilar from *Sfpi1*, we observed *Etv5* mRNA was dependent on STAT6 (Fig. 2D). *Etv5* mRNA was also decreased in Th9 cultures by the absence of IRF4, suggesting Etv5 expression is downstream of a STAT6/IRF4 network. In contrast, *Etv5* expression was increased in Th9 cultures that lacked expression of BATF or PU.1, suggesting that IL-4–dependent expression does not require BATF (Fig. 2D).

Specificity of ETS proteins in the promotion of IL-9 production

The similarity of phenotypes in Th9 cultures ectopically expressing ETV5 or PU.1 raised the question whether any ETS protein had the capability to induce IL-9 production. To test this, we examined microarray and RNA-seq data sets (6, 28) to determine which ETS family members are expressed in appreciable amounts in Th9 cells. We examined the function of Elk3 and Etv6, both having enriched expression in Th9 cultures (Fig. 3A, 3B), using the ectopic expression assay. Ectopic expression of either cDNA increased mRNA levels at least 10-fold (data not shown). We observed that although PU.1 and ETV5 had similar abilities to induce IL-9 production (8, 9) (Fig. 1), Elk3 and Etv6 had no effect in this assay (Fig. 3C, 3E). Etv6 has previously been linked to IL-17 production in Th17 cells (29), but we did not see induction of

FIGURE 1. ETV5 promotes IL-9 and inhibits Th2-associated cytokine production. (**A–D**) Wild type or *Etv5*^ΔCD4 naive T cells were cultured in vitro under Th2 or Th9 conditions for 5 d. (A) Representative FACS profiles of IL-9– and IL-4–producing cells after stimulation with PMA/ionomycin for 6 h. Depicted cells were gated as FSC^lo^SSC^lo^CD4^+^. (B) Cytokines secreted after stimulation with anti-CD3 for 24 h. (C) *Il9* and *Il4* expression after stimulation with anti-CD3 for 6 h. Data were normalized to *β2m* expression. *Il9* expression was relative to wild type Th2, and *Il4* was relative to wild type Th9. (D) Wild type naive T cells were cultured for 5 d in Th9 conditions and were transduced with control or *Etv5*-expressing retrovirus (RV) on day 2 of culture. Representative FACS profiles of IL-9– and IL-4–producing transduced cells. Cells stimulated with PMA/ionomycin. Depicted cells were gated as FSC^lo^SSC^lo^CD4^+^Thy1.1^+^. Data are represented as mean ± SEM. (A–C) Data depicted are from two independent experiments (four to five mice per experiment) (D) Data depicted are from three independent experiments (two mice per experiment). A two-tailed Student *t* test was used for single comparisons. *$p < 0.05$, **$p < 0.01$.
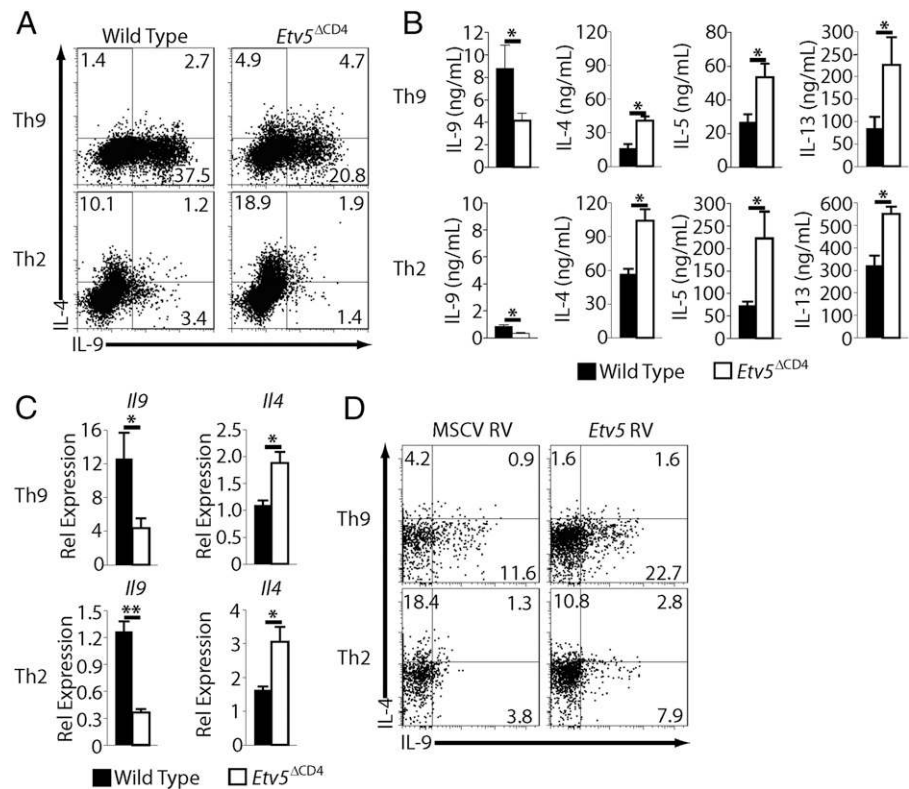
IL-17, which was expressed in only a small percentage of the Th9 cultured cells (Fig. 3D). Thus, the ability to induce IL-9 expression is not shared among all ETS family proteins.

Chromatin remodeling at the Il9 locus by ETV5

Previous reports indicated that PU.1 bound to the *Il9* promoter and recruited Gcn5-containing complexes to alter the histone modifications at the *Il9* locus (8, 9). We first tested whether ETV5 also bound to the *Il9* locus at three conserved noncoding sequence (CNS) regions: CNS0, CNS1 (promoter), and CNS2 (30). ETV5 binding was significantly enriched at the CNS0 and CNS2 regions (Fig. 4A). This was in contrast with PU.1 binding that is enriched only at the CNS1 region (5, 8). Moreover, ETV5 binding at CNS2 was significantly greater in Th9 cells than in either Th2 or Th17 cells, and binding at CNS0 was significantly greater in Th9 than Th17, with a trend toward increased binding compared with Th2 cells (Fig. 4B). Binding of ETV5 to a nonconserved sequence in the *Il9* locus was barely detectable (Fig. 4B). PU.1 and ETV5 also had differential effects on the recruitment of HAT proteins to the locus. In contrast with PU.1-deficient Th9 cells that had increased p300 association and decreased Gcn5 association with the *Il9* locus (9), ETV5-deficient cells had normal Gcn5 recruitment but significantly decreased p300 recruitment, correlating with significant decreases in the overall histone H3 acetylation and H4K16 acetylation at the *Il9* promoter (Fig. 4E, 4F). The histone acetylation events were specific because there was no difference in the acetylation of H4K5 or H4K8 between control and ETV5-deficient Th9 cells (Fig. 4G, 4H). These results suggest that although PU.1 and ETV5 have overlapping biological functions, they promote the expression of *Il9* by acting on the *Il9* locus through distinct binding sites.

Parallel effects of PU.1 and ETV5 in vitro

These results suggested that ETV5 has distinct and overlapping functions with PU.1 in the development of Th9 cells. To test this

directly, we mated mice with a conditional PU.1-expressing allele (*Sfpi1*) with the *Etv5* fl/fl mice, in the context of the CD4-Cre transgene. Naive CD4 T cells were isolated from control, *Etv5* fl/fl CD4-Cre, *Sfpi1* fl/fl CD4-Cre, and *Sfpi1/Etv5* fl/fl CD4-Cre
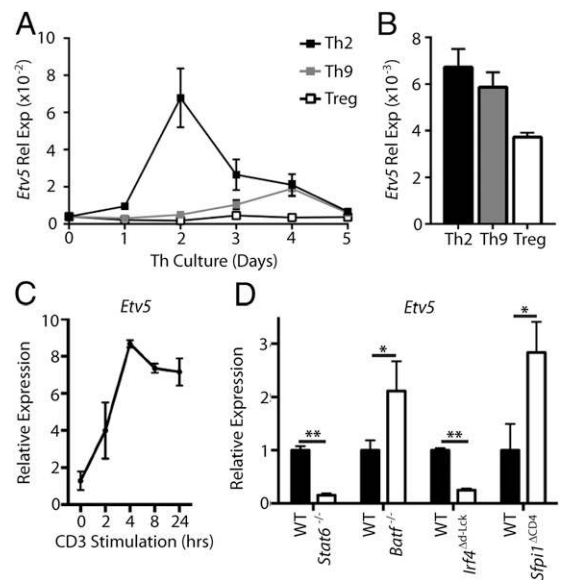

FIGURE 2. *Etv5* expression in Th cell subsets. (**A–C**) Wild type naive T cells were cultured in vitro under Treg, Th2, or Th9 conditions for 5 d. (A) Every 24 h during the culture period RNA was isolated to assay for Etv5 expression by qRT-PCR in each culture condition. (B) Comparison of Etv5 expression among the subsets at day 5 of culture. (C) Etv5 mRNA levels in Th9 cells activated with anti-CD3 for the indicated time points. (**D**) T cells from mice of the indicated genotypes were cultured in vitro under Th9 conditions over 5 d. Etv5 expression was measured using qRT-PCR at day 5 of culture. A two-tailed Student *t* test was used for single comparisons. *$p < 0.05$, **$p < 0.01$. (A–D) Data are represented as mean ± SEM from at least two independent experiments.
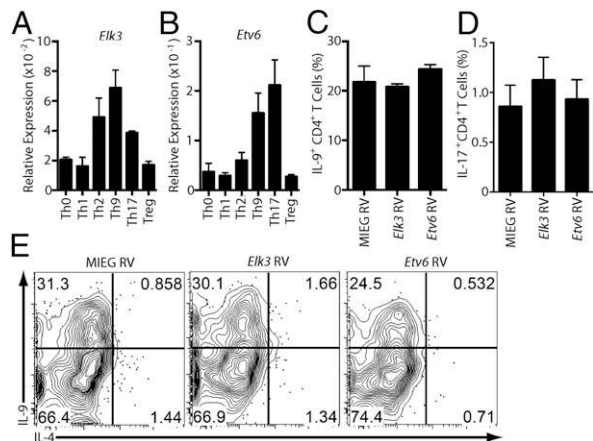
FIGURE 3. ELK3 and ETV6 do not promote IL-9 production in Th9 cells. (**A** and **B**) *Elk3* and *Etv6* expression in T cells cultured in depicted Th subset conditions for 5 d. Data were normalized to *β2m* expression. (**C–E**) Naive T cells were cultured for 5 d in Th9 conditions and were transduced with control, *Elk3*-expressing, or *Etv6*-expressing retrovirus (RV) on day 2 of culture. Cells were restimulated with PMA/ionomycin. Accumulated data (C) and representative FACS profiles (E) of IL-9–producing transduced cells. Accumulated data (D) of IL-17–producing transduced cells from Th9 cultures. Depicted cells were gated as FSC^{lo}SSC^{lo}CD4$^+$GFP$^+$. Data are represented as mean ± SEM from two to three independent experiments (three mice per experiment).

mice, and cultured under Th9 conditions. Consistent with our previous reports, Th9 cultures that were deficient in PU.1 had diminished IL-9 production (Fig. 5A). Similarly decreased levels of IL-9 were observed in Etv5-deficient Th9 cultures (Fig. 5A). This pattern was similar when IL-9 secretion was analyzed (Fig. 5B). Importantly, Th9 cultures that were doubly deficient in PU.1 and ETV5 showed an additive effect of the deletion of each gene and had the lowest percentages of IL-9$^+$, the lowest concentration of secreted IL-9, and the lowest *Il9* mRNA among the cultures (Fig. 5A, 5B). Interestingly, although deficiency in either PU.1 or ETV5 increases the production of IL-4 (Fig. 5B), there

was not an additive effect of deficiency in both factors, with double-deficient Th9 cultures having intermediate percentages of IL-4$^+$ cells and concentrations of secreted IL-4 (Fig. 5A, 5B).

To determine whether PU.1 and ETV5 have distinct effects on the expression of other genes enriched within Th9 cells, we performed quantitative PCR analysis of cDNA from each of the Th9 cultures. Consistent with our previous report, PU.1 deficiency impacted a small subset of Th9 genes relative to those affected by STAT6 and BATF deficiencies in almost all genes indicated in Fig. 5C (6). We observed no significant effect of PU.1 or ETV5 deficiency on the expression of *Irf4*, *Maf*, *Gata3*, *Erg*, *Crem*, *Tnfsf13b*, *Ahr*, *Ccr4*, or *Fasl* (Fig. 5C and data not shown). Expression of *Sfpi1* (PU.1) was increased in the absence of ETV5, indicating that the phenotype observed in ETV5-deficient Th9 cells is not due to diminished PU.1 expression (Fig. 5C). This does suggest that increased PU.1 expression might be at least partially compensating for ETV5 deficiency in the expression of Th9 genes. Deficiency in both PU.1 and ETV5 was required to see decreases in *Batf* and *Cxcl3* (Fig. 5C). In contrast, we observed Etv5 had significant effects on *Il1rn* expression, and PU.1 was somewhat repressive to Ccr8 (Fig. 5C). Thus, PU.1 and ETV5 have distinct effects on gene expression in Th9 cells.

Parallel effects of PU.1 and ETV5 in vivo

We then tested whether combined deficiency in PU.1 and ETV5 had distinct effects on IL-9 production when tested in vivo. We used the OVA/alum allergic airway inflammation model that has previously been shown to be partially dependent on IL-9 and PU.1 expression in T cells (8). Mice were sensitized and challenged as portrayed in Fig. 6A. Overall, we observed that there were parallel effects of deficiency in both PU.1 and ETV5 in T cells, with each affecting distinct aspects of inflammation. We observed that PU.1 alone had a greater effect on total inflammation as assessed by histology and eosinophils in the lung (Fig. 6B, 6D, 6H). ETV5 deficiency resulted in significant decreases in mucous staining, PMN infiltration, and mast cell accumulation (Fig. 6C, 6F, 6H, 6I). Among these parameters, the double-deficient mice were not significantly different from the respective single-deficient mice, suggesting that
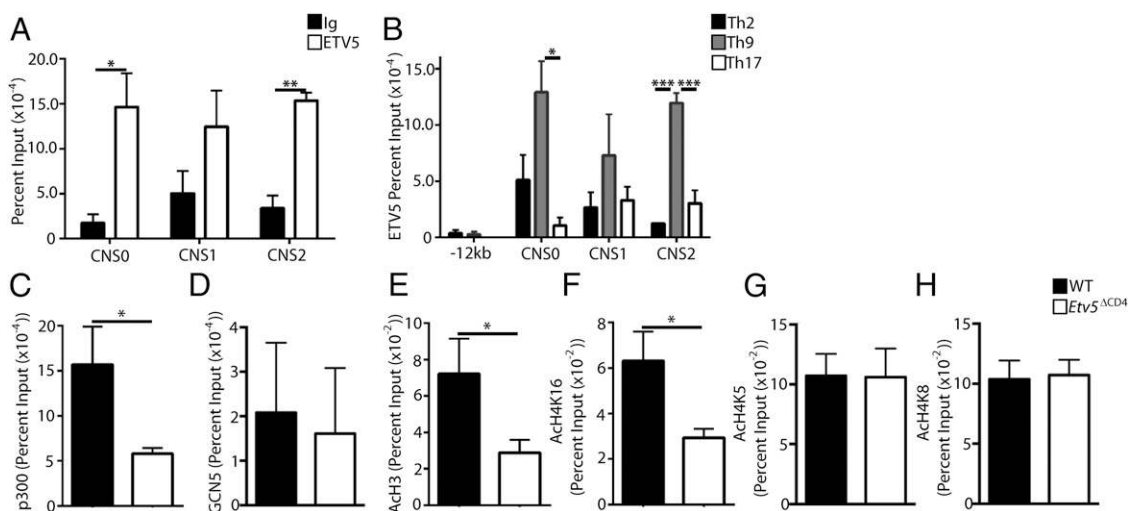

FIGURE 4. ETV5 binds and enhances histone acetylation at the *Il9* CNS regions (**A–H**) Wild type or *Etv5*^{ΔCD4} naive T cells were cultured in vitro under Th9 conditions, unless depicted otherwise, for 5 d. (A) ETV5 binding, with respective isotype controls, at the *Il9* CNS regions. (B) ETV5 binding within indicated Th cultured cells at the *Il9* CNS regions and a −12-kb region of the *Il9* locus (negative control). (C and D) p300 or GCN5 binding to the *Il9* CNS1. (E–H) Depicted Ac-histone levels at the *Il9* CNS1. (B–H) Percent input depicted are the ETV5 ChIP values after subtraction of the control Ig ChIP values. Data are represented as mean ± SEM from two to three independent experiments (three mice per experiment). A two-tailed Student *t* test was used for single comparisons. One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
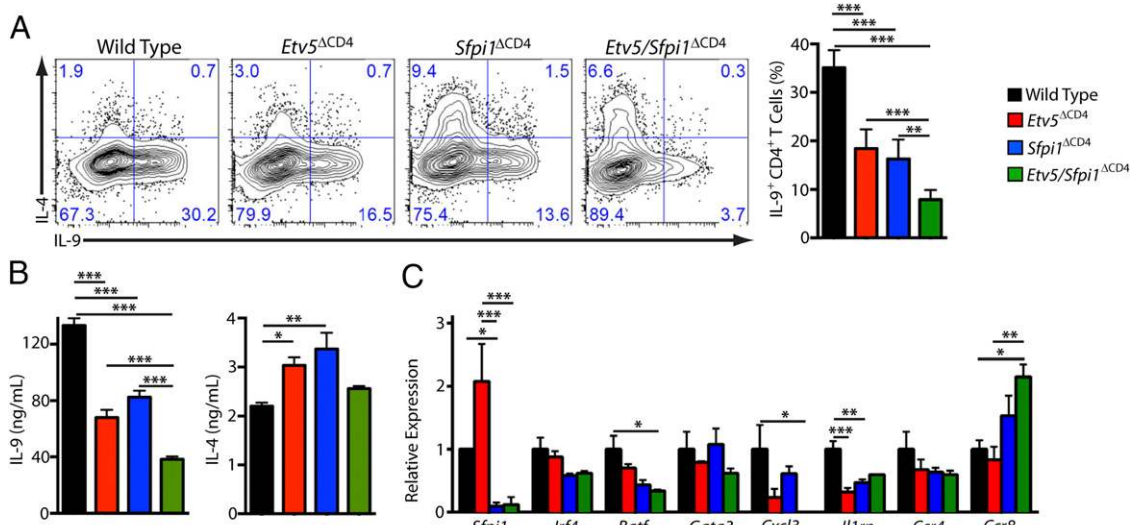
FIGURE 5. Effect of deficiency in *Etv5* and *Sfpi1* on Th9 differentiation. (**A–C**) Wild type, *Etv5*$^{\Delta CD4}$, *Sfpi1*$^{\Delta CD4}$, or *Etv5/Sfpi1*$^{\Delta CD4}$ naive T cells were cultured in vitro under Th9 conditions for 5 d. (A) Representative FACS profiles of IL-9– and IL-4–producing cells. Cells stimulated with PMA/ionomycin. Depicted cells were gated as FSC^{lo}SSC^{lo}CD4$^+$. (B) Cytokine production after stimulation with anti-CD3 for 24 h. (C) Gene expression in day 5 cultured cells. (C) Data were normalized to *β2m* expression and are relative to wild type cell expression. (B and C) Data are represented as mean ± SEM from three independent experiments (two to three mice per experiment). One-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

PU.1 and ETV5 were controlling separable aspects of inflammation. However, only the double-deficient mice were significantly lower than wild type mice in total lung cell accumulation, *Muc5ac* expression, and *Mcpt2* expression (Fig. 6E, 6G, 6J). Moreover, double-deficient mice demonstrated *Mcpt1* expression that was significantly lower than wild type and either of the single-deficient mice (Fig. 6J).

We then tested whether this corresponded to decreased IL-9 production in vivo. Although there was a similar reduction in total CD4 T cells in the lung in each single- or double-deficient mouse (Fig. 7A), there was an overall decrease in the number and percentage of IL-9$^+$ T cells in the lung, with T cells from double-deficient mice demonstrating IL-9 reduced to background amounts (Fig. 7B, 7C), although these values were not significantly decreased compared with mice with PU.1-deficient T cells. This is in contrast with the numbers and percentages of lung Th2 cytokine–producing T cells that were, respectively, modestly decreased or unaffected (Fig. 7B, 7C). The number of IL-13–producing T cells in the lung was decreased in double-deficient mice, although the percentages in the lung were not decreased, suggesting a link to overall inflammation (Fig. 6). Moreover, in the periphery, Th2 responses developed normally. OVA stimulation of
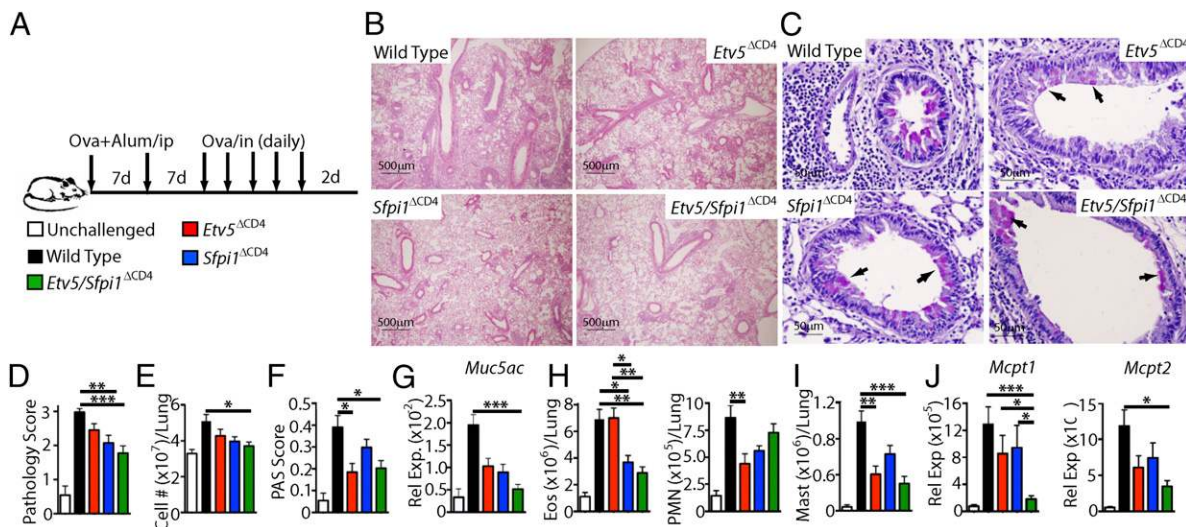

FIGURE 6. Pathology of mice deficient in both *Etv5* and *Sfpi1* in an allergic disease model. (**A**) Schematic of OVA/Alum model used to promote allergic inflammation. Unchallenged mice were sensitized with OVA/Alum but did not receive OVA challenge intranasally. Representative images of H&E (**B**) and PAS staining (**C**) of inflamed lung tissue. (**D**) Tabulated pathology scores from H&E-stained tissues. (**E**) Absolute cell numbers of the lungs. (**F**) Tabulated PAS scores. (**G**) Muc5ac expression in the lung. Data were normalized to *β2m* expression. (**H–I**) Eosinophil (FSC^{lo}SSC$^{int/hi}$SigLecF$^+$CD11c^{negt}), neutrophil (FSC^{lo}SSC^{int}Ly6G$^+$CD11b^{hi}), and mast cell (FSC^{int}SSC^{int}FcεR1$^+$cKit$^+$) absolute numbers in the lung. (**J**) *Mcpt1* and *Mcpt2* expression in the lung. Data were normalized to *β2m* expression. (D–J) Data represented as mean ± SEM from two independent experiments (five to six mice per treatment per experiment). For values from challenged mice, one-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 7. CD4$^+$ T cell responses in *Etv5*- and *Sfpi1*-deficient mice during allergic inflammation. Pulmonary allergic inflammation was generated as in Fig. 6. (**A**) Absolute number of CD4$^+$ T cells (FSC^{lo}SSC^{lo}CD3ε$^+$CD4$^+$CD8^{negt}) in the lung. Numbers (**B**) and percentage (**C**) of cytokine-producing CD4$^+$ T cells in the lung. (**D**) Cells were collected from the lung draining lymph nodes in allergic mice. Equivalent numbers of cells were cultured with exogenous Ova for 36 h. Supernatants were analyzed for depicted cytokines. Data are represented as mean ± SEM from two independent experiments (five to six mice per treatment per experiment). For values from challenged mice, one-way ANOVA with a post hoc Tukey test was used to generate *p* values for all multiple comparisons. *$p < 0.05$, **$p < 0.01$.
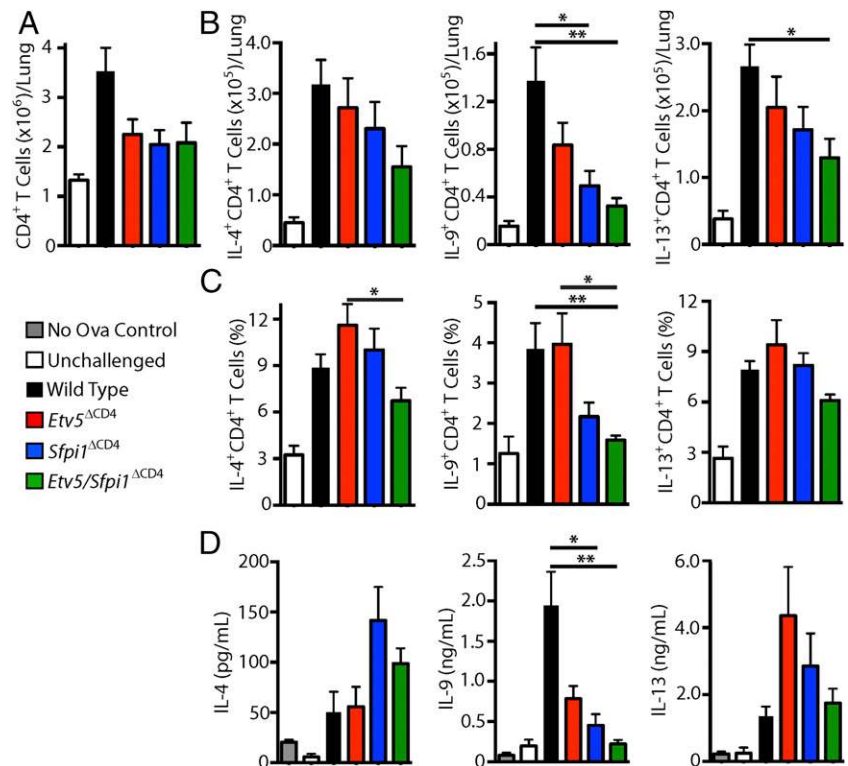
draining lymph node cells yielded normal or slightly increased production of Th2 cytokines (Fig. 7D). This was in contrast with OVA-stimulated IL-9 concentrations that were significantly decreased in cultures from mice with PU.1-deficient or double-deficient T cells (Fig. 7D). Together, these data demonstrate a role for ETV5 in regulating IL-9 production and Th9 development in vitro and regulating allergic inflammation in parallel with PU.1.

Discussion

Transcription factors play an obligate role in defining the phenotype and function of Th cells. In this report, we have defined the contribution of the ETS family transcription factor ETV5 to the Th9 phenotype. ETV5 shares many functions with the ETS family factor PU.1 that we previously demonstrated was required for Th9 development (8, 9). Both ETV5 and PU.1 regulate IL-9 production, although they appear to work through different regulatory elements in the *Il9* gene. Apart from IL-9, PU.1 and ETV5 regulate some common and some distinct genes associated with the Th9 phenotype. Moreover, T cells that lacked both ETV5 and PU.1 had decreased IL-9 production in vitro.

The concept of transcription factors from the same family having overlapping functions is observed in other T cells. In Th17 cells, the loss of IL-17 production is more severe in mice that lack both RORγt and RORα than either factor individually (31). Similarly, Tc17 cells develop from CD8 T cells that are doubly deficient in the T-box factors T-bet and Eomesodermin (32). This paradigm is also seen among ETS family factors that are divided into four classes based on binding sequence and nine subfamilies based on homology (33). The partial redundancy between PU.1 and Spi-B, two factors in the same subfamily, has been examined in several models including myeloid and lymphoid cell development, B cell function, and the development of leukemia (34–37). Other examples of factors from the same subfamily having overlapping function include ETV1 and ETV4 that cooperate in prostate cancer, ELK1 and ELK4 in thymocyte development, and FLI-1 and ERG in hematopoiesis (38–40). However, PU.1 and ETV5 are

from different subfamilies. This is not without precedent and ETS2 and ELF5, factors from distinct subfamilies, cooperate in development (41). Some of this redundancy is clearly dependent on overlapping binding specificities of the family-defining DNA binding domain (42). Yet, the protein sequences outside of the DNA binding domain are divergent and suggest that the partial redundancies observed are limited by the ability of each ETS family member to interact with other transcription factors at gene-regulatory elements (33).

Although ETV5 and PU.1 were clearly cooperating on IL-9 production within in vitro–derived cells, we observed a trend but not a significant effect of ETV5 deficiency on IL-9 production in vivo. This could be caused by several reasons including compensation by increased expression of PU.1 (Fig. 5) or other signals in vivo that might bypass the requirement for ETV5 in the OVA/alum model. Despite these data, we observed distinct effects of PU.1 and ETV5 on the overall allergic inflammation, suggesting that PU.1 and ETV5 are not exerting effects in vivo strictly through IL-9. This is consistent with the effects of deficiency of either factor or both in T cells during the development of allergic inflammation. Although deficiency in either factor results in diminished inflammation, PU.1 deficiency had a slightly greater effect on eosinophil accumulation during allergic inflammation than did deficiency in ETV5. Conversely, ETV5 deficiency had a greater effect on neutrophil recruitment, consistent with our previous studies (22). Importantly, although we and others have shown that the HDM model of airway inflammation is dependent on IL-17 (22, 43), the OVA-alum model is largely IL-17 independent (44), so that the effects of ETV5 deficiency on IL-17 should not impact the total cellular inflammation in our studies. Both ETV5 and PU.1 contribute to the ability of Th9 cells to promote accumulation and gene expression of mast cells in lung tissue, again consistent with our previous results on the role of Th9 cells in mast cell accumulation in multiple models of allergic airway disease (45). We did observe that IL-13–producing T cells in the lung were also decreased in mice with T cells lacking both

ETV5 and PU.1, although the proportion of these cells was not different, suggesting the decrease in number was linked to decreased overall inflammation. IL-13 production from draining lymph node cells in mice with T cells lacking ETV5 and PU.1 was normal, leading to the conclusion that there is normal development of the Th2 response in these mice. Still, it is possible that the observed decrease in IL-13–producing T cells might contribute some aspect of the diminished inflammation observed in vivo.

Although we have focused on conventional CD4 T cells in this report, it is possible that ETV5 might be functioning in other types of cells. Other sources of IL-9 in the immune system include NKT cells, γδ T cells, and innate lymphoid cells (4). Among these, the CD4-Cre should only affect expression in NKT cells. We previously suggested that PU.1 was not required for IL-9 production by NKT cells (45), but it is not clear whether ETV5 might play a role within these cells. The role of NKT cells in asthma models is still debated, and what controls IL-9 within these cells is not yet clear.

As noted earlier, the ETS transcription factor family is divided based on binding specificity and homology (33). As we have shown that PU.1 and ETV5 preferentially bind different sites in the *Il9* locus and recruit different HATs to the locus, they appear to have distinct functions at a convergent target gene. It is not clear whether this is a common theme among cooperating ETS factors. The ability of PU.1 and ETV5 to cooperate across subfamilies suggests that redundancy of function is not restricted to family members that have similar binding site specificities or similar structures outside of the common ETS DNA binding domain. Further work will help to elucidate the rules governing the development of evolutionary cooperation among this family of transcription factors.

Disclosures

The authors have no financial conflicts of interest.

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